# Phytochemical Accumulation in Coffee (*Coffea arabica* L.) Plants as a Photoprotective Mechanism during Oxidative Stress Conditions

# （酸化ストレス条件下における光保護メカニズムとしてのコーヒー(*Coffea arabica* L.)へのファイトケミカルの蓄積）

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**THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES, TOTTORI UNIVERSITY**

# Phytochemical Accumulation in Coffee (*Coffea arabica* L.) Plants as a Photoprotective Mechanism during Oxidative Stress Conditions

**A DOCTORAL THESIS**

**BY**

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**Dedication**

*This thesis is dedicated to my family*

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## Table of contents

## List of tables

## List of figures

## List of equations

## Acronyms and abbreviations

| | |
|---|---|
| A | − Net Assimilation Rate |
| ABTS | − 2,2−anzinobis−(3−ethylbenzothiazoline−6−sulfonic acid) |
| ANOVA | − Analysis of Variance |
| BL | − Brown Coffee Leaves |
| $C_a$ | − Extracellular $CO_2$ Concentration |
| CAF | − Caffeine |
| Car | − Carotenoids |
| CGAs.s | − Chlorogenic Acids |
| Chl | − Chlorophylls |
| $C_i$ | − Intracellular $CO_2$ Concentration |
| CQA | − Caffeoylquinic Acid |
| Cy3G | − Cyanidin 3−glucoside |
| DPPH | − 2,2 Diphenyl−1 Picrylhydrazyl Radical Assay |
| DW | − Dry weight |
| ELI | − Electrolyte Leakage Index |
| ETR | − Electron Transfer Rate |
| Fo | − Photorespiratory $CO_2$ release |
| FRAP | − Ferrous Reducing Antioxidant Power Assay |
| $F_v/F_m$, $F_v'/Fm'$ | − Maximal Efficiency of PSII Photochemistry during Dark and Light-adapted States, respectively |
| $F_v/F_o$, $F_v'/F_o'$ | − Minimal Efficiency of PSII Photochemistry during Dark and Light-adapted States, respectively |
| GA | − Gross Assimilation Rate |
| GAE | − Gallic Acid Equivalent |
| GB | − Green Coffee Beans |
| $g_s$ | − Stomatal Conductance |
| HPLC | − High-performance Liquid Chromatography |
| HS | − Herbaceous Stem |
| $IC_{50}$ | − Half Maximal Inhibitory Concentration |
| ICO | − International Coffee Organisation |
| $J_{max}$ | − Maximum rate of electron transport |
| Kin | − Kinetin |
| Mel | − Melatonin |
| NPQ | − Non-Photochemical Quenching |

| | |
|---|---|
| NPs | − Nanoparticles |
| $^{o}C$ | − Degrees Celsius |
| ODS | − Octadecyl silyl |
| $OH^{-}$ | − Hydroxyl Ion |
| $OH^{\bullet -}$ | − Hydroxyl Radical |
| PAR | − Photosynthetically Active Radiation |
| $P_N$ | − Net Photosynthetic Rate |
| PPFD | − Photosynthetic Photon Flux Density |
| $q_L$ | − Proportion of Open PSII centres |
| $q_N$ | − Fluorescence Quenching |
| RB | − Raw Coffee Beans |
| RE | − Rutin Equivalent |
| RI | − Refractive Index |
| RBOH | − Respiratory Burst Oxidase Homologs |
| ROS | − Reactive Oxygen Species |
| RuBisCO | − Ribulose-1,5-bisphosphate carboxylase/oxygenase |
| RUBP | − Ribulose 1,5-Bisphosphate |
| SA | − Salicylic Acid |
| SEM | − Scanning Electron Microscope |
| TAC | − Total Anthocyanin Content |
| TEAC | − Trolox Equivalent Antioxidant Capacity |
| TPC | − Total Phenolic Content |
| TPTZ | − 2,4,6-Tri(2-pyridyl)-s-triazine |
| Trmmol, E | − Transpiration Rate |
| UV | − Ultra-violet |
| $V_c$ | − RuBisCO Carboxylation Rate |
| $V_{c,max}$ | − Maximum Rate of Carboxylation |
| $V_o$ | − Rubisco Oxygenation Rate |
| WS | − Woody Stem |
| WUE | − Water Use Efficiency |
| XRD | − X−Ray Diffraction |
| $Y_{(II)}$ | − Quantum yield of PSII |
| YGM−5b | − 3−(6−Caffeoylsophoroside)−5 Glucoside |
| $\Phi_{PSII}$ | − Actual Photochemical Efficiency of PSII |

# Chapter one

# General Introduction

## 1.1 Introduction

Coffee (*Coffea*.) is a genus of tribe *Coffeea* DC in the family *Rubiaceae* and sub−family *Ixoroideae* that comprises of over 124 species (appendix 1) all of which are indigenous to the African continent (Davis *et al.,* 2006; Stoffelen *et al.,* 2008). The three main centres of species diversity of *Coffea* are; the evergreen, humid forests of eastern Madagascar, Cameroon, and the eastern arc mountains of Tanzania. (Davis and Mvungi, 2004; Davis *et al.,* 2006). All *Coffea* spp. originate from Madagascar, Mainland Africa and Mascarenes. In fact, there is no known species of *Coffea* that naturally occurs outside tropical Africa. By quantity, 16, 14, 1 and 1 species of *Coffea* originate from Tanzania, Cameroon, Ethiopia and Mascarenes respectively while the rest originate from Madagascar where there is a variety of forest types ranging from littoral evergreen, riverine, mixed deciduous, dry xerophytic and high altitude mossy forest whose diversity in habitat conditions relates to a high *Coffea* spp. diversity on the island (Krishnan *et al.,* 2013). However some species such as *C. arabica* L. and *C. canephora* Pierre ex A. Froehner have been introduced on all continents across the globe where they have consequently been naturalized in the tropical or subtropical regions outside their natural range so long as the climate is not significantly different from the original habitats (Teketay, 1999).

The *Coffea* genus contains the three most commercially important coffee species used in the production of the beverage. These include; *C. arabica* (arabica coffee), *C. canephora* (robusta coffee) and *C. liberica* (Liberian/ Liberica coffee or excelsa coffee). Out of these, *C. arabica* is the most economically important species contributing about 60% on the global trade of coffee (ICO, 2019). Arabica coffee is believed to have originated from the south western highlands of Ethiopia (Charrier and Berthaud, 1985; Teketay, 1999; Davis *et al.,* 2006) which is also the species centre of diversity. This species inhabits humid evergreen forests in the North east Tropical Africa (South west Ethiopia – west of the Great Rift Valley) and has also been naturalized in many parts of the Tropics such as the East Tropical Africa (In Kenya near Mt Marsibit). The knowledge of the beverage processed from this *C. arabica* was known from Arabia hence the name Arabica coffee. *C. arabica* L. is the only allotetraploid (2n=4x=44) coffee species. In addition, besides *C. heterocalyx* and *C. anthonyi* Stoff. & F. Anthony, *C. arabica* L. is the only other autogamous species of *Coffea*. That notwithstanding, there are a

number of interspecific hybrids since *C. arabica* L. can be crossed with most other diploid (2n=22) species of *Coffea* (Lashermes et al., 2000).

In cultivation, *C. arabica* L. has a low genetic diversity compared to wild populations in North−East tropical Africa where it is indigenous and other parts of Africa where it has been naturalised (Teketay, 1999; Anthony *et al.,* 2002). Domestication of Coffea arabica followed major colonial routes (France, Great Britain, The Netherlands, Germany, Spain, Belgium and Portugal) using materials with a narrow genetic base that was originally obtained from Ethiopia by the Arabs around 651 AD (Wellman 1961; Teketay, 1999; Anthony *et al.,* 2002). By the 20$^{th}$ century, there were more than 200 existing Arabica varieties grown in over 80 countries (Teketay, 1999). This is because of its superior coffee taste combined with low caffeine content which stimulates both commercial and scientific interest. Bourbon is among the most popular cultivars of *C. arabica* L. whose distribution worldwide started from the Reunion islands formerly known Bourbon, then a French colony. To date, *C. arabica* L. is mainly cultivated in highland areas with moderate temperature. Cultivated *Coffea* species have slightly different climatic requirements. *C. arabica* L. grows well in areas with an annual rainfall ranging between 1400 mm and 2400 mm although the species can grow in conditions of less or higher rainfall conditions between 762 mm and 4200 mm. In Africa, South East Asia, Latin America; tropical areas with the best coffee growing conditions receive 1800 mm of rainfall which is either well distributed throughout the year or rains continuously for about 7–8 months and with only a short distinct dry season of about 2 to 3 months. According to Hilten *et al.* (2011), the nature of the rainy season in terms of length, intensity and distribution is a key ecological factor determining the phenology of coffee. At the onset of the dry season, growth slows followed by lignification of the young stems as well as flower bud initiation. Fruit−bearing coffee plants need a significant period of dry season for seed maturation to occur. *C. arabica* L. is highly susceptible to leaf diseases and pests whose incidence and severity increases with high amounts of annual rainfall over 3000 mm. In regions with less rainfall, coffee is cultivated under irrigation regimes and under shade trees that conserve the subsoil moisture content. *C. canephora* tolerates high moisture levels due to its resistance to majority of the fungal diseases. It however needs a distinct dry season to initiate flowering. Contrariwise, *C. liberica* needs heavier rainfall than the aforementioned species.

Oxidative stresses induced by extreme environmental conditions pose a serious threat to coffee cultivation globally (Wang *et al.,* 2003; DaMatta and Ramalho, 2006). It has been suggested that oxidative stress induced by abiotic pressures is the primary source of crop loss including

coffee worldwide causing over 50% loss of potential agricultural yield (Bray *et al.,* 2000). Abiotic stresses conditions stimulate a series of biochemical, physiological, molecular and morphological changes that adversely affects plant growth. Such conditions are associated with disruption of the osmotic and the ionic homeostasis which cause disruption of osmotic and ionic homeostasis culminating into damage of functional and structural proteins and membranes (Zhu, 2001; Mittler, 2002; Demidchik, 2015 ). The osmosensors such as AtHK1; phospholipid cleaving enzymes such as PLD; second messengers such as $Ca^{2+}$, PtdOH, ROS; MAP kinases, $Ca^{2+}$ sensors such as SOS3, and calcium−dependent protein kinases such as CDPKs after perceiving the stimuli trigger signal sensing and transduction and transcription control. The transcription control factors such as CBF/DREB, ABF, HSF, bZIP, and MYC/MYB activate stress response mechanisms through gene activation to re−establish homeostasis in an attempt to protect and repair the damaged membranes and tissues (Wang *et al.,* 2003).

Photosynthesis is one of the most highly regulated metabolic process in plants but also significantly affected by changes in the ambient environmental conditions (Takahashi and Badger, 2010). Using their leaves, plants convert light energy into chemical energy in a two stepwise reaction. The first step of these two processes is the light−dependent capture of the light energy and the subsequent electron transport chain reactions whereas the second is the light−independent carbon fixation also known as the Calvin Benson cycle. The primary reactions of photosynthesis are mediated by three protein complexes embedded in the thylakoid membranes of chloroplasts namely; PSII, the cytochrome $b_6$f complex (Cyt$b_6$f), and PSI, which are connected in series through the photosynthetic electron transport chain (Rochaix, 2011). These photosynthetic complexes consist of numerous chloroplast and nucleus−encoded subunits pigments such as chlorophylls and xanthophylls as well as hemes, quinones, and iron−sulphur (Fe−S) centres that act as redox cofactors (Nevo *et al.,* 2012). Hence the biogenesis of the photosynthetic apparatus involves a concerted interplay between the chloroplast and nucleocytosolic genetic systems as well as a tight coordination between protein and pigment (chlorophylls and carotenoids) synthesis and insertion into the thylakoid membranes (Barkan and Goldschmidt−Clermont, 2000; Eberhard *et al.,* 2008; Tripathy and Pattanayak, 2012; Nisar *et al.,* 2015).

Excess energy resulting from high light intensities damages the photosynthetic machinery especially the PSII and causes photoinhibition which result into limited photosynthetic activity,

growth and productivity. Photoinhibition characterized by a decline in the photosynthetic capacity occurs when oxygenic organisms are exposed to prolonged illumination with high light intensities which cause functional impairment of PSII, electron transport and structural damage of the PSII reaction center (RC) D1 protein (Yruela, 1996). The extent of such a damage depends on the balance between the rate of photodamage and its repair. Several studies have shown that light absorption by PSII causes primary damage whereas excess light absorbed by LHCs inhibits the PSII repair process mainly through generation of reactive oxygen species, ROS (Takahashi and Badger, 2010). Therefore, it can be suggested that PSII photodamage and inhibition of repair can be alleviated by photoprotection mechanisms including avoidance of light absorption by photosynthetic pigments and through consuming or dissipating of excess excitation energy (Jahns and Holzwarth, 2012).

Coffee plants have evolved to protect their leaves from damages induced by excess light intensity by employing several protection mechanisms. Such mechanisms range from behavioral, anatomical or physiological adaptations intended to halt photoinhibition (Asada, 1999; Logan et al., 2006; Partelli et al., 2010; Gill and Tuteja, 2010; Foyer, 2018). Under conditions of excess light, production of different types of reactive oxygen species (ROS) is accelerated at PSI and PSII in chloroplasts. In PSI, electron transfer to oxygen causes production of hydrogen peroxide ($H_2O_2$) via the superoxide anion radical ($O_2^{\bullet-}$), whereas in PSII, the excitation of oxygen by triplet excited state chlorophyll ($^3$Chl*) causes production of singlet state ($^1O_2$) oxygen (Krieger−Liszkay, 2004). To avoid oxidative stress, chloroplasts scavenge ROS effectively using multiple enzymes such as superoxide dismutase, ascorbate peroxidase and peroxiredoxin as well as nonenzymatic antioxidants compounds. The latter includes both the hydrophilic and substances such as water−soluble ascorbate and lipophilic substances such as membrane−bound α−tocopherol and carotenoids, such as zeaxanthin, neoxanthin and lutein in chloroplasts (Havaux *et al.,* 2005; Li *et al.,* 2012). ROS are highly reactive and therefore accelerate photoinhibition through direct oxidative damage to PSII and also through inhibition of the repair of photodamaged PSII rather than participating in direct damage processes (Nishiyama *et al.,* 2001; Murata *et al.,* 2007; Foyer*,* 2018).

To maintain growth and productivity, adaptation to excessive ROS is paramount. Therefore, coffee plants also respond to abiotic stress by stimulating a series of complex cellular signalling and responses that involve various genes and biochemical−molecular mechanisms (Ramalho et al., 2018). These include; production of stress proteins, up−regulation of antioxidant

compounds and accumulation of compatible solutes (Cushman and Bohnert, 2000). Inadequate response at one or several steps in the signalling and gene activation may result in irreversible changes of cellular homeostasis and in the destruction of functional and structural proteins and membranes leading to cell death. Adaptation to stress subsequently leads to activation of detoxification genes that control the production of antioxidants that scavenge the toxic compounds (Mittler, 2002). Coffee plants regulate ROS and RNS using both enzymatic and nonenzymatic antioxidant systems (Ashihara, 2006; Ramalho et al., 2018). These components prevent or delay the oxidation of oxidizable molecules by scavenging free radicals which would otherwise trigger a series of detrimental chemical reactions within the cells. Antioxidant compounds are utilised by the plants defence systems to neutralize the ROS and other reactive radicals. Antioxidants are substances that even when present in low concentrations significantly delay or reduce oxidation of cell contents (Gupta and Sharma, 2006). A variety of antioxidants exist in many plants with different composition and therefore different chemical and physical properties. This gives antioxidants different mechanisms as well as different sites of action.

There are various enzymatic antioxidants compounds in the plant kingdom of which some are limited to a few species whereas others are ubiquitous in distribution (Pratt, 1972). In addition to other peptides and/or protein hydrolysates that protect lipids against oxidation possibly due to their metal complexing ability (Lim and Shipe, 1972), isolation of antioxidant enzymes in coffee plants has been investigated especially in vitro using cultured cells. The enzymatic antioxidant system is comprised of three major primary intracellular antioxidant enzymes in the cells. Superoxide dismutases (SOD) catalyses the conversion of the superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide ($H_2O_2$). Catalase (CAT) and peroxidase (GPX) subsequently convert $H_2O_2$ into water ($H_2O$). GPx requires several secondary enzymes such as glutathione reductase (GR) and glucose−6−phosphate dehydrogenase (G−6−PDH), and cofactors such as glutathione (GSH), NADPH and glucose 6−phosphate to function at high efficiency. If GR is inhibited, cells cannot remove $H_2O_2$ through the glutathione peroxidase system and the levels of glutathione disulphide (GSSG) increase. If glutathione synthesis is inhibited, either by inhibiting glutathione synthetase (GS) or by γ−glutamyl cysteine synthetase (γ−GCS), glutathione will be depleted and GPx will not be able to remove $H_2O_2$. If catalase is inhibited, cells will not be able remove $H_2O_2$. Finally, glucose uptake will be inhibited creating a chemically induced state of glucose deprivation and also inhibition of $H_2O_2$ detoxification.

A study on the immediate antioxidant response of *C. arabica* L. cells as reported by Gomes−junior *et al.* (2006a; 2006b) when exposed to heavy metal stress revealed the existence of superoxide dismutases (SOD). Consequently, SOD activity staining following procedures described by Beauchamp and Fridovich (1971) and Azevedo *et al.* (1998), indicated the existence of several isoenzymes of SODs in the cells including 6 Mn−SODs and 3Fe−SODs of which 2 major Mn−SODs exhibited an increase in response to heavy contamination. According to Larson (1988), SODs catalyse the conversion of the radical anion of oxygen ($O_2^-$) to less reactive $H_2O_2$ and oxygen. Catalase (CAT), a known Metalloenzyme was also observed in coffee cells. Subsequent staining revealed the existence of only one isoenzyme in the coffee cell suspension cultures (Gomes−junior *et al.,* 2006b). Catalase is one the most known protein catalysts that promotes the redox of $H_2O_2$ to water and oxygen (Larson, 1987). Cells of coffee plants also contain ascorbate peroxidase (APX) which catalyses the conversion of $H_2O_2$ to water in the ascorbate−glutathione cycle using ascorbate as a specific electron donor (Asada, 1992; Shigeoka *et al.,* 2002; Gomes−junior *et al.,* 2006b; Caverzan *et al.,* 2012).

The nonenzymatic antioxidant system is composed of several compounds with inherent ability to scavenge ROS and RNS. Studies have indicated that under oxidative stress conditions, the content of such compounds increase as a tolerance mechanism against oxidative stresses (Das and Roychoudhury, 2014). Recent studies have indicated that this system is of tremendous important in the young leaves of coffee plants whose enzymatic antioxidant system is underdeveloped (Campa *et al.,* 2017; Ramalho et al., 2018). In addition, due to a repressed enzymatic antioxidant system during cold stress conditions, the reliance of coffee plants on the nonenzymatic antioxidant system for ROS scavenging has been demonstrated (Ramalho *et al.,* 2018). This system comprises of several classes of compounds as shown here.

Coffee plants accumulate high amounts of phenolic compounds, commonest of which are the hydroxycinnamic acid esters also known as chlorogenic acids (Bertrand *et al.,* 2003; Mondolot *et al.,* 2006). Other phenolic compounds such as flavonoids, flavonols, xanthonoids and mozambioside have also been identified in some species of in the genus *Coffea.* Chlorogenic acids (CGAs.s) are products of phenylpropanoid metabolism whose conjugates are involved in the biosynthesis of lignin and plant defence mechanisms against scavenging molecules (Harbone, 1980). These polyphenolic compounds are composed of two or more monocyclic aromatic units linked by an ester bond. They are limited only to esters of quinic acid with caffeic acid, namely caffeoylquinic acids (CQA) and dicaffeoylquinic acid (DiCQA). However,

to a lesser extent they include other hydroxycinnamoyl conjugates such as ferulic or *p*−coumaric acid derivatives (Clifford and Jarvis, 1988; Clifford *et al.,* 2008). Chlorogenic acids are abundant in many plants including all species of the *Coffea* genus though in differing concentrations. Ky *et al.,* (2001) observed high values of CGAs.s. in seeds of *C. canephora* 11.3% dry matter basis (dmb) compared to 4.1% dmb in *C. arabica* L. This could have been caused existence of *C. canephora* in more abiotically stressed environments and therefore evolution of high amounts of these antioxidant compounds as a mechanism of adaptation. On degradation at high temperatures during coffee processing, CGA converts to phenol derivatives which gives a characteristic bitter taste. Recent studies have therefore concentrated on identification of low CGA containing varieties and even species to provide a genetic base for genetic breeding for the desired qualities. (Ky *et al.,* 2000; Bertrand *et al.,* 2003).

Mangiferin is another phenolic compound also known as C−glucosylxanthone that was initially isolated from *C. pseudozanguebariae* (Talamond *et al.,* 2008). Mangiferin is an antioxidant compound that was fast isolated from *Mangifera indica* L. (Barreto *et al.,* 2008). Franklin *et al.* (2009) reported that mangiferin provides antimicrobial protection against biotic stress. Besides isolation from other *Coffea* spp. (Talamond *et al.,* 2008; Campa *et al.,* 2012), little is known about the role of this xanthonoid compounds towards adaptation to abiotic stress conditions despite suggestions that it might be involved in protection against UV stress. This is owed to the fact that, in *Coffea* spp. mangiferin mainly accumulates in those naturally occurring in high altitude areas and absent in their low−lying habitats.

Coffee contains high amounts of flavonoids due to their role is ROS scavenging (Mazur *et al.,* 1997). Flavonoids are classified into nine major subgroups; flavanols, flavonols, flavanones, anthocyanidins, flavones, isoflavones, chalcones, dihydrochalcone and dihydroflavonols (Ferreyra *et al.,* 2012) and a minor group aurones has been observed in some plants (Winkel−Shirley, 2001; 2006). Coupling of anthocyanidins with sugars at different places results into formation of anthocyanins which play a vital role in adaptation to cold stress conditions during winter conditions in some plant species (Zhang *et al.,* 2019). During plant growth, flavonoids accumulate is leaves, flower tissues including pollen and woody parts of the plant. Exposure of plants to supplemental levels of UV stress significantly increased the content of flavonoids (Anderson and Kasperbauer, 1971) where they have been implicated in protection against oxidation of ascorbic acid. Amongst the flavones, quercetin, kaemferol, myricetin, robetin, rutin and morin were suggested to have high antioxidant activity by inhibiting *in vitro* $O_2^{\bullet-}$ promoted redox reactions within the chloroplasts (Takahama, 1983;

Teramura, 1983). Nevertheless, their role in adaptation to both abiotic and biotic stress in whole coffee plants remains unstudied.

Coffee contains mainly two kinds of alkaloids synthesized from nucleotides. The first group, purine alkaloids comprises of caffeine (1, 3, 7−N−trimethylxanthinne) and theobromine (3, 7−N−dimethylxanthine). The second group, pyridine alkaloid is made up of 1−N−methylnicontinc acid also known trigonelline (Ashihara, 2006). Caffeine is the most common purine alkaloid in majority of the plants including most coffee species, however according to Ashihara and Suzuki (2004), some species such as *Theobroma cacao* L. and special Chinese tea plant possess theobromine or methyluric acid as the main purine alkaloid. The caffeine content within the *Coffea* genus varies greatly. In the coffee beans, most cultivars of *C. arabica* L. contain about 1% caffeine per dry weight whereas *C. canephora* contains up to 2% (Mazzafera and Carvalho, 1991). Other non−commercial species of coffee also contain relatively low caffeine content, *C. salvatrix* (0.7% dmb), *C. eugenioides* (0.4% dmb) and *C. racemosa* (0.8% dmb) (Mazzafera and Carvalho, 1991). *C. pseudozanguebariae* has been identified as a non−caffeine containing coffee species reported (Anthony, 1993; Ky *et al.,* 1999). Caffeine is distributed within all parts of the coffee plant including coffee seeds, pericarp and leaves (Zheng and Ashihara, 2004). Aerts and Bauman (1994) speculated that caffeine is deposited in vacuoles of coffee leaves as chlorogenic acid complexes after its biosynthesis. Caffeine is synthesized from xanthosine a purine nucleotide derivative (Ashihara, 2006). Xanthosine acts as a substrate for the methyl group donated by S−adenosyl−1−methionine (SAM). Using tracer experiments with labelled precursors and leaf discs from coffee plants, Ashihara (2006) showed a possible major synthesis route to caffeine from xanthosine, 7−methylxanthosine, 7−methylxanthine, theobromine and finally caffeine in this corresponding order. According to Fujimori and Ashihara (1994), caffeine biosynthesis occurs in young leaves and in fact biosynthetic activity from adenine in fully developed leaves was not detected. However according to Ashihara *et al.* (1996) even though biosynthesis of caffeine form guanine and adenosine was found in only young leaves, conversion to caffeine from theobromine was still present in mature and aged coffee leaves. Caffeine biosynthesis was also reported to occur in coffee fruits mainly during the immature green stage of coffee development particularly in the pericarps and seeds (Koshiro *et al.,* 2006) although expression of caffeine synthase gene was found in all stages of coffee fruit development up to the pre−maturation. Because of its bitter unpalatable taste, Frischknecht *et al.* (1986) proposed that the synthesis of caffeine in buds and young leaves is intended to prevent predation by animals.

Caffeine as an antioxidant compound after consumption has been a centre of controversy. Several studies have indicated negative effects of over consumption of caffeine. Nevertheless Yashin *et al.* (2013) discussed positive antiradical and antioxidant properties of coffee components including caffeine that help in adaptation to stress by scavenging ROI. While caffeine content is expected to be increased under stress conditions due to its antioxidant capacity, Kumar *et al.* (2015b) observed low caffeine contents of *C. canephora* plants that were subjected to both saline and drought stress.

Like caffeine, trigonelline is widely distributed within the plant kingdom and some animal species including sea urchins and jelly fish (Scheline, 2017). In coffee seeds according to Mazzafera (1991), the concentration of trigonelline varies within the genus as well as within the varieties of a given species. *C. arabica* L. varieties have been reported to have the highest concentration of trigonelline up to 2% of their seed dry weight. (Clifford, 2012; Mazzafera, 1991). Trigonelline is synthesized from nicotinic acid which is produced as a degradation product of nicotinamide adenine dinucleotide (NAD) (Zeng and Ashihara, 2004) and the synthesis has been reported to occur in all parts of coffee seedlings. During biosynthesis, the nicotinamide and the nicotinic acid formed by NAD degradation pathways are re−utilized for NAD synthesis. In fact according to Zheng *et al.* (2004), in addition to the *de novo* synthesis, NAD is also synthesized in the six membered pyridine nucleotide cycle (PNCVI), in corresponding order: nicotinamide adenine dinucleotide (NAD), nicotinamide mononucleotide (NMN), nicotinamide, nicotinic acid, nicotinic acid mononucleotide [NaMN], nicotinic acid adenine dinucleotide (NaAD) and finally nicotinamide adenine dinucleotide (NAD) which is utilised in the trigonelline biosynthesis. Other minor pathways for NAD synthesis have been suggested. Trigonelline is synthesized by S−adenosyl−L−methionine (SAM) dependent nicotinate N−methyltransferase mostly in developing leaves where both the de novo and the pyridine nucleotide cycle may both be active (Zheng and Ashihara, 2004; Zheng *et al.,* 2004). Little biosynthetic activity has been reported in reported in mature senescent leaves due to a fall in pyridine nucleotide cycle activity while on the other hand high biosynthetic activity of trigonelline has been reported in the pericarp with less activity in the seeds. As a result, Zheng *et al.* (2004) and Koshiro *et al.* (2006) suggested that, the trigonelline which accumulates in the coffee fruits during growth is transported to the seeds. It has been suggested that the nicotinic acid formed from NAD via nicotinamide may be preferentially utilised for NAD formation and only the reminder converted to trigonelline.

These plant pigments mainly act as photoreceptive antenna pigments for photosynthesis whose principal role is to gather wavelength of light that are not absorbed by chlorophyll (Larson, 1987). Several studies have indicated a protective function of *β*−carotene against oxidative damage. By growing some coffee seedlings in the shade and the others under full sunny conditions, Pompelli *et al.* (2010) observed an increased carotenoid content in the leaves of *C. arabica* L. that was subjected to full sunlight. In addition, full sunlight conditions led to qualitative changes in the carotenoid composition by increasing both antheraxanthin and zeaxanthin pools on a chlorophyll basis in what is increasingly known as de−epoxidation of the xanthophyll cycle. Furthermore, Ledford and Niyogi (2005) reported in addition to light collection for photosynthesis, carotenoids protected the photosystem against photo−oxidation by reactive oxygen species produced by excited triplet state of chlorophyll during photosynthesis.

Carotenoids play a more comprehensive role in protection of the photosystems (Josse *et al.,* 2000). Therefore, any block in carotenoid biosynthesis induced either by mutation or by herbicide may result into severe photobleaching thus tremendously decreasing the photosynthetic ability of the plants (Sandmann *et al.,* 2006). A number of studies have therefore focused on the biosynthesis of carotenoids and their role in adaptation to changing environment light conditions (Sandmann *et al.,* 2006; Simkin *et al.,* 2008). These biosynthetic processes have largely been found to be dependent of the nitrogen availability. In coffee plants, Carelli *et al.* (2006) discussed the relationship between nitrogen uptake and photosynthetic rates of coffee plants under different light conditions. In their review, it was concluded that besides light, nitrogen was a major limiting factor for photosynthesis and therefore plant metabolism due its effect on the synthesis of photosynthetic pigments (Hikosaka and Terashima, 1995; Ramalho *et al.,* 1998; 1999; 2000).

Plants contain several vitamins such as E, C and A which are natural nonenzymatic antioxidant compounds (Moussa *et al.,* 2019). In coffee plants, the tocopherol content of green coffee beans was first determined by Gonzalez *et al.* (2001) using reversed phase high chromatography resolution liquid to differentiate coffee varieties and species by using tocopherols as a biochemical indicator. Tocopherols are naturally occurring compounds with vitamin E activity. They consist of a group of closely related phenolic benzochroman derivatives with an extensive alkylation (Larson, 1988). Amongst the four major tocopherols, α−tocopherol is one of the most in vitro chain breaking antioxidants tested (Burton *et al.,* 1983). Vitamin C (AsA) is another organic compound with redox buffer functions. In addition to neutralising ROS, AsA

is cofactor for enzymes involved in regulating photosynthesis, hormone biosynthesis as well as regeneration of other antioxidants (Pehlivan, 2017). AsA is one of the potent reducing agents and scavenges free oxidizing radicals and harmful oxygen−derived species such as hydroxyl ($OH^{\bullet-}$) radical, $H_2O_2$ and $^1O_2$ (Das and Roychoudhury, 2014).

Glutathione (GSH) is one of the most ubiquitous nonenzymatic antioxidant compounds in the plants and animal cells. Its staining has revealed the presence of four isoenzymes whose activity increases with exposure to oxidative stress conditions (Gomes−junior *et al*., 2006b). Glutathione is a common tripeptide in cells that reacts with a range of oxidants such as $H_2O_2$ to form the oxidized form disulphide (GSSG) (Larson, 1987). GSH is involved in the first line of defence against ROS by utilizing its inherent thiol group which protects proteins against oxidative damage (Moussa *et al.,* 2019). The thiol group complexes the ROS and in so doing, its oxidized to the sulfonic acids. GSH has been reported to scavenge ROS during both enzymatic and nonenzymatic reactions and also functions to regenerate other oxidized antioxidants such as vitamins C and E (Chen *et al.,* 2003). Other compounds have also been suggested to have high antioxidant activity. These include minerals such as selenium, copper, manganese, zinc and chromium amongst others (Shirwaikar *et al.,* 2004) These inorganic minerals, together with the known metalloenzymatic compounds were categorised as the first line of defence (Gupta and Sharma, 2006) in response to environmental stress. Organic compounds such as phenolic compounds, carotenoids, and tocopherols make up the second defence line whereas more complex group of enzymes such as DNA repair enzymes, transferase, methionine reductase and/or phytohormones such as salicylic acid, melatonin were categorised as the third line of defence against oxidative stress (Irshad and Chaudhuri, 2002).

In plants and coffee in particular, studies focusing on the characterisation of the enzymatic antioxidant systems are largely available. On the other hand, despite the existing knowledge on presence of high amounts of phytochemicals in some coffee plant organs and their potent antioxidant activities, a study profiling accumulation of such compounds in whole coffee plants compared to that in green and roasted beans is still unavailable.

Moreover, the level of accumulation of these protective molecules is highly dependent on the ambient environmental conditions such as light and temperature which may vary daily or over a long-term. Light is a survival quotient for all photosynthetic plants including coffee for it regulates several biochemical metabolic processes in plants (Johnson, 2003; Moraes, 2019). Coffee is a low-light adapted specie, having evolved in the understorey habitats of tropical

Africa and therefore grows best under moderate light and temperature conditions (Charrier and Berthaud, 1985). Cultivation under direct sunlight has on the other hand been associated with increased yield over a short period of time (DaMatta, 2004). Direct sunlight is associated with rapid fluctuations in temperature that weakens the coffee plant thereby predisposing the plants to diseases (Teketay, 1999). In addition, exposure of the plants to high irradiance increases the intercepted light energy that surpasses the requirements of the photosynthetic machinery resulting in photo−inhibition of photosynthesis and sometimes photooxidation of the photosynthetic pigments associated with the breakdown of lipid membranes (Adams *et al.,* 2013). If not remedied early, this might lead to death of the vegetative plant parts and whole trees.

Absorption of excess light by the LHC results into a singlet−state excitation of Chl *a* molecule which can return to the ground state via one of several pathways (Muller *et al.* 2001). This excitation energy can either be re−emitted as Chl fluorescence, transferred to reaction centres and used to drive photochemistry and/or be de−excited by thermal dissipation processes (NPQ), or it can decay via the triplet state. Excess excitation energy resulting from high light energy leads to production of ROS that oxidizes the pigments of the photosynthetic machinery (Tikkanen *et al.,* 2012). Despite the fact that coffee is an understorey species, coffee trees are reported to have enough plasticity to be cultivated in both direct sunlight and shaded environments (DaMatta, 2004). These environments are characterised with a combination of low night temperatures and high diurnal solar radiation depending on the season. Stress conditions resulting from such environments are associated with creation of excess excitation energy in the photochemical apparatus (Chaves *et al.,* 2008) that subsequently cause marked decreases in vegetative growth and photosynthetic rates and therefore induce several biochemical adaptation mechanisms (Barros *et al.,* 1997; DaMatta *et al.,* 1999, Silva *et al.,* 2004; Partelli *et al.,* 2009; Pompelli *et al.,* 2010).

In addition, *C. arabica* L. prefers a climate characterized by high altitude with temperatures ranging between 18˚C and 24˚C and with contrasting seasons. According to Teketay (1999), high temperatures above optimum force rapid growth characterised by early bearing, overbearing, early exhaustion, dieback and susceptibility to diseases. High temperatures also cause flower shedding, reduce fruit formation, accelerate development and ripening of fruits which ultimately cause loss of physical beverage quality. Cold temperatures on the other hand, result in slow stunted and uneconomic growth, high production of secondary and tertiary

branches as well as high incidences of 'hot and cold' disease (Barros *et al.,* 1997; Teketay, 1999. All coffee species cannot withstand frost although *C. arabica* L. is more resistant to cold than *C. canephora*. *Coffea* species therefore low tolerance to extreme temperature conditions. This is attributed to the limitation in functioning of antioxidant systems provoked particularly by decreased respiratory activity associated with temperatures extremes (Queiroz *et al.,* 1998; Chaves *et al.,* 2008; Ramalho *et al.,* 2014). Nevertheless, in addition to long−term seasonal acclimatory responses, the role of antioxidant compounds in adaptation to circadian rhythm changes and/or to high light intensity remain un–investigated.

The failure of antioxidative enzymes to protect lipid membranes culminates into rapid peroxidation of membrane lipids ultimately causing tissue damage and membrane rigidity. However, some coffee species and/or genotypes are able to withstand low positive temperatures without significant impacts on the cell membrane. Icatau a hybrid of *C. arabica* L. and *C. canephora* and Catuai a popular cold resistant variety of *C. arabica* L. enhance the synthesis of some classes of lipids and unsaturation of the membrane as a measure to survive cold stress (Campos *et al.,* 2003). These changes in lipid synthesis have been associated with not only cold stress but with also other conditions that promote oxidative stress such exposure to high irradiance and drought (Gigon *et al.,* 2004; Ramalho *et al.,* 1998). Coffee plants also adapt to cold stress by increasing the FA saturation as well as decreasing the ratio of galactolipids monogalactosyldiacylgycerol (MGDG) to digalactosyldiacylglyerol (DGDG) as a result preferential DGDG synthesis ( Ramalho *et al.,* 1998).In addition, coffee especially *C. arabica* L. contains high amounts of phenolic and alkaloids compounds such as chlorogenic acids, mangiferin, trigonelline, caffeine whose role in adapting to abiotic stress conditions is becoming of interest recently (Campa *et al.,* 2017; Ramalho *et al.,* 2018).

Given the effect of oxidative stress on growth and productivity of plants, their mitigation has been the main focus of research for many generations (Sharma *et al.,* 2012). In plants, oxidative stresses are induced by over−production an accumulation of ROS by NADPH oxidases and peroxidases in the chloroplast and mitochondria among other organelles due to presence of several electron chain reactions (Shapiguzov *et al.,* 2012; Gupta and Sharma, 2015; Kohli *et al.,* 2019). In addition to abiotic or biotic stress factors, over−production and accumulation of ROS is enhanced by either an inefficient photosynthetic apparatus or diminished mechanism of ROS scavenging respectively. Oxidative stresses can therefore be mitigated through maintenance of an efficient photosynthetic system as well as an efficient ROS scavenging mechanism.

Nitrogen plays an important role in the metabolism of both chlorophylls and carotenoids and is particularly the most limiting factor in cultivation of many crops including coffee, due to high rates of nitrogen mining at harvesting (Wrigley, 1988). Inorganic nitrogen is always highly abundant in agricultural soils to a concentration as high as 20 mol $m^{-3}$ mainly in form of nitrates as compared to ammonium form (Andrews, 1986). Plants absorb nitrogen mainly in of nitrates. In fact, Vaast *et al.* (1998) reported significant reduction in uptake of ammonium form of nitrogen in presence of nitrate when coffee seedlings were fed on the two forms in hydroponic solution. Nevertheless, within the plants the nitrates undergo reduction by NR reductase to ammonium before being incorporated into amino acids and subsequent organic molecules (Hewitt and Cutting, 1977). The significance of Nitrogen metabolism and its role in *C. arabica* L. physiology has been a subject of tremendous research (Carelli *et al.,* 2006; Bote *et al.,* 2018). Several reports indicating the plasticity of coffee to different light regimes have indicated that this high adaptability of coffee is dependent on the change in the content and/or composition of the photosynthetic apparatus a process directly related to nitrogen partitioning (Long *et al.,* 1994; Ramalho, 1997; 1999; 2000). A close association between light−saturated photosynthetic rate and nitrogen contents of leaves due to allocation of high amounts nitrogen to photosynthetic elements has been reported (Evans, 1989). On the other hand, nitrogen deficiency would cause a reduction in the cellular concentration of pigments, photosynthetic units and enzymes hence causing a decline in carbon assimilation hence increasing susceptibility to photo−inhibition (Sukenik *et al.,* 1987).

Moreover, nitrogen deficiency amplifies oxidative stress resulting from even mild deflections from the normal environmental conditions (Verhoeven *et al.,* 1997; Carelli *et al.,* 2006). *C. arabica* L. can sustain high growth rates in poor environmental conditions so long as the nitrogen content of the growing media is high (Ramalho *et al.,* 1998). This is due to the photo−protective mechanisms (Ramalho *et al.,* 1999; 2000), osmotic adjustments and an increase in long−term water use efficiency (DaMatta *et al.,* 2002) that are triggered by high Nitrogen levels. In fact, similar results have been observed in many plants (Bennet *et al.,* 1986; Lawlor *et al.,* 1987; Osmond, 1987). Young (1.5 years old) plants of *C. arabica* L. that were fed on varying rates of nitrate were subjected to high irradiance for a short period of time before re−establishment of the normal conditions. Those with high Nitrogen availability were able to recover from the shock and this was associated with higher contents of zeaxanthin, lutein and β−carotene (Ramalho *et al.,* 1997). Furthermore, several enzymatic antioxidants well known for quenching ROS were all reinforced by nitrogen nutrition (Ramalho *et al.,* 1998).

Nevertheless, the effect of foliar nitrogen supply on the recovery of the photosynthetic performance and its concomitant effects on the nonenzymatic antioxidant system of coffee plants remains to be investigated.

On the other hand, exogenous foliar application of several substances has recently been explored as a possible means of mitigating as well as conferring tolerance to abiotic stress conditions (Kul *et al.,* 2019; Singh *et al.,* 2019; Sun *et al.,* 2012; Comotto *et al.,* 2014; Farooqui *et al.,* 2016). Substances such as phytohormones and nanoparticles have been used to reinforce the antioxidant system in plants under environmental stress.

Kinetin ($N^6$−furfuryladenine) was first isolated from autoclaved herring sperm DNA (Miller *et al.,* 1956). This compound has recently received tremendous attention for its oxidative stress mitigation and growth promotion effects (Ahanger *et al.,* 2018; Kaya et al., 2018; Singh *et al.,* 2019). Moreover, due to its abundance amongst the cytokinins, its commercial applications particularly in agricultural science has been implored (Hamayun *et al.,* 2015). Exogenous application of kinetin has been reported to increase growth rate, yield and crop quality and tolerance to abiotic stress conditions such as salinity, heavy metal stress, and drought among others in several crops (Sawan *et al.,* 2000; Ahanger *et al.,* 2018; Kaya *et al.,* 2018; Hamayun *et al.,* 2015; Kaya *et al.,* 2010). Although the mechanism of kinetin action in mitigating oxidative stresses is not yet clearly understood, it has been suggested that kinetin upregulates both the enzymatic and the nonenzymatic antioxidative systems hence reinforcing the ROS scavenging abilities of plants (Ahanger *et al.,* 2018). Therefore, due to the repressed enzyme activity during cold stress conditions, amplification of the nonenzymatic antioxidative system through kinetin application might offer a necessary tolerance mechanism to enhance growth of plants such as coffee. It is largely reported that exogenous kinetin activates and stimulates the biosynthesis of secondary metabolites through upregulation of the corresponding transcripts or increasing the activities of the biosynthetic enzymes in a number of plants (Hamayun *et al.,* 2015; Jalaluddin *et al.,* 2015; Weremczuk−Jeżyna *et al.,* 2018).

Salicylic acid also known as 2−hydroxybenzoic acid is a plant phenolic compound normally present in plant cells where it regulates a number of physiological processes that affect growth and development of plants (Rivas−San Vicente and Plasencia, 2011). Among the many biological process mediated by salicylic acid, its role in signalling for modulation of responses to abiotic and biotic stresses is becoming more of interest to science (Shabanian *et al.,* 2019). Increased concentration of cellular salicylic acid is normally associated with improved

tolerance or alleviation of abiotic stresses including salinity (Miura and Tada, 2014), heavy metal stress (Kovács *et al.,* 2014; Singh *et al.,* 2017; Lu *et al.,* 2018), high light stress (Wang *et al.,* 2014), low temperature (Min *et al.,* 2018; Shin *et al.,* 2018; Wang *et al.,* 2019) drought (Tang *et al.,* 2017) and pesticide toxicity (Wang and Zhang, 2017) among others in several plants.

Melatonin (N–acetyl–5–methoxy tryptamine) is a pleiotropic ubiquitous hormone with multifunctional effects in both animals and plants (Arnao and Hernández−Ruiz, 2019). Although first discovered in the bovine pineal gland as a secretory molecule (Lerner *et al.,* 1958), the subsequent discovery of melatonin in several plant tissues suggested its involvement in modulation of several physiological, metabolic and biochemical processes necessary for plant growth and development (Lerner *et al.,* 1958; Hattori *et al.,* 1995; Sharif *et al.,* 2018). Besides, its regulatory role under normal cellular functioning (Hardeland 2016; Arnao and Hernández−Ruiz, 2006), melatonin accumulation has been reported to increase as a result of exposure to abiotic stress conditions where it directly scavenges reactive oxygen species (Arnao and Hernández−Ruiz, 2013; Yu *et al.,* 2018).

The advent of nanotechnology as a novel discovery of the 21$^{st}$ century is associated with tremendous possibilities of application in various scientific disciplines including agriculture (Siddiqui *et al.,* 2015). Nanotechnology refers to the manipulation of matter on nanoscale resulting into substances generally known as nanomaterials with at least a dimension of 5 to 100 nm in diameter. In addition to their inadvertent effects in the environment, nanomaterials are recently being applied in form of nano−fertilizers, pesticides as well as abiotic stress protectants also known as nanoagrochemicals. Use of these nanomaterials is normally intended for improved fertilizer use efficiency, molecular management of diseases or pests and enhanced tolerance against abiotic stress conditions (Farooqui *et al., 2016;* Jampílek and Kráľová 2018; Hayles *et al.,* 2017; Kah *et al.,* 2018). Nanomaterials facilitate crop growth and development by improving germination, root growth and improvement in several physiological and metabolism aspects of plants under optimum and during abiotic stress conditions (Yasmeen *et al.,* 2015; Wang *et al.,* 2016; Liu *et al.,* 2016; Marslin *et al.,* 2017).

Although, several studies have indicated that exogenous application of these compounds upregulate the activity of the enzymatic antioxidant system with concomitant improvements of photosynthetic physiology in several plants during abiotic stress conditions, their evaluations

in reinforcement of the nonenzymatic antioxidant system and photosynthetic performance of coffee plants during cold stress has not been done.

Despite having been known to damage cellular components during abiotic stress conditions, the ROS in triggering acclimation response is gaining attention recently (Demidchick, 2015). ROS play a key role in the acclimation process of plants to several abiotic stresses through their role in activation of several downstream metabolic pathways as well influencing the expression of a number of genes that may affect antioxidant response (Das and Roychoudhury, 2014). Amongst the ROS, $H_2O_2$ has emerged as the major redox metabolite operative in redox sensing, signaling and redox regulation (Sies, 2017). It has been suggested that $H_2O_2$ together with $Ca^{2+}$ and ATP are at the forefront of transcription−independent signal molecules (Sies, 2014). Produced from the superoxide radical by activities of SOD in the chloroplasts or mitochondria and other organelles (Alscher *et al.,* 2002; Fahnenstich, 2008; Pilon and Tapken, 2010), $H_2O_2$ acts as a messenger molecule and diffuses through cells and tissues to initiate immediate cellular effects, such as cell shape changes, initiation of proliferation and recruitment of immune cells (Sies, 2015; 2017). It is increasingly becoming clear that $H_2O_2$ serves fundamental regulatory functions in metabolism beyond the role as damage signal (Smirnoff and Arnaud, 2019). Particularly, $H_2O_2$ serves as a key molecule in the Third Principle of the Redox Code, which is: "Redox sensing through activation/deactivation cycles of $H_2O_2$ production linked to the NAD and NADP systems to support spatiotemporal organization of key processes" (Jones and Sies, 2015; Mittler, 2017).

Plants cells mediate ROS homeostasis and redox signalling to enhance their proliferation and survival under abiotic stress conditions (Huang *et al.,* 2019). Mitigation of oxidative stresses through exogenous application of several substances relies on priming by ROS application in order to stimulate the antioxidant system in plant cells (Kerchev *et al.,* 2020). Increased $H_2O_2$ content resulting from priming interacts with Cys thiolate anions (Cys–S) at physiological pH and oxidizes them to their sulfenic form (Cys–SOH), causing structural changes within the target protein and hence altering its function. These redox−derived changes in protein function can affect transcription, phosphorylation, and other important signalling events, and/or alter metabolic fluxes and reactions in the cell by altering enzymatic properties that might contribute to enhanced ROS scavenging capacity (Mittler, 2017).

Despite increasing knowledge that the leaves of coffee plants rely on the nonenzymatic antioxidant system especially during cold−induced oxidative stress, no studies have attempted

to profile, reinforce and also determine the role of ROS or RBOH in modulation of the secondary metabolites that also make up the nonenzymatic antioxidant system of coffee plants.

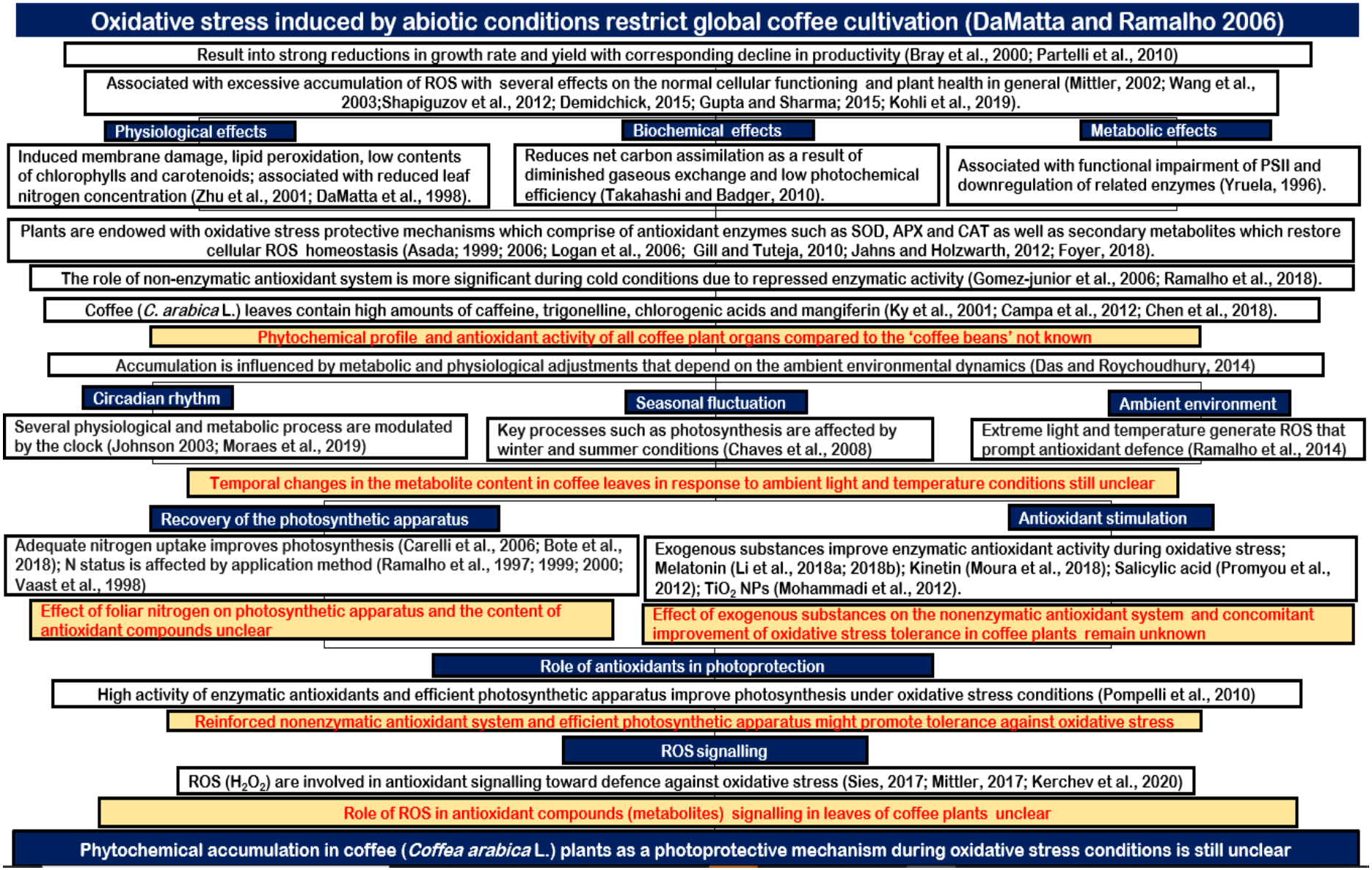


Fig. 1. Literature review flow chart

## 1.2 Overall scope

This study aimed at profiling and reinforcing the nonenzymatic antioxidant system in young coffee plants through exogenous applications of several elicitor compounds to enhance coffee cultivation under cold stress conditions.

## 1.3 Main objectives of the study

The main objectives of the research in this thesis were;

1. To determine the phytochemical profile and corresponding antioxidant activities of different coffee plant organs
2. To characterize the temporal changes in the leaf phytochemical profile of coffee in responses to fluctuations in the ambient environmental conditions.
3. To facilitate the recovery of the photosynthetic apparatus through foliar supply of nitrogen and determine the concomitant effect on the selected leaf metabolites
4. To evaluate the response of the nonenzymatic antioxidant systems and the photosynthetic physiology of coffee plants to foliar sprays containing elicitor compounds such as phytohormones and nanoparticles.
5. Understand the effect of a combination of nitrogen and phytohormone (melatonin) on the photosynthetic physiology and the nonenzymatic antioxidant system during cold stress conditions.
6. To clarify the role of ROS and RBOH in modulation of the nonenzymatic antioxidant system in coffee leaves during oxidative stress conditions.

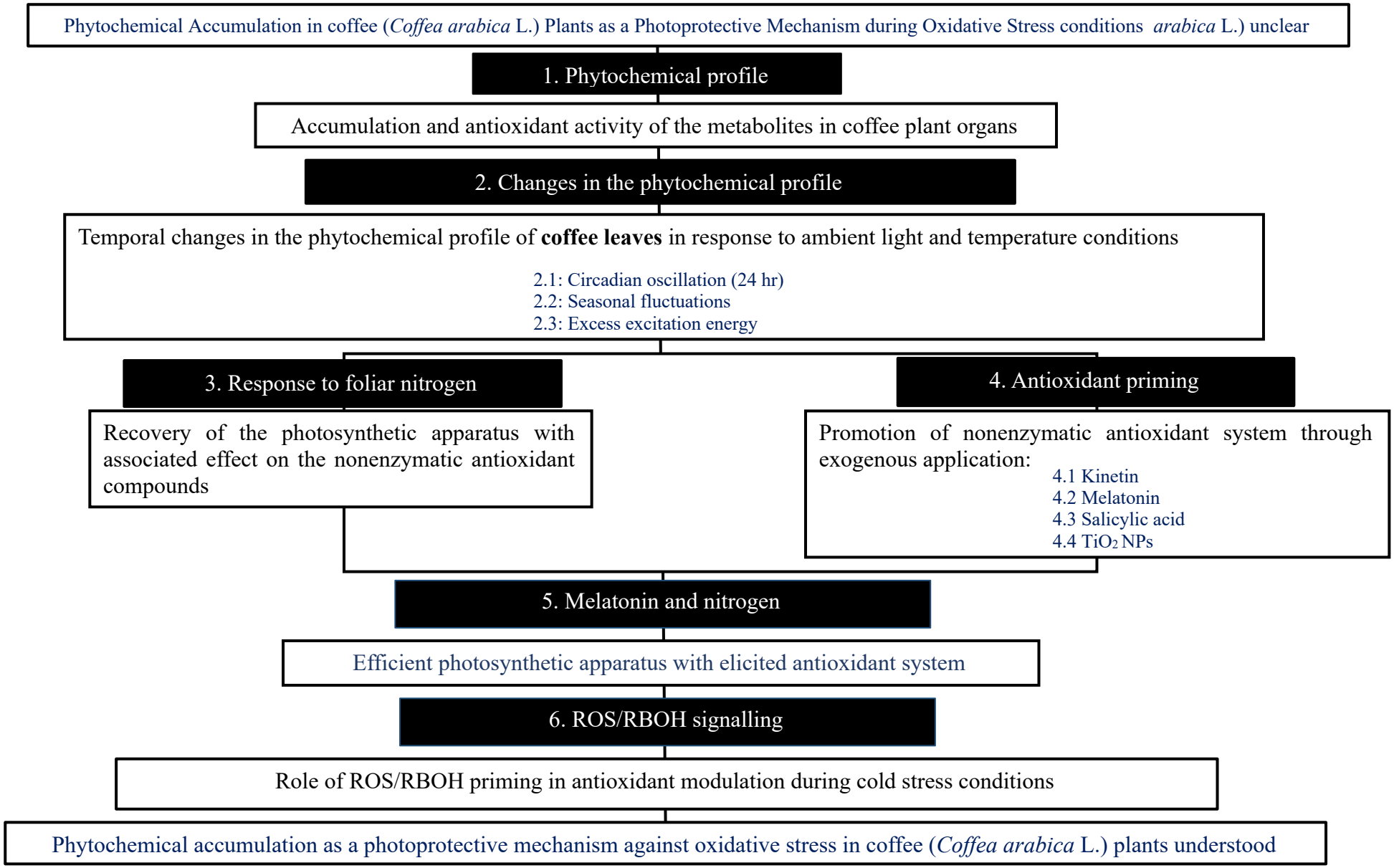


Fig. 2. Summary of the experiments flow chart

## Chapter two

## Phytochemical profile and antioxidant capacity of coffee plant organs compared to green and roasted coffee beans

### 2.1 Introduction

Coffee remains the most valuable primary product globally employing over 26 million people along the chain from cultivation mainly in developing countries to consumption in developed countries (ICO, 2019). By 2017, 160 million bags of coffee were exported, of which arabica (*C. arabica* L.) accounted for 63.3% of the total and the rest being mainly robusta (*C. canephora* Pierre ex A. Froehner). It has been estimated that the coffee industry value is over $100 billion dollars worldwide with up to $20 billion dollars involved in export alone. Coffee is mainly traded as raw or processed beans. Therefore it is not surprising to give considerable attention to the fluctuations in the phytochemical profile that occur during fruit growth and maturation (Farah and Donangelo, 2006; Koshiro *et al.*, 2006; Koshiro *et al.,* 2007; Farah *et al.,* 2008; Patay *et al.,* 2016) as well as changes induced during processing such as fermentation and roasting of the coffee beans after harvest (Pereira *et al.,* 2019).

In mature coffee beans, accumulation of sucrose, caffeine, chlorogenic acids and trigonelline has been well investigated due to the involvement of these compounds in flavour formation and the characteristic bitter taste of the coffee beverage (Poisson *et al.,* 2018; Pereira *et al.,* 2019). Sucrose and its derivatives fructose and glucose provides the carbonyl group that combines with the hydrazyl group from amino acids to form a number of odorants during caramelisation in Strecker and Maillard reactions (Poisson *et al.,* 2018). The thermal degradation of trigonelline into pyrroles and pyridine derivatives has also been reported to contribute to coffee aroma (Amrani-Hemaimi *et al.,* 1995; Stadler *et al.,* 2002). On the other hand, products of chlorogenic acids thermal degradation as well as caffeine which is barely affected by high temperatures during roasting are responsible for the characteristic bitter taste of the beverage (Fuller and Rao, 2017). Moreover, the ratio of chlorogenic acids to caffeine has also been described as an important parameter that might have an effect on taste, antioxidant potential and preference of a given type of the beverage (Ky *et al.,* 2001).

On the other hand, previous reports have suggested that coffee leaves may contain all the major metabolites present in the seeds (Patay *et al.,* 2016). In addition, they contain mangiferin, a

xanthonoid with great antioxidant potential as well as therapeutic and pharmacological properties and although present in the fleshy fruit parts, this compound is absent in the endosperm (Talamond *et al.,* 2008; Campa *et al.,* 2012; Imran *et al.,* 2017). As a result, the use of coffee leaves as a dietary source of antioxidants in form of 'coffee leaf tea' might become more common. Moreover, several processing methods like those used in tea processing have since been suggested (Chen *et al.,* 2018). Coffee leaves also contain carotenoids and chlorophyll pigments whose antioxidant value for dietary intake can be harnessed by using appropriate processing methods such as in green tea processing (Stahl and Sies, 2003; Lanfer-Marquez *et al.,* 2005; Wangcharoen and Phimphilai, 2016; Chen *et al.,* 2018). It has been demonstrated that food processing generally affects the content of the phytochemicals and the corresponding antioxidant capacities in foods (Alvarez−Jubete *et al.,* 2010). Until today, coffee beans and recently leaves are the main sources of antioxidants from coffee plants. These undergo either roasting or some form of drying before utilization. The two processes may result into significant changes in the biochemical composition and/or antioxidant capacities of the resultant beverage (Chen *et al.,* 2018; Pereira *et al.,* 2019). For example, Strecker and Maillard reaction may increase oxidant scavenging abilities due to formation of new compounds (Lindenmeier and Hofmann, 2004).

Although it is likely that other organs of the coffee plant such as roots or stems may accumulate similar phytochemicals, their utilization and or investigations has not received similar attention. In fact, only, a few reports have elucidated accumulation of some alkaloids and phenolic compounds in other organs such roots of seedlings or juvenile coffee plants (Aerts and Baumann, 1994; Zheng and Ashihara, 2004). Moreover, reports on the accumulation of the major phytochemicals in all coffee plant organs in comparison with the content in green and roasted coffee beans is still lacking. In addition, studies comparing the relationship between this phytochemical composition and the antioxidant capacities in the organs compared to the typical dietary antioxidant sources (beans and leaves) remain un–investigated whatsoever. Similarly, phytochemical changes with corresponding effects on the antioxidants capacities resulting from bean or leaves processing still remain unclear.

This study therefore aimed at assessing the phytochemical composition and antioxidant capacities of coffee plant organs such as coffee seeds, leaves, stems and roots. In addition, the changes induced by roasting and leaf senescence on the phytochemical composition and antioxidant capacities of beans and leaves respectively were investigated. Coffee is mainly

consumed as a beverage processed from coffee beans, therefore we also investigated weather other organs especially leaves might have high antioxidant activities and/or phytochemical content in relation to roasted coffee beans. Finally, relationships amongst plant organs in terms of phytochemical composition and antioxidant capacities were evaluated.

## 2.2 Materials and Methods

### 2.2.1 Seeds

Seeds of *C. arabica* were imported as dried depulped cherries (green beans) from Indonesia for commercial roasting by Sawai Coffee Limited, Tottori, Japan. From these, raw and roasted bean samples were obtained and used in the current study. Some of the raw seeds were germinated and raised into plants for further investigations.

### 2.2.2 Plant material and growing conditions

The seeds were soaked in running water for 3 days and pre−germinated on moist paper towels in an incubator at 30˚C in the dark (Gebreselassie *et al.,* 2010). After sprouting, each seedling was transplanted into a 3−litre pot containing a mixture of peat moss, perlite and humus at a ratio of 5:3:2 respectively and grown in a naturally lit vinyl–house at temperatures above 20˚C. The seedlings were irrigated, first with half strength nutrient solution for the first 6 months and then with the full−strength solution prepared according to Hoagland and Arnon (Hoagland and Arnon, 1950) with a few modifications. The nutrient solution composition included in mmol/L, 5.7 N–$NO_3^-$, 1N−$NH_4^+$, 0.1 P–$H_2PO_4^-$, 2.4 $K^+$, $Ca^{2+}$, 0.6$Mg^{2+}$, 0.7S–$SO_4^{2-}$, and in µmol/L 35 Fe III EDTA, 0.8 Cu, 1.5 Zn, 5 Mn, 17 B, and 0.1 Mo. Coffee plants were grown for one and a half years from May, 2017 until they attained 6 pairs of fully expanded leaves on the main stem.

### 2.2.3 Sample preparation

All the samples were thoroughly washed, rinsed with distilled water and immediately flash frozen in liquid nitrogen before storing under –80˚C in a freezer awaiting further processing.

#### *2.2.3.1 Green and roasted beans*

Both green seeds (GB) and roasted seeds (RB), characteristically known as green and roasted coffee beans, respectively, were obtained in triplicates and treated as independent samples.

*2.2.3.2 Leaves*

Each leaf pair was sampled independently and labelled from L1 (youngest) to L6 (oldest). In addition, to determine the phytochemical changes that occur after sun drying, naturally dried leaves corresponding to the first leaf pair were sampled from the dried plants and labelled as brown leaves (BL).

*2.2.3.3 Stems*

The stem was divided in to two parts:

i. The herbaceous stem (HS) consisting of the green parts of the main stem and the green branches.

ii. The woody stem (WS) consisting of the ligneous brown part of the stem above the root collar.

*2.2.3.4 Roots*

Roots were thoroughly cleaned off of all the soils before further processing.

**2.2.4. Extraction and analysis**

*2.2.4.1. Chlorophylls and carotenoids analysis*

Leaf pigments were extracted with 2.5 mM sodium phosphate 80% buffered acetone (pH 7.8) and quantified spectrophotometrically (Hitachi ratio beam spectrophotometer U–500, Japan). Both pigments were extracted from about 0.25 g fresh leaf samples with liquid nitrogen and centrifuged at 29300 ×g. Both chlorophylls and carotenoids were assayed according to Porra *et al.* (1989) and determined using equations (Eqn. 1) developed by Lichtenthaler and Buschmann (2001).

$$\text{Chl } a = 12.25\ A_{663.6} - 2.55\ A_{646.6} \quad \text{.....A}$$

$$\text{Chl } b = 20.31\ A_{646.6} - 4.91\ A_{663.6} \quad \text{.....B}$$

$$\text{Chl } a + b = 17.76\ A_{646.6} + 7.34\ A_{663.6} \quad \text{.....C}$$

$$\text{Car } (x + c) = \frac{1000\ A470 - 1.82\ \text{Chl a} - 85.02\ \text{Chl b}}{198} \quad \text{.....D}$$

Equation 1. Determination of photosynthetic pigments

*2.2.4.2. Sample processing for phytochemical and antioxidant capacity determination*

All the samples were freeze dried at –12ºC (Eyela DRC 1000–FDU 1110, Tokyo, Japan) to a constant moisture content for 72 hours. The dried samples were milled into a fine powder using wonder blender (Osaka chemical Co., Ltd., Osaka, Japan). All the processed samples were kept in air−tight self−seal poly bags with silica gel awaiting extraction and analysis.

*2.2.4.3. Sample extraction*

Extraction proceeded as described by Chen *et al.* (2018) with a few modifications. The sample powders were infused in 25 mL boiling water in 50 mL Falcon conical centrifuge tubes (Thermo Fisher Scientific, Massachusetts, USA) for 8 minutes. The samples were thereafter cooled under room temperature then centrifuged at 29300 ×g at 25ºC for 15 minutes. The supernatants were collected in 50 ml volumetric flasks and to the residue was added 10 mL cold water for re−extraction. For each sample, all the supernatants were pooled together, volumetric flasks filled to the mark and the extracted samples then filtered using 0.45 µm Millipore (Sigma and Aldrich, Tokyo, Japan) into 50 ml glass vials. From each sample, an aliquot of about 2 mL was obtained for HPLC phytochemical analysis. The extracted samples in the glass vials were freeze dried at –20˚C (Eyela DRC 1000–FDU 1110, Tokyo, Japan) for 7 days. Extraction yield was calculated as the difference in the weights between the sample powder and the extracted freeze−dried powder, multiplied by 100.

*2.2.4.4. Alkaloids and phenolic compounds determination*

Alkaloids and phenolic compounds were determined simultaneously using a high–performance liquid chromatography (HPLC) system equipped with a UV detector at a wavelength of 270 nm (Hitachi L–2490, Hitachi, Japan) from a 10 µL sample extract. Separation of the analytes was performed on a TSKgel ODS–100 C18 column (5 µm particles size, 4.6 x 150 mm) in a thermostatic oven at a temperature of 40˚C (Sigma–Aldrich, Tokyo, Japan) with a binary phase mobile gradient at a total flow rate of 0.4 mL $min^{-1}$. The mobile phase consisted of two filtered (0.22µm Millipore), sonicated and degassed solvents A (methanol, 100%) and B (acetic acid: $H_2O$, 98:2, v/v) with linear evolution of the gradient profile according to a set elution program (Table 1). Calibration curves were obtained from three replicate points for the standard compounds (trigonelline, 5–caffeoylquinic acid, caffeine and mangiferin). Each analyte from the samples was then identified by peak position and thereafter quantified by peak area measurement using regression equations developed from calibration curves for the standard

compounds (Eqn. 2). All the analytical standards and the organic solvents used were of HPLC grade (Sigma–Aldrich, Tokyo, Japan).

| | |
|---|---|
| Trigonelline | y = 38230x……A |
| Chlorogenic acid | y = 375x……B |
| Caffeine | y = 75067x……C |
| Mangiferin | y = 10018x……D |

Equation 2. Determination of the selected metabolites

*2.2.4.5 Sucrose extraction and determination*

Extraction was done from 0.5 g freeze dried powder in Falcon tubes using 30 mL of ultrapure water. The contents were sonicated for 30 minutes, centrifuged at 29300 ×g. Extraction was repeated 3 more times and all the supernatants pooled together. The final extract was filtered (0.22 µm) and thereafter all the solvent evaporated off using a rotary evaporator (Rotavapor R–300 Buchi, Flawil, Switzerland). The residue was re–dissolved, first in 3 mL ultrapure water and then to this solution was added 3 mL acetonitrile solvent. The new solution was re−filtered (0.22 µm) and analysed using HPLC. Sucrose content was determined using the same HPLC system described above but with an RI detector at a temperature of 35$^{o}$C and a shodex (R spak DC–613) column (5 µm, 150 mm x 4.6 µm made by Sigma–Aldrich, Tokyo, Japan) at a temperature of 55$^{o}$C. The flow liquid consisted of acetonitrile and water at 75 and 25 % (v/v) respectively. Elution was done at a flow rate of 1.0 mL min$^{-1}$ resulting into a retention time of 9.30 min for sucrose standards and the samples. Other sugars such as glucose and fructose were undetectable due to their low concentration in the samples. Sucrose content was thereafter determined from the peak area measurement of sucrose standard (Eqn. 3).

| | |
|---|---|
| Sucrose | y = 6E -06x + 0.198 |

Equation 3. Determination of sucrose

Table 1. Time−course changes in the composition of the mobile phase during linear gradient analytical HPLC.

| Time (min) | Methanol (100%) | Acetic acid (2%) |
|---|---|---|
| 0 | 15 | 85 |
| 4 | 15 | 85 |
| 10 | 35 | 65 |
| 22 | 43 | 57 |
| 24 | 60 | 40 |
| 28 | 60 | 40 |
| 30 | 15 | 85 |
| 40 | 15 | 85 |

*2.2.4.6 Total phenolic content (TPC) determination*

Total phenolic content was determined from 0.5 mL of the collected aliquots described in subsection *2.4.3* according to Chen *et al.* (2017) with slight modifications. Each sample was diluted 10–fold from which 1 mL was transferred to a new test tube. The samples and/or gallic acid standards were then incubated with 5 mL of 10% Follin–Ciocalteu's reagent for one minute and then reacted with 4 mL of 20% (w/v) sodium carbonate solution for 30 minutes at room temperature. Absorbance was then read at 765 nm using Hitachi ratio beam spectrophotometer (U–500 Hitachi, Tokyo, Japan) and phenol content determined accordingly from the calibration curve for gallic acid standard (Eqn. 4).

Gallic acid $y = 10.227x - 0.1322$

Equation 4. Determination of phenolic content using gallic acid standard

*2.2.4.7. Antioxidant capacity*

The ability of coffee sample extracts to scavenge reactive species was determined by dissolving the freeze−dried powder in ultrapure water followed by dilution with methanol as described by Chen *et al.* (2018). Each sample was divided into 4 subsets of serial dilutions and stored under –80°C until further analysis.

2.2.4.7.1 2,2–diphenyl–1–picryl hydrazyl (DPPH) radical assay

The DPPH radical was used to determine the free radical scavenging capacity of the coffee sample extracts following procedures described by Chen *et al.* (2018). For analysis, the frozen samples were adjusted to room temperature, from which 1 mL was transferred to a new test tube, diluted 10–fold with methanol and then incubated with freshly prepared 0.1 mmol/L DPPH solution for 10 minutes in the dark in a total volume of 10 mL. In addition, freshly prepared Trolox (6–hydroxy–2, 5, 7, 8–tetramethyl–chroman–2–carboxylic) acid standards of serial concentrations were treated in the same way as the samples. Absorbances of both the standard and samples were determined at 519 nm using the Hitachi ratio beam spectrophotometer. The concentration that caused a 50% decrease in the initial concentration of the DPPH radical defined as $IC_{50}$ was determined for both the standard and the samples from the percentage inhibition of the DPPH radical which was calculated as % inhibition = $(Abs_{control} - Abs_{sample}) / (Abs_{control} - Abs_{blank}) \times 100$ where $Abs_{control}$ = absorbance of the 0.1 mmol/L DPPH methanol solution, $Abs_{sample}$ = absorbance of the 0.1 mmol/L methanol solution after fading induced by addition of sample or Trolox; and $Abs_{blank}$ = absorbance of the methanol solvent

only. Antioxidant capacity of the samples as determined using the DPPH scavenging radical was calculated as ($IC_{50\ (Trolox)}$ / $IC_{50\ (sample)}$) x $10^5$ and expressed as µmol Trolox/g sample of Trolox equivalent antioxidant capacity (TEAC).

2.2.4.7.2 2,2–azino bis (3–ethyl benzothiazoline–6–sulphonic acid) radical (ABTS) assay

The ABTS radical scavenging capacity was determined as modified by Chen *et al.* (2018) with a few changes. ABTS solution of 7 mmol $L^{-1}$ was prepared and mixed with $K_2S_2O_8$ solution of 140 mmol/L at a ratio of 5 mL: 88 µL respectively. The solution was then incubated at room temperature in the dark overnight. On the ABTS measurement day, the stock solution was diluted with ultra−pure water at a ratio of 0.6: 40 mL respectively and the absorbance adjusted to 0.7 ± 0.02 at 734 nm by spectrophotometry. Serial dilutions of freshly prepared Trolox standard and coffee sample extracts of 0.5 mL were then incubated with 9.5 mL of the new solution for 10 minutes at room temperature in the dark after which absorbance was read at the same wavelength. ABTS antioxidant capacity was then calculated as gradient of Trolox standard/ gradient of sample and expressed as µmol Trolox $gDW^{-1}$ of TEAC.

2.2.4.7.3 Ferric ion reducing antioxidant power (FRAP) assay

The FRAP assay was carried out following procedures modified by Alvarez−jubete *et al.* (2010). The FRAP oxidant solution consisted of 0.2 M sodium acetate buffer (pH 3.6), 20 mM Ferric chloride solution and 10 mM TPTZ (2,4,6 tris (2–pyridyl)–s–triazine) solution in 40 mM HCl mixed at a ratio of 10:1:1 respectively. The serially diluted samples and Trolox standard of 0.5 mL were incubated with 9.5 mL of freshly prepared FRAP oxidant solution in a water bath at 37°C for 40 minutes after which absorbance was read at 593 nm. The FRAP antioxidant capacity was then calculated as the gradient of Trolox standard / gradient of sample and expressed as µmol Trolox/g sample of TEAC.

### 2.2.5. Statistical analysis

Experimental data were analysed using Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). Group means of the parameters were analysed using one−way analysis of variance (ANOVA) and compared for statistical significance using the Tukey's test at $p \leq 0.05$. Correlations and relationships amongst the parameters and amongst the plant organs were evaluated using Pearson's correlation co–efficient and principal component analysis (PCA) in Minitab v14, Minitab Inc. (State College, PA, USA). All analyses were performed in triplicates and data are expressed as mean ± S.D, n = 3.

## 2. 3. Results

### 2.3.1. HPLC Phytochemical composition

Typical HPLC chromatograms of the samples displaying the coeluted compounds (trigonelline, 5–CQA, caffeine and mangiferin) detected under UV and sucrose detected under RI are shown in Fig. 3A and B respectively.

*2.3.1.1 Coffee beans*

The HPLC phytochemical profile of coffee beans was significantly different between GB and RB (Table 2). This pronounced difference was caused more by changes in sucrose and 5–CQA content which were also the most abundant compounds in the raw beans. On the other hand, due to the degradation of sucrose and 5–CQA during roasting, the 2 alkaloids, caffeine and trigonelline were the most abundant in RB. In fact, roasting reduced the total content of the phytochemicals by 66%. Moreover, the HPLC profile revealed that mangiferin was absent in the GB and definitely in RB.

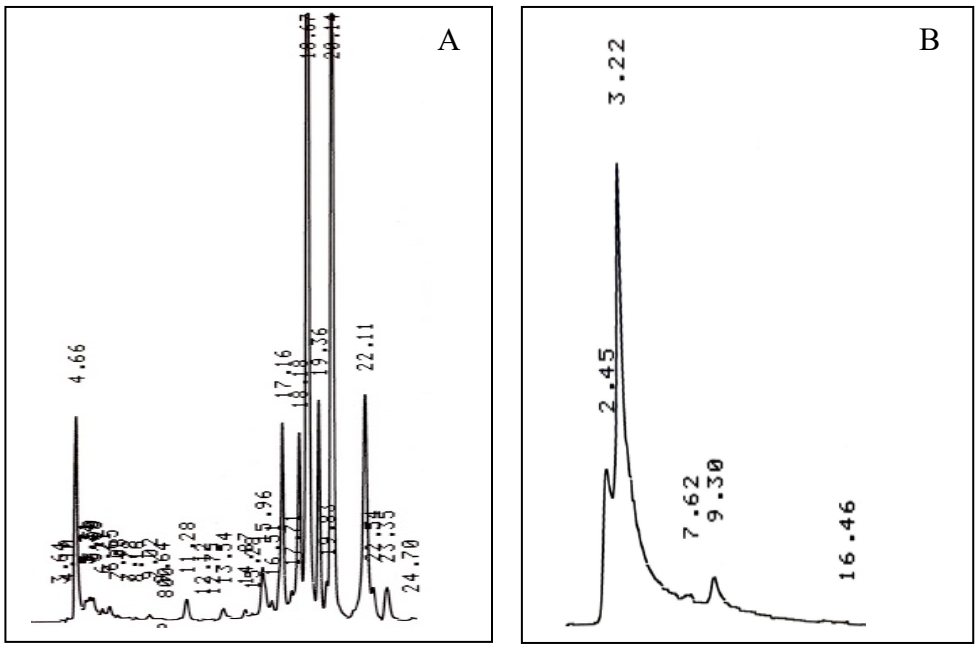


Fig. 3. HPLC chromatograms of the extracted coffee sample (A) displaying peaks for trigonelline, 5–CQA, caffeine and mangiferin at 4.66, 18.67, 20.14 and 22.11 min respectively using UV detector at 270 nm; (B) displaying sucrose peak at 9.30 min using RI detector.

*2.3.1.2 Leaves*

The HPLC phytochemical profile of the leaves indicated in Table 2 revealed that the most abundant isomer of chlorogenic acid (5–CQA) was the most abundant phytochemical determined while mangiferin was the least contained in the coffee leaves. 5–CQA, sucrose, trigonelline and mangiferin were highest in the youngest leaves at 3.97, 2.63, 0.67, and 0.09 mg $gDW^{-1}$ respectively. These phytochemical compounds were accordingly least in the oldest leaves 2.48, 1.51, 0.47 and 0.06 mg $gDW^{-1}$ respectively. On the contrary, caffeine accumulated more with increase in leaf age and therefore was highest in the oldest leaves compared to the youngest ones at 1.05 and 0.85 mg $gDW^{-1}$ respectively. Nevertheless, the youngest leaves contained the highest amount of the total phytochemicals analysed in the current study at 8.23 mg $gDW^{-1}$ while oldest leaves contained the least at 5.57 mg $gDW^{-1}$. Moreover, the ratio 5–CQA to caffeine also reduced gradually with increasing leaf age from 4.58 to 2.36 in the youngest and the oldest leaves respectively. There was a decline in the content of the phytochemicals induced by leaf senescence as observed in BL compared to L1. Trigonelline content was least affected by senescence despite reducing by around 50%. The content of caffeine, 5–CQA and sucrose reduced by over 60% while mangiferin was completely degraded by senescence. On the other hand, there was an increase in the ratio of 5–CQA to caffeine due to a more pronounced decline in the content of caffeine than that of 5–CQA, 63 and 61%, respectively.

The concentrations of chlorophylls and carotenoids in the leaves are indicated in Table 3. The concentration of chlorophyll *a*, total chlorophylls and total carotenoids showed no significant differences ($P \leq 0.05$) with leaf age. On the other hand, that of chlorophyll *b* tended to increase as the leaves mature hence resulting into an increase in the chlorophyll *a*/*b* ratio in the older leaves. Contrastingly, although the ratio of total chlorophylls to total carotenoids which is an indicator of the status of the photosynthetic apparatus tended to reduce in the older leaves, it showed no significant difference.

*2.3.1.3 Stems*

There was a dramatic variation in the content of the phytochemicals within the stem (Table 2). The herbaceous stem (HS) contained more phytochemicals compared to the WS. In fact, the current study revealed that the content of the phytochemicals decreased by over 70% when the herbaceous stem tissues were lignified into woody tissues. In addition to total decline of caffeine and mangiferin, there was a decrease of 54, 55 and 75% in the contents of sucrose,

trigonelline and 5–CQA respectively. The relatively higher content of 5–CQA in both HS and WS coupled with a low content of caffeine resulted into an amazingly high 5–CQA/caffeine with the ratio being highest in WS.

*2.3.1.4 Roots*

Coffee roots accumulated all the phytochemicals determined by the HPLC as in other organs with the exception of caffeine and mangiferin (Table 2). Sucrose was the highest at 1.01%, followed by 5–CQA at 0.71% whereas trigonelline was least at 0.14%.

### 2.3.2 Total phenolic content and antioxidant capacities of the coffee plant organs

The extract yield, total phenolic content and antioxidant capacities of the different coffee organs showed great variation amongst the samples (Table 4). The current study revealed that RB and the youngest leaves (L1) had the highest extraction yield at 36.4 and 26.6% respectively. This was followed by GB at 25.8% and older leaves at 25.6% accordingly. The yield was least for HS, roots and WS at 16.9, 14.6 and 6.4% respectively. Similarly, TPC of the extracts was highest in the leaves and more so increasing with increase in leaf age from 65.1 to 71.5 mg GAE $gDW^{-1}$ In addition, senescence did not significantly affect the content of the total phenols in the youngest leaves despite showing a decline to 60.4 mg GAE $gDW^{-1}$ in BL. In beans however, roasting caused an increase in total phenolic content of 20.8% from 29.3 to 35.4 mg GAE $gDW^{-1}$. The total phenolic content of the stems was low at 27.8 and 8.7 mg GAE $gDW^{-1}$ in HS and WS respectively while that of roots was 15.8 mg $gDW^{-1}$ (Table 4).

Table 2. HPLC Phytochemical profile of coffee plant organs expressed as unit weight per freeze−dried sample (mg $gDW^{-1}$)

| Sample (Plant organ) | | Caffeine | Trigonelline | 5 – CQA | Mangiferin | Sucrose | 5–CQA/ caffeine | HPLC total |
|---|---|---|---|---|---|---|---|---|
| **Seeds** | GB | 0.97 ± 0.09bc | 0.65 ± 0.05abc | 3.13 ± 0.33abc | 0.00 ± 0.00c | 4.95 ± 0.40a | 3.23 ± 0.03c | 9.70 ± 0.73a |
| | RB | 1.30 ± 0.13ab | 0.85 ± 0.01a | 1.00 ± 0.02e | 0.00 ± 0.00c | 0.14 ± 0.05g | 0.77 ± 0.07c | 3.29 ± 0.20gh |
| Leaves | L1 | 0.87 ± 0.03c | 0.67 ± 0.22abc | 3.97 ± 0.61a | 0.09 ± 0.03a | 2.63 ± 0.55b | 4.58 ± 0.85c | 8.23 ± 0.18b |
| | L2 | 0.78 ± 0.13c | 0.72 ± 0.26ab | 3.35 ± 0.37ab | 0.05 ± 0.01b | 2.30 ± 0.16bc | 4.46 ± 1.26c | 7.19 ± 0.15bc |
| | L3 | 0.90 ± 0.37c | 0.68 ± 0.20abc | 3.21 ± 0.24abc | 0.05 ± 0.01b | 2.35 ± 0.34b | 4.15 ± 2.10c | 7.19 ± 0.43bc |
| | L4 | 1.35 ± 0.07a | 0.76 ± 0.12ab | 2.83 ± 0.23bc | 0.05 ± 0.00b | 1.95 ± 0.08bcd | 2.10 ± 0.06c | 6.93 ± 0.20cd |
| | L5 | 1.12 ± 0.00abc | 0.44 ± 0.03bcd | 2.35 ± 0.11cd | 0.04 ± 0.01b | 1.85 ± 0.16bcd | 2.09 ± 0.09c | 5.81 ± 0.06de |
| | L6 | 1.05 ± 0.01abc | 0.47 ± 0.01bcd | 2.48 ± 0.30bcd | 0.06 ± 0.02ab | 1.51 ± 0.12cde | 2.36 ± 0.28c | 5.57 ± 0.46ef |
| | BL | 0.32 ± 0.00d | 0.34 ± 0.04cd | 1.55 ± 0.32de | 0.00 ± 0.00c | 0.82 ± 0.12efg | 5.44 ± 0.67c | 3.00 ± 0.42hi |
| Stem | HS | 0.01 ± 0.00d | 0.45 ± 0.04bcd | 2.67 ± 0.29bc | 0.00 ± 0.00c | 1.24 ± 0.35def | 190.2 ± 58.3b | 4.37 ± 0.60fg |
| | WS | 0.00 ± 0.00d | 0.20 ± 0.08d | 0.66 ± 0.23e | 0.00 ± 0.00c | 0.57 ± 0.11fg | 369.4 ± 193.2a | 1.42 ± 0.32j |
| Roots | Roots | 0.00 ± 0.00d | 0.14 ± 0.00d | 0.71 ± 0.44e | 0.00 ± 0.00c | 1.01 ± 0.30ef | 0.00 ± 0.00c | 1.85 ± 0.69ij |

Data are expressed as means ± S.D, n = 3. Within a column, data means followed by the same letter are not statistically different by Tukey's test ($P \leq 0.05$). GB = green beans, RB = roasted beans, L1 to L6 = leaf pairs ($1^{st}$ – $6^{th}$) from top to bottom of the coffee plant, BL = brown leaves of the first pair, HS = herbaceous stem, WS = woody stem.

Table 3 Concentration of chlorophylls and carotenoids in the leaves of coffee plants expressed as mg $gFW^{-1}$)

| Leaf position | Chlorophyll *a* | Chlorophyll *b* | Total Chlorophyll | Total Carotenoids | Chlorophyll *a*/*b* | Chlorophyll/Carotenoid |
|---|---|---|---|---|---|---|
| L1 | 1.09 ± 0.28a | 0.37 ± 0.09b | 1.46 ± 0.36a | 0.26 ± 0.06a | 2.93 ± 0.20ab | 5.48 ± 0.27a |
| L2 | 1.02 ± 0.06a | 0.35 ± 0.05b | 1.37 ± 0.09a | 0.24 ± 0.02a | 2.90 ± 0.26ab | 5.70 ± 0.02a |
| L3 | 1.31 ± 0.12a | 0.48 ± 0.01ab | 1.79 ± 0.13a | 0.31 ± 0.04a | 2.73 ± 0.20ab | 5.84 ± 0.38a |
| L4 | 1.28 ± 0.17a | 0.52 ± 0.03a | 1.80 ± 0.19a | 0.30 ± 0.04a | 2.45 ± 0.24b | 5.96 ± 0.22a |
| L5 | 1.27 ± 0.05a | 0.40 ± 0.07ab | 1.67 ± 0.11a | 0.37 ± 0.09a | 3.20 ± 0.48a | 4.72 ± 1.23a |
| L6 | 1.08 ± 0.02a | 0.36 ± 0.01b | 1.44 ± 0.02a | 0.28 ± 0.01a | 3.02 ± 0.09ab | 5.06 ± 0.14a |

Data are expressed as means ± S.D, n = 3. Within a column, data means followed by the same letter are not statistically different by Tukey's test ($P \leq 0.05$).

L1 to L6 represents leaf pairs from top (youngest) to bottom (oldest) of the coffee plant.

Table 4 Total phenolic content (TPC) and antioxidant capacity of the coffee plant organs

| Sample (Plant organ) | | Yield (%) | TPC content (mg GAE $gDW^{-1}$) | DPPH $IC_{50}$ (µg $mL^{-1}$) | DPPH | ABTS | FRAP |
|---|---|---|---|---|---|---|---|
| | | | | | TEAC (µmol Trolox $gDW^{-1}$) | | |
| Seeds | GB | 25.8 ± 4.40ab | 29.3 ± 1.40c | 146.8 ± 12.0c | 199.7 ± 16.1abc | 220.7 ± 22.4ab | 974.2 ± 83.4ab |
| | RB | 36.4 ± 11.9a | 35.4 ± 1.49bc | 87.0 ± 5.73c | 337.0 ± 22.2a | 325.5 ± 81.0a | 1104.4 ± 323.3a |
| Leaves | L1 | 26.6 ± 0.23ab | 65.1 ± 14.1a | 90.6 ± 12.1c | 328.0 ± 43.9a | 345.7 ± 53.3a | 1097.4 ± 132.8a |
| | L2 | 24.2 ± 1.54b | 66.7 ± 19.0a | 94.0 ± 38.5c | 302.5 ± 141.7a | 323.8 ± 72.1a | 1016.6 ± 271.2a |
| | L3 | 23.1 ± 0.53b | 63.6 ± 10.2a | 119.0 ± 34.7c | 268.3 ± 78.3ab | 306.1 ± 92.3a | 1024.2 ± 229.2a |
| | L4 | 22.5 ± 2.96b | 66.4 ± 3.95a | 115.3 ± 15.8c | 258.2 ± 35.4ab | 267.0 ± 48.4a | 969.1 ± 173.8ab |
| | L5 | 18.1 ± 2.89bc | 72.0 ± 15.6a | 125.5 ± 10.1c | 228.7 ± 29.1ab | 238.9 ± 8.12ab | 618.6 ± 247.5abc |
| | L6 | 18.9 ± 0.94b | 71.5 ± 2.47a | 132.6 ± 15.7c | 223.4 ± 26.5ab | 208.8 ± 15.7ab | 624.1 ± 104.1abc |
| | BL | 25.6 ± 3.04ab | 60.4 ± 8.71ab | 99.8 ± 22.6c | 304.6 ± 79.0a | 294.5 ± 17.7a | 755.1 ± 156.4ab |
| Stem | HS | 16.9 ± 0.55bc | 27.8 ± 2.57c | 263.8 ± 10.5c | 110.9 ± 4.42bcd | 124.8 ± 1.42bc | 469.7 ± 13.0bc |
| | WS | 6.4 ± 0.27c | 8.7 ± 1.17c | 1403.1 ± 406.0a | 21.9 ± 5.59d | 24.9 ± 7.44c | 110.0 ± 34.0c |
| Roots | Root | 14.6 ± 3.16bc | 15.8 ± 3.43c | 916.1 ± 370.4b | 36.8 ± 18.6cd | 41.5 ± 21.2c | 156.7 ± 85.6c |

Data are expressed as means ± S.D, n = 3. Within a column, data means followed by the same letter are not statistically different by Tukey's test ($P < 0.05$). GB = green beans, RB = roasted beans, L1 to L6 = leaf pairs ($1^{st}$ – $6^{th}$) from top to bottom of the coffee plant, BL = brown leaves of the first pair, HS = herbaceous stem, WS = woody stem.

Similar to the variation in TPC, both leaves and beans had consistently the highest TEAC values as determined by DPPH, ABTS and FRAP and lowest DPPH $IC_{50}$ values (Table 4). This is an indicator of higher antioxidant capacity in these samples. Nevertheless, within beans, there was a consistent increase in the antioxidant capacity when green beans were roasted and hence resulting into an increment of 68.8, 47.5 and 13.4% in DPPH, ABTS and FRAP values respectively. This was accompanied by lower $IC_{50}$ values in RB compared to GB at 87.0 and 146.8 µg $mL^{-1}$ respectively. Despite the variation being statistically non−significant within the leaves, there was a consistent decrease in the antioxidant capacities induced by leaf maturity. The TEAC of the youngest leaves (L1) was 328.0, 345.7 and 1097.4 µmol Trolox $gDW^{-1}$ as determined by DPPH, ABTS and FRAP assays respectively while the same assays showed less values in the oldest leaves (L6) at 223.4, 208.8 and 624.1 µmol Trolox $gDW^{-1}$. The younger leaves (L2 to L5) consistently showed intermediate values of antioxidant capacity. In addition, senescence somewhat caused a consistent decline in the antioxidant capacity of the youngest leaves which was more perceptible in the FRAP TEAC values (31.2% decline) compared to 7.13 and 14.8% for DPPH and ABTS respectively. Antioxidant capacity was least in WS, showing a remarkable increase of 80.3, 80.0 and 76.6% in DPPH, ABTS and FRAP TEAC values respectively in HS. This was accompanied with lower $IC_{50}$ values in HS compared to WS at 263.8 and 1403.1 µg $mL^{-1}$, respectively (Table 4). The antioxidant capacity of roots was intermediate between WS and HS with $IC_{50}$ value of 916.1 µg $mL^{-1}$ while DPPH, ABTS and FRAP values were 36.8, 41.5 and 156.7 µmol Trolox $gDW^{-1}$, respectively.

### 2.3.3 Relationships amongst phytochemicals and plant organs

Principal component analysis of the different coffee plant organs and 19 variables including pigments, phytochemicals, TPC and antioxidant capacity revealed that the 2 principal components explained 77.4% of the total variance in the data with a contribution of 59.3% and 18.1% for PC1 and PC2, respectively (Fig. 4A and B). Principal component 1 clearly separated the samples in to 2 groups based on the presence or absence of chlorophylls, carotenoids and mangiferin whereas PC2 tended to separate the samples according to the content of the other phytochemicals, TPC and antioxidant capacities. The score plots for PC1 and PC2 revealed that RB, GB and BL contained high amounts TPC and antioxidant capacities and hence loaded highest on PC2 followed by leaves (L1–L6) especially the youngest (L1) and then HS respectively (Fig. 4A). On the other hand, both roots and WS contained low amounts of caffeine and hence had a very high 5–CQA/caffeine ratio. Moreover, accumulation of low phytochemicals and low TPC in these samples meant very low antioxidant capacities and very

high $IC_{50}$ values hence positioning of these samples in the bottom right corner of the PCA chart. Variation amongst the leaves in terms of leaf pair positioning was less significant and therefore samples L1–L6 were clustered on the left−hand side of the PCA chart. This was due to presence of chlorophylls, carotenoids and mangiferin which were barely present in other samples. Similarly, these samples had relatively similar antioxidant capacities with corresponding DPPH $IC_{50}$ values. Basing on PCA (Fig. 4) and correlation coefficient results (Table 5), there was a significant positive correlation ($P \leq 0.01$) amongst the different measures of the antioxidant capacity (DPPH, FRAP and ABTS). In addition, this antioxidant capacity correlated positively with TPC which also amongst the phytochemicals correlated more strongly with mangiferin and 5–CQA content. On the other hand, DPPH $IC_{50}$ correlated negatively with both the antioxidant activities as wells as the phytochemical content in the plant organs. Amongst the phytochemicals, caffeine correlated more strongly with trigonelline whereas 5–CQA correlated more strongly with mangiferin ($P \leq 0.01$). In addition, these phytochemicals correlated strongly with all the chlorophylls and carotenoids parameters. Nevertheless, the correlation between these pigments was most significant with mangiferin than with the rest of the phytochemicals.

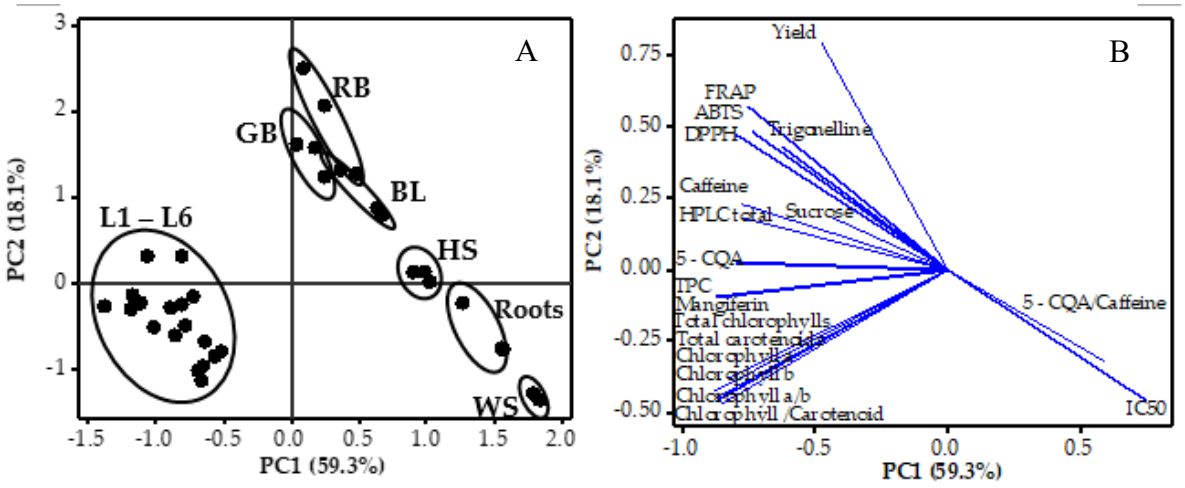


Fig. 4 Principal component analysis summarizing the relationships among variables listed in Tables 2, 3 and 4; (**A**) Score plot of phytochemical contents according to plant organ; (**B**) Loading plot of variables; sucrose, caffeine, trigonelline, 5–CQA, mangiferin, HPLC total, chlorophyll a, chlorophyll b, total chlorophyll, total carotenoids, chlorophyll a/b, chlorophyll/carotenoids and antioxidant capacity; DPPH, $IC_{50}$, ABTS and FRAP. GB = green beans, RB = roasted beans, L1 to L6 = leaf pairs ($1^{st}$ – $6^{th}$) from top to bottom of the coffee plant, BL = brown leaves of the first pair, HS = herbaceous stem, WS = woody stem.

Table 5. Pearson's correlation coefficients amongst the measured parameters shown in tables 2, 3 and 4.

| | Yield | TPC | IC50 | DPPH | ABTS | FRAP | Trigonelline | 5 – CQA | Caffeine | Mangiferin | Sucrose | 5 – CQA/Caffeine | HPLC Total | Chl a | Chl b | Total Chl | Total Car | Chl a/b |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TPC | .35* | | | | | | | | | | | | | | | | | |
| IC50 | −.69** | −.69** | | | | | | | | | | | | | | | | |
| DPPH | .76** | .75** | −.76** | | | | | | | | | | | | | | | |
| ABTS | .80** | .74** | −.79** | .94** | | | | | | | | | | | | | | |
| FRAP | .84** | .57** | −.75** | .87** | .92** | | | | | | | | | | | | | |
| Trigonelline | .63** | .30 | −.63** | .52** | .61** | .69** | | | | | | | | | | | | |
| 5 – CQA | .29 | .63** | −.65** | .51** | .59** | .64** | .48** | | | | | | | | | | | |
| Caffeine | .58** | .58** | −.64** | .61** | .65** | .68** | .77** | .43** | | | | | | | | | | |
| Mangiferin | .12 | .76** | −.40* | .46** | .51** | .44** | .31 | .71** | .48** | | | | | | | | | |
| Sucrose | .18 | .17 | −.36* | .17 | .24 | .41* | .34* | .69** | .32 | .25 | | | | | | | | |
| 5 – CQA/Caffeine | −.57** | −.54** | .57** | −.56** | −.57** | −.53** | −.39* | −.33 | −.56** | −.34* | −.31 | | | | | | | |
| HPLC Total | .39* | .50** | −.64** | .48** | .56** | .68** | .62** | .90** | .61** | .56** | .88** | −.44** | | | | | | |
| Chl a | .07 | .80** | −.43** | .42* | .48** | .40* | .36* | .64** | .61** | .88** | .24 | −.38* | .55** | | | | | |
| Chl b | .08 | .79** | −.43** | .42* | .48** | .41* | .39* | .63** | .61** | .86** | .24 | −.37* | .56** | .99** | | | | |
| Total Chl | .074 | .80** | −.43** | .42* | .48** | .40* | .37* | .64** | .61** | .87** | .24 | −.38* | .56** | .1.0** | .99** | | | |
| Total Car | .06 | .77** | −.42* | .39* | .45** | .37* | .33* | .59** | .61** | .84** | .23 | −.37* | .53** | .98** | .95** | .98** | | |
| Chl a/b | .07 | .79** | −.44** | .43** | .48** | .39* | .35* | .63** | .57** | .88** | .26 | −.38* | .55** | .97** | .93** | .96** | .97** | |
| Chl/Car | .10 | .81** | −.44** | .46** | .51** | .42* | .41* | .67** | .57** | .90** | .27 | −.38* | .58** | .97** | .98** | .98** | .93** | .95** |

*. Correlation is significant at the 0.05 level (2−tailed).

**. Correlation is significant at the 0.01 level (2−tailed).

## 2.4 Discussion

*Coffea* species contain several phytochemicals such as caffeine, trigonelline, chlorogenic acids, mangiferin, sucrose (Fig. 3) which render their wide exploitation for pharmacological and health promoting benefits (Patay *et al.,* 2016). Utilization of coffee plants has mainly focused on a single organ, the coffee seeds as the source of the phytochemicals with related health benefits (Pereira *et al.,* 2019). However, utilization of other organs including those evaluated in the current study is also increasingly becoming common because of their possible therapeutic values (Chen *et al.,* 2018). Moreover, organs such as leaves have always been used traditionally in many coffee growing regions for mitigation of a number of illnesses including cardiovascular, gastro−intestinal, cancer, diabetes, dermatological and obesity amongst others (Patay *et al.,* 2016). In addition to containing all the known phytochemicals in the coffee seeds, leaves contain exclusive compounds such as mangiferin (1,3,6,7−tetrahydroxyxanthone−C2−β−D−glucoside) and pigments like chlorophylls and carotenoids whose antioxidant potency is also widely reported (Stahl and Sies, 2003; Lanfer-Marquez *et al.,* 2005; Talamond *et al.,* 2008; Campa *et al.,* 2012; Wangcharoen and Phimphilai, 2016; Imran *et al.,* 2017;Chen *et al.,* 2018).

The current study revealed that raw beans contained the highest amounts of the phytochemicals especially 5–CQA, sucrose, caffeine and trigonelline. Coffee seeds remain the most important organ in coffee trade because of their extensive use in the coffee beverage processing (Pereira *et al.,* 2019). Phytochemicals in seeds accumulate as a result of metabolism within the fleshy parts of the fruits during maturation but also due to deposition having been processed from leaves and young buds of the coffee plant (Farah and Donangelo, 2006; Koshiro *et al.,* 2006; Koshiro *et al.,* 2007; Farah *et al.,* 2008). Coffee seeds contain mainly carbohydrates, sucrose being the main constituent whose role is to provide nourishment for the embryo in case the seeds germinate (Xu *et al.,* 2010). Also, similar to the findings in the current study (Table 2), high amounts of phenolic compounds have been reported in the coffee fruits and seeds. Chlorogenic acids are the main phenolic compounds that accumulate in the beans during maturation of coffee fruits and seeds (Farah and Donangelo, 2006; Farah *et al.,* 2008). Of these, 5–CQA forms the main constituent of these hydroxycinnamic acid esters (Campa *et al.,* 2012). On the other hand, although mangiferin another phenolic compound was reported in the fruits, its accumulation in the seeds has been disputed (Campa *et al.,* 2012) which explains its absence in all the beans in our study (Table 2). Phenolic compounds in the seeds and fleshy parts of the young coffee fruits are associated with their role in defence against oxidative stress and for

future use in the synthesis of cell wall–bound phenolic polymers after seed germination and during seedling development (Aerts and Baumann, 1994). However, as the fruits mature, a decline in the total chlorogenic acids content from between 5–7 mg $gDW^{-1}$ by up to 7% or more occurs as a result of development of an elaborate enzymatic antioxidant system and reduced activities of polyphenol peroxidase and oxidase activities hence warranting less role of the phytochemicals in defence against reactive oxygen species (Mazzafera and Robinson, 2000; Montavon *et al.,* 2003). In the current study, the content of 5–CQA in the mature GB was 3 mg $gDW^{-1}$ (Table 2) which is consistent with the above findings. Before utilization as a beverage, coffee beans normally undergo processing which includes roasting under high temperatures (Pereira *et al.,* 2019). During such processes, a number of reactions such as Maillard, Strecker's and caramelisation occur at temperatures over 200°C during roasting which result into the characteristic aroma and bitter taste of the coffee beverage (Maria *et al.,* 1994; Poisson *et al.,* 2018). In the current study, sucrose content reduced by 97% when the green beans were roasted due to its participation in the above reactions (Table 2). During roasting, free amino acids in the coffee beans react with fructose and glucose produced from sucrose digestion beforehand during fermentation to form a number of odorants such as 2–furfurylthiol, 2, 3 butanedione amongst others whose identity is rather determined by the type of amino acid involved in the reaction (Amrani-Hemaimi, 1995; Poisson *et al.,* 2018). Additionally, coffee flavour is also determined by pyrroles and pyridines from trigonelline degradation (Amrani-Hemaimi, 1995; Stadler *et al.,* 2002). However, only a small fraction of trigonelline is involved in this reaction hence minor reductions in the content between raw and roasted beans occurs, similar to what was observed in the current study (Table 2). On the other hand, coffee taste is determined by caffeine which is thermally stable and phenolic derivatives from chlorogenic acid degradation into melanoidins (Fuller and Rao, 2017). The content of 5–CQA dropped sharply when the beans were roasted supporting their degradation in to melanoidins and other related compounding at high temperatures (Table 2). However, the slight increase in caffeine in the roasted samples could have resulted from the differences in the moisture content when the GB were roasted.

Leaves are associated with high rates of metabolism due to their role in photosynthesis. For this, they contain chlorophylls to facilitate photosynthetic activity. The concentration of total chlorophylls in the leaves or proportions of their respective types (*a* and *b*) varies with leaf age or position. Chlorophyll *a* is normally highest in the youngest leaves whereas chlorophyll *b* is highest in mature leaves. The latter is normally found in the reaction centres of photosystem I,

II and in the pigment antenna system whereas the former is found only in the pigment antenna system (Lichtenthaler and Buschmann, 2001). In the current study, there was a general increase in the concentration of the chlorophyll *b* in the older leaves (Table 3). It is suggested that this is meant to maximize light capture because of the quaternary arrangement of leaves on the orthotropic stem, which dictates older leaves to receive less incident light than their younger counterparts (Sakiyama *et al.,* 2017). Chlorophylls normally are unable to utilize all the photosynthetically active radiation (PAR) and therefore plants have evolved mechanisms to avoid or detoxify ROS that result from excess excitation energy. In addition to energy evasion, by accumulating less amounts of chlorophylls (Revatipadale *et al.,* 2019), leaves contain carotenoids which serves to protect the chlorophylls against oxidative stresses (Wangcharoen and Phimphilai, 2016). These pigments have also been reported to contribute to health benefits such as decreasing disease risk due to their high antioxidant activities when consumed (Wangcharoen and Phimphilai, 2016; Fiedor and Burda, 2014). Presence of high amounts of other phytochemicals such as alkaloids, phenolic compounds and sugars have also been reported in the coffee leaves (Talamond *et al.,* 2008; Campa *et al.,* 2012; Chen *et al.,* 2018). Our results also showed similar findings especially in the youngest leaves. Like carotenoids, these compounds protect the leaves against ROS that are by−products of aerobic metabolism more so in the young leaves (Mittler, 2002; Asada, 1999; Ende and Valluru; 2009). These compounds normally compliment the enzymatic defence system in detoxifying the ROS (Brewer, 2011). It has recently been shown that unlike the older counterparts, young coffee leaves have a poorly developed enzymatic antioxidant defence system and hence the reliance on oxidant scavenger compounds is inevitable (Das and Roychoudhury, 2014; Ramalho *et al.,* 2018). In addition to defence, some phytochemicals in the current study have other functions in coffee plants. Sucrose, a highly soluble disaccharide is synthesized in the leaf cytosol and hence its accumulation is directly related to photosynthesis (Lunn *et al.,* 2016). By virtue of their position, the youngest leaves accumulated the highest content of sucrose which reduced with leaf maturity (Table 3). It is also a storage reservoir molecule and a transportation solute which is readily broken down to provide energy for growth and other cellular functions (Hammond and White, 2008).

In the current study, relatively higher amounts of sucrose accumulated in the HS compared to roots while WS had the least content (Table 2). This could be due to the presence of active meristems in both the HS and the roots that require the energy for growth (Hammond and White, 2008). Accumulation of phytochemicals such caffeine, 5–CQA and mangiferin in other

organs of the coffee plants like the stem (especially WS) and roots is less reported. Nevertheless, this study confirmed presence of high amounts of sucrose and 5–CQA in the roots (Table 2). Although evidence of chlorogenic acids metabolism in the roots remains un–investigated, their accumulation has been suggested to be because of their regulatory role in root hair formation (Franklin and Dias, 2011). Mangiferin and caffeine were essentially absent in the WS and the roots. This observation is in agreement with similar findings that have suggested that mangiferin, a bioactive xanthonoid compound accumulates in the photosynthetic tissues so as to protect the organs against ultra–violet stress (Campa *et al.,* 2012). On the other hand, caffeine is known to protect against herbivory and therefore accumulates only in the forage tissues especially leaves and beans and hence less in lignified tissues such as WS and roots (Lindenmeier and Hofmann, 2004). Though in less amounts, trigonelline was present in the HS, roots and WS in that order. This pyridine alkaloid accumulates in coffee organs as a reservoir for nicotinamide adenine dinucleotide (NAD) biosynthesis which plays a key role in sub–cellular energy metabolism (Koshiro *et al.,* 2006).

Biosynthesis of these phytochemicals is normally limited to specific organs. Phenolic compounds accumulation mainly occurs via the phenylpropanoid biosynthetic pathway (Sreekumar and Soniya, 2017). However, just like in Campa *et al.* (2012), this study found no correlation between chlorogenic acids (5–CQA) and mangiferin accumulation in the plant organs (Table 5). This is owed to the absence of metabolite competition for the two phenolic compounds and the silencing of the gene that encodes 3–ketoacyl–CoA thiolase (PhKAT1) protein which catalyses the committed step for benzoic acid production in the benzenoid biosynthetic pathway (Moerkercke *et al.,* 2009) from which mangiferin biosynthesis proceeds. Moreover, unlike chlorogenic acids that are distributed in all organs of the coffee plant (Aerts and Baumann, 1994), recent reports have only reported presence of mangiferin only in the photosynthetic tissues of the coffee leaves and the receptacle of the young fruits of arabica coffee, which is in agreement with our findings (Campa *et al.,* 2012). The two phenolic compounds are however degraded during senescence which could explain the decrease in the content of mangiferin and 5–CQA in BL (Table 2). On the other hand, alkaloids are metabolised in young leaves and the growing tips of the coffee plants and therefore accumulation of caffeine in older leaves is as a result of deposition rather than active biosynthesis where they protective against herbivory (Zheng and Ashihara, 2004). Although it was earlier suggested that trigonelline too acts as a chemical defence against herbivory (Shimizu and Mazzafera, 2000), recent reports have suggested that trigonelline biosynthesis

results from detoxification of excess nicotinic acid and therefore is reconverted to the required substrate whenever the need for NAD biosynthesis arises (Ashihara and Watanabe, 2014). Moreover, trigonelline accumulation was rather almost equally distributed in all the plant organs especially those with active meristems. Ours results agree with Ashihara and Watanabe (2014) who have also reported presence of trigonelline in all coffee plant organs with higher amounts especially in the upper stem and relatively lower amounts in the roots. Metabolism of the two alkaloid compounds occurs through two pathways; the *de novo* pathway and the salvage pathway. These two pathways for the alkaloids have been reported in the occur simultaneously in the youngest buds and expanding leaves hence resulting into high accumulation of alkaloids in such organs. On the other hand, the mature leaves contain only the salvage pathway which is further constrained by reduced endogenous supply of the necessary substrates during biosynthesis (Zheng and Ashihara, 2004). Caffeine and trigonelline are degraded by demethylation into xanthine and nicotinic acid in mature plant organs. Our results suggest that caffeine degradation could be occurring at higher rates compared to that of trigonelline hence a higher degradation percentage in BL due to loss in biological value in dried leaves (Heaton and Marangoni, 1996; Zheng and Ashihara, 2004). The pattern of biosynthesis and accumulation of the two main alkaloid compounds; caffeine and trigonelline in coffee seeds especially the pericarp follows a similar trend (Koshiro *et al.,* 2006). It has been reported that, largely the two alkaloids are biosynthesized elsewhere and transported to the fruits and the seeds during maturation (Koshiro *et al.,* 2006). Therefore, the difference in caffeine and trigonelline content in the seeds corresponded with the difference in the youngest leaves which are the main sites of alkaloid biosynthesis.

Coffee plants are an important source of dietary antioxidants. Antioxidant capacity of several foods including coffee is reported to be as a result of polyphenol accumulation (Alvarez−Jubete *et al.,* 2010) which include mangiferin and 5–CQA. The current study revealed that coffee leaves contained the highest of total phenolic content compared to other organs (Table 4). This could due to exposure of the leaves to oxidative stresses resulting from ultraviolet radiation and/or pathogens which the polyphenols protect against (Harbone and Williams, 2000). In coffee beans, roasting significantly increased the total phenolic content which could be due to thermal degradation of complex phenolic compounds such as chlorogenic acids into simpler ones like melanoidins with several hydroxyl components and glycosylic linkages (Fuller and Rao, 2017). As a consequence, the ROS scavenging capacity determined by DPPH, FRAP and ABTS was highest in the leaves, followed by beans, HS, roots and least in WS. It's presumed

that this order is dependent on the risk of ROS accumulation and hence increase in total phenolic content. Moreover, Alvarez−jubete *et al.* (2010) also reported a strong positive correlation between TPC and oxidant scavenging capacity while these parameters strongly negatively correlates DPPH $IC_{50}$ as indicated in Fig. 4. Phenolic compounds contain hydroxyl components and glycosylic linkages that scavenge ROS (Das and Roychoudhury, 2014). Antioxidant capacity and related benefits on human heath are however dependent on bioavailability of the phytochemicals after consumption which in turn is dependent on the soluble parts of the sample also known as extraction yield (Farah *et al.,* 2008; Chen *et al.,* 2018). The results in the current study suggest that in addition to coffee beans, other coffee organs especially the leaves are also a major source of phytochemicals and bio–available antioxidant compounds.

### 2.5 Conclusions

The phytochemicals determined in this study were generally distributed in all organs of the coffee plant in different amounts. Nevertheless, mangiferin was only present in the leaves and caffeine which was barely present in WS and roots. Although GB contained the highest content of the total phytochemicals analysed, coffee leaves contained higher amounts of the phytochemicals than RB. Leaves also contained mangiferin, chlorophylls and carotenoids which were absent in the both GB and RB. In addition, leaves contained the highest total phenolic content compared to other samples. Amongst other organs, relatively higher amounts of phytochemicals accumulated in HS than was present in roots while WS had the least content. Of the phytochemicals that were studied, sucrose and 5–CQA were the main compounds followed by caffeine and trigonelline while mangiferin was least in the organs when present. Due to thermal degradation of 5–CQA and reaction of sucrose during roasting coupled with relative stability of the alkaloids at high temperatures, it was revealed that caffeine and trigonelline were the main metabolites present in RB. Nevertheless, caffeine was more degraded during senescence compared to trigonelline and the other phytochemicals in the leaves. Accumulation of high amounts of phytochemicals in the leaves and beans resulted into the highest antioxidant capacities as measured by DPPH, ABTS and FRAP assays in the respective samples. Other organs such as HS, roots and WS had the least antioxidant activities in that order. Therefore, on the account of the phytochemicals, TPC and antioxidant capacity examined, coffee leaves might have the highest therapeutic and pharmacological value and therefore their utilization as a health beverage shouldn't be ignored. Moreover, exploitation of coffee leaves will certainly add extra income to coffee farmers when materials arising from

managerial practices such as de–suckering in which new shoots are regularly pruned are economically utilized for ‘coffee leaf tea’ processing.

## Chapter three

## Temporal changes in phytochemical profile of the leaves of young coffee plants in response to ambient temperature and light dynamics

### 3.1 Introduction

Following up on the previous chapter where high amounts of the phytochemicals were observed in the leaves especially the youngest, this chapter assessed the changes that occur in this phytochemical content in response to the ambient environmental conditions.

Being sessile organisms, plants normally often grow in environments with widely fluctuating ambient conditions. Despite having evolved as an understorey species in the forests of the tropical climate with moderate temperature and light conditions (Charrier and Berthaud, 1985), cultivation of coffee plants has extensively increased in regions with relatively adverse climatic conditions because its high economic value (ICO, 2019). These conditions normally pose abiotic limitations which are predicted to increase further given the current trends in climatic variations (DaMatta *et al.*, 2010; Camargo, 2010; Davis *et al.*, 2012). Environmental factors regulate several metabolic and biological processes and therefore their disruption from the optimal results into abiotic stresses in plants(Damatta and Ramalho, 2006; Camargo, 2010). Conditions such as extreme temperatures and high light intensity among others are normally associated with rapid accumulation of reactive species compared to normal values (Mittler, 2002; Gill and Tuteja, 2010; Foyer, 2018).

Reactive oxygen species are generated mainly from chloroplasts, mitochondria and peroxisomes due to presence of several electron transport reactions such as in the thylakoid membranes in the latter (Asada, 2006; Foyer, 2018). Subcellular generation of ROS such as singlet ($^1O_2$) and superoxide molecules ($O_2^{\bullet-}$) in chloroplasts is very detrimental to plant cells due to the vicinity of chlorophylls molecules which can rapidly be oxidized to their triplet and singlet ($^3$Chl* and $^1$Chl respectively) states (Asada, 2006). This results in to disintegration of the chlorophylls, changes in the pigment complexes as well as reduction in the fluidity of the lipid matrix of membranes (Damatta and Ramalho, 2006; Ramalho *et al.*, 2014; 2018). In addition to other limitations, these biochemical responses reduce the efficiency of several physiological processes that cause further generation of ROS (Das and Roychoudhury, 2014; Foyer, 2018). Moreover, despite being part of normal cellular respiration, excessive accumulation of ROS cause severe damage to many other cellular molecules including proteins, carbohydrates and DNA molecules which lead to oxidative stresses in plants and might result

into cell death (Del Río *et al.,* 2002; Gill and Tuteja, 2010). Nevertheless, ROS have also been implicated in playing positive roles for cell survival such as in biological stimuli for systemic signalling of several plant reposes aimed at restoring cellular metabolism (Dalton *et al.,* 1999; Mittler, 2002; Gill and Tuteja, 2010; Foyer, 2018; Huang *et al.*, 2019). Moreover, the role of ROS in signalling scavenger molecules and related antioxidant defence mechanisms is increasingly becoming understood (Del Río *et al.,* 2006; Das and Roychoudhury, 2014; Singh *et al.,* 2016; Waszczak *et al.,* 2018; Huang *et al.,* 2019).

Plants have evolved several mechanisms for homeostatic control of ROS accumulation. Such mechanisms range from avoidance of excess excitation through reducing the concentrations of chlorophylls (Adams *et al.*, 2004) to scavenging mechanisms when the ROS are generated (Asada, 2006). Antioxidant scavenging enzymes such as superoxide dismutase which scavenges the superoxide radical, together with peroxidases and catalase which scavenge the resultant hydrogen peroxide are widely distributed in all the cellular organelles (Asada, 2006). Leaves also possess protective carotenoids molecules which dissipate excess excitation energy away from the PSII in chlorophylls through de-epoxidation of the xanthophyll cycle in which violaxanthin is converted to xanthophyll (Demmig-Adams and Adams, 2004; Verhoeven *et al.,* 1997). Plants such as coffee whose young leaves have a poorly developed enzymatic antioxidant system, normally rely on nonenzymatic antioxidant compounds for ROS scavenging (Chaves *et al.,* 2008; Campa *et al.,* 2017; Ramalho *et al.,* 2018). This nonenzymatic antioxidant system is more significant during cold stress conditions when enzyme activity is repressed by low temperatures (Ramalho *et al.,* 2018). The nonenzymatic antioxidant system is composed of compounds such as phenolic compounds (chlorogenic acids and mangiferin) and alkaloids (caffeine and trigonelline) among others. These compounds contain glycosylic linkages and hydroxyl components that detoxify the ROS (Das and Roychoudhury, 2014).

Leaves deal with excess excitation energy both on a short–term such as during a natural circadian rhythm and/or on long–term basis due to diurnal and seasonal fluctuations on in the ambient conditions. During a 24 hrs cycle, the metabolic sensors in plant leaves modulate the circadian clock in plants in a light dependent manner (Shin *et al.,* 2017). This is associated with cyclical relative expression of dawn, day, dusk and evening genes with related metabolic and/or physiological adjustments which result in to generation of ROS (Johnson *et al.,* 2003; Florian *et al.*, 2014). This circadian oscillation has been studied in model crops such as Arabidopsis

(*Arabidopsis thaliana*) in regard to some physiological processes such as starch turnover (Sulpice *et al.,* 2014; Florian *et al.,* 2014; Moraes *et al.,* 2019).

In addition, cool summer conditions can be associated with higher rates of metabolism in coffee plants when the temperatures are favourable compared to winter conditions (Chaves *et al.,* 2008). Nevertheless, extreme high temperatures such as those above 24˚C in coffee plants cause increased mesophyll resistance with concomitant reduction in photosynthetic efficiency in the leaves (Kumar and Tieszen, 1980; Marias *et al.,* 2017; Rodrigues *et al.*, 2018). On the other hand, low positive temperatures below 17–18˚C during winter impose several biochemical and diffusive limitations which reduce photosynthetic efficiency and result into generation of ROS resulting into oxidative stress (Pompelli *et al.,* 2010; Ramalho *et al.,* 2014). The ability of coffee plants to grow in such stressful environments is owed to the plasticity of the photosynthetic apparatus (Matos *et al.,* 2009). The photosynthetic apparatus has evolved to efficiency use between 300 to 700 µmol $m^{-2}$ $s^{-1}$ photosynthetic active radiation (PAR) and therefore any light energy incident leads to excess excitation energy (Clifford, 2012; Ramalho *et al.,* 2014). This adaptive range is also dependent on the ambient conditions since abiotic stress such as extreme temperature and or light conditions are known to reduce photosynthetic efficiency with concomitant effects on pigment and the antioxidant status of the plants (Damatta and Ramalho, 2006). Moreover, oxidative stress conditions with their physiological and metabolic effects are likely worsened when previously low light acclimated plants are suddenly exposed to excess excitation. Nevertheless, in addition to long−term seasonal acclimatory responses, the role of antioxidant compounds in adaptation to circadian rhythm changes and/or to high light intensity remain un–investigated.

Therefore, we hypothesized that ambient environmental dynamics have an effect on the content of photosynthetic pigments nonenzymatic antioxidants compounds. This phenomenon was investigated by carrying out three sub–experiments. Fluctuations in the above parameters (i) during a 24–hr cycle (ii) over long–term, from summer to winter season (iii) and (iii) in response to high light intensity were monitored in young leaves of one–year old coffee plants in a naturally conditioned vinyl–house.

### 3.2 Materials and methods

#### 3.2.1 Plant material and growing conditions

Plant materials and growing conditions have been described in the previous chapter (see section 2. 2).

### 3.2.2 Experimental treatments

*3.2.2.1 Seasonal fluctuations*

To monitor the role of the nonenzymatic antioxidants in natural seasonal acclimations, leaf samples were taken every after two weeks from one−year old plants from summer (August 2018) through autumn to the onset of winter (December 2018). The leaf discs were collected at around midday on each sampling date from the plants growing in the dimmed greenhouse. Changes in the ambient temperatures in the greenhouse were monitored with a thermohygrometer (RTR 500B1, T & D corporation, Japan).

*3.2.2.2 Diel fluctuations*

The plants were transferred and acclimated in a different greenhouse under natural light conditions (PAR 1800 µmol $m^{-2}$ $s^{-1}$ at midday) for 10 days. Thereafter, samples were collected every after four hours covering a period of 24 h to monitor the natural circadian oscillations of the pigments and the nonenzymatic antioxidant compounds in order to understand their response to rapid changes in the temporal dynamics of light and dark conditions of day and night respectively.

*3.2.2.3 Changes in response to excess excitation energy*

Low light intensity acclimated plants were transferred to high illumination (PAR 1800 µmol $m^{-2}$ $s^{-1}$ at midday) to understand the role of the nonenzymatic antioxidants in light acclimations. Thereafter, leaf discs were collected every after 2 days for 10 days for both the low light intensity plants and the sun grown plants.

### 3.2.3 Sample preparation

Leaves for biochemical analyses were collected from the first fully expanded pair, thoroughly washed with tap water and then rinsed with reverse osmotic water. They were then flash frozen in liquid nitrogen before being kept under –80$^{o}$C in a refrigerator until further analysis. All the samples were then freeze dried at –20$^{o}$C (Eyela DRC 1000–FDU 1110, Tokyo, Japan) for 72 hours and then processed into a fine powder by milling with a blender (Wonder blender, Osaka,

Japan). The processed samples were kept in air−tight vinyl bags and stored together with silica gel to prevent moisture contamination throughout the experimentation period.

### 3.2.4 Pigments analysis

Chlorophylls and carotenoids were extracted from the freeze−dried powder using 80% chilled acetone in 50 mL Falcon tubes (Thermo Fisher Scientific, Massachusetts, USA) with minimum light conditions. The mixture was sonicated in ice−cold water bath for 1 hour and thereafter centrifuged at 29300 ×g at a temperature of 4ºC. Extraction was repeated 3 times until all the green colour from the residue was extracted. The supernatants for each sample were merged together and then filtered using 0.45µm Millipore (Sigma and Aldrich, Tokyo, Japan) for analysis. Quantification was done using a spectrophotometer (Hitachi ratio beam spectrophotometer, U−5100, Japan) according to Porra *et al.* (1989). Chlorophylls and carotenoid concentrations were calculated according to Porra*et al.,* (1989) and Lichtenthaler and Buschmann (2001), respectively.

### 3.2.5 HPLC analysis of the selected metabolites

The selected alkaloid and phenolic compounds were determined following procedures described in the previous section (2.2.4.4).

### 3.2.6 *Statistical analysis*

All the experimental data were statistically examined using one−way analysis of variance (One–way ANOVA) for each sub−experiment following a completely randomized design. The parameters were compared for statistical significance amongst the treatments using the Newman–Keuls test at $P \leq 0.05$. Assumptions of normality were tested using the Kolmogorov–Smirnov test and data transformed accordingly to attain a normal distribution whenever necessary. Statistical analyses were done using Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). Correlations amongst parameters and/ or relationships amongst the treatments for each sub–experiment were established independently using principal component analysis using Minitab version 17 software (Minitab Inc. State college PA, USA). Data are expressed as means ± SD, n = 5. Error bars represent standard deviation of the mean.

## 3.3 Results

The seasonal variations from summer through autumn to winter were associated with consistent decreases in the ambient temperature conditions (Fig. 5). Summer temperatures where highest

reaching a maximum of 34°C during the day and a minimum of 24°C at night at the beginning of September. On the other hand, the temperatures in the autumn season from late October to late November were moderate with an average maximum and minimum of 22.5°C and 13.5°C during day and night respectively. The onset of winter season from late November was characterised by a remarked drop in the ambient diurnal maximum average temperature to 13°C and an average minimum of 11°C during the night. The slight increase in the average temperatures recorded in December were due to installation of artificial heating system to avoid extreme low temperatures which would otherwise induce senescence of the coffee plants with progression of winter.

### 3.3.1 Changes in the pigment content

The response of the concentrations of the photosynthetic pigments to diel fluctuations seemed not to be statistically different as a result of temporal dynamics of circadian clock (Fig. 6). Nevertheless, the highest concentrations of chlorophyll *a* was observed at dusk whereas that of *b* was observed at dawn. The lowest concentration of the chlorophyll pigments was observed at midday. On the other hand, carotenoids tended to accumulate during day and plummeting during the dark conditions at night. Hence total chlorophylls and carotenoids varied antagonistically. Although the ratio of chlorophyll *a* to *b* ratio showed stability in the leaf tissues, that of total chlorophylls to total carotenoids was reduced during daylight with least ratio being recorded at the end of the day at 1600 hrs. In addition, a gradual recovery was observed from dusk with highest values observed at dawn. Therefore, onset of the day light was generally associated with a decrease in the ratio of chlorophyll to carotenoids.

Seasonal fluctuations in the ambient temperatures cued various effects on the metabolism of the photosynthetic pigments (Table 6). The concentrations of both chlorophylls *a* and *b* and their total were highest during summer when the ambient temperatures were highest and thereafter reducing gradually through autumn with winter conditions recording significantly least concentrations. Similarly, the concentrations of total carotenoids measured as sum of xanthophylls and β–carotene, although showed less variations, were highest during summer and gradually reduced through autumn. However, the onset of winter, in the early November, a remarked increase in the concentrations of carotenoids in the coffee leaves was observed. On the other hand, although the ratio of chlorophylls *a* to *b* showed no significant changes, that of chlorophylls to carotenoids were affected by seasonal changes (Table 6). The ratio of total

chlorophylls to total carotenoids were highest during summer season though autumn only significantly dropping during winter seasons during low ambient temperatures.

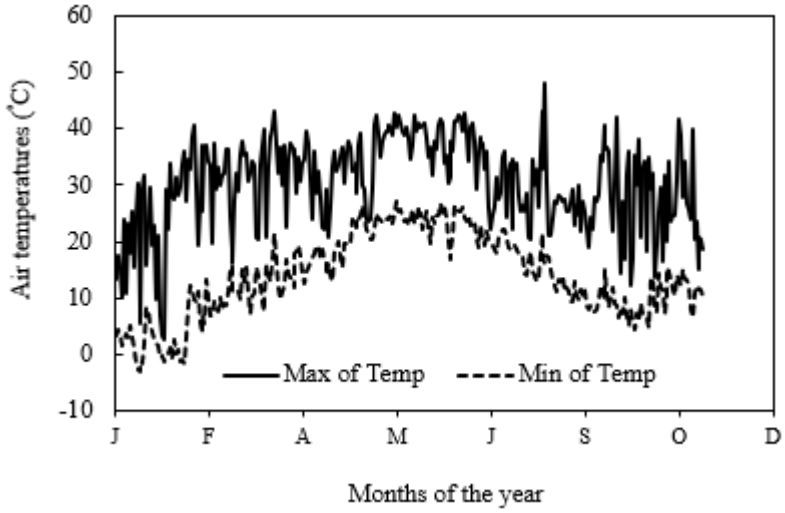


Fig. 5. Time–course changes in the monthly average of the ambient temperatures during a one–year period in the natural conditioned greenhouse at Tottori University (35° 30' 32.39" N, 134° 10' 10.80" E; 20 m a.s.l.) Western Japan.

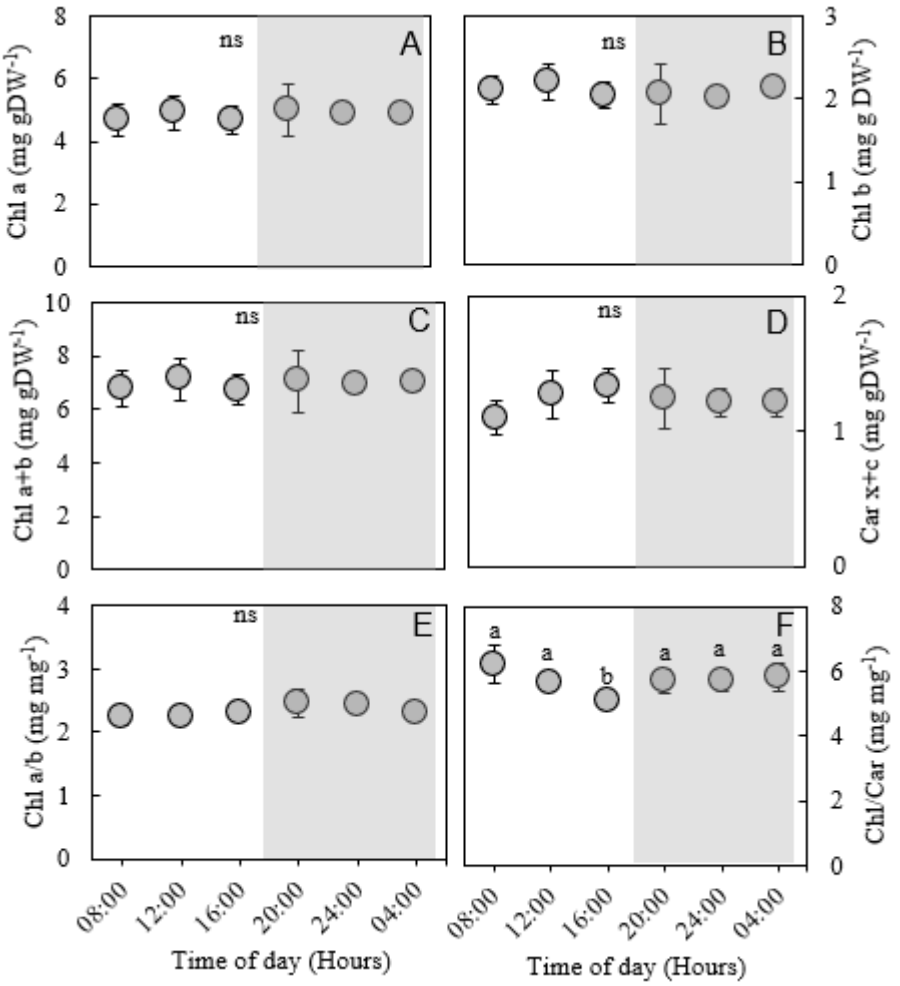


Fig. 6. Diel fluctuations in the concentration of chlorophylls and carotenoid pigments. Samples were collected every after 4 hours starting at 08:00 hrs in the morning. Gray portion represents night−time. Different letters represent significant differences between shade and sun leaves; ns denotes non−significance in the treatment means, $p \leq 0.05$ (Newman−Keuls test). The results are represented as mean ± SD, n = 5.

Table 6. Seasonal changes in the contents of the photosynthetic pigments, chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophylls (Chl a + b), total carotenoids ( Car x + c), and the ratios of chlorophyll a to b (Chl a / b) and chlorophylls to carotenoids (Chl / Car) in the leaves of coffee plants in response to changes in ambient environmental conditions over a period of 4 months from summer (August) to winter (December ).

| | | Seasonal fluctuations | | | | | |
|---|---|---|---|---|---|---|---|
| | **Temperature (day/night)** | **Chl a** | **Chl b** | **Chl a + b** | **Car x + c** | **Chl a / b** | **Chl / Car** |
| **Sampling date** | | **(mg gDW$^{-1}$)** | | | | | |
| 20th−Aug | 32/23ºC | 6.82 ± 0.81 b | 2.76 ± 0.33 b | 9.58 ± 1.12 b | 1.48 ± 0.12 b | 2.47 ± 0.08a | 6.47 ± 0.29 a |
| 3rd − Sept | 34/24ºC | 8.50 ± 0.28 a | 3.42 ± 0.14 a | 11.91 ± 0.38 a | 1.73 ± 0.03 a | 2.49 ± 0.09a | 6.91 ± 0.28 a |
| 24th − Sept | 23/18ºC | 6.30 ± 0.74 bc | 2.46 ± 0.34 bc | 8.77 ± 1.08 bc | 1.30 ± 0.09 b | 2.56 ± 0.07a | 6.71 ± 0.38 a |
| 08th − Oct | 26/12ºC | 6.59 ± 0.72 b | 2.57 ± 0.28 bc | 9.16 ± 0.98 bc | 1.34 ± 0.13 b | 2.57 ± 0.08a | 6.84 ± 0.10 a |
| 22nd − Oct | 20/12ºC | 5.37 ± 0.97 bcd | 2.19 ± 0.35 bcd | 7.55 ± 1.31 bcd | 1.19 ± 0.13 bc | 2.45 ± 0.12a | 6.33 ± 0.48 a |
| 5th − Nov | 21/12ºC | 4.11 ± 1.34 d | 1.56 ± 0.57 d | 5.67 ± 1.91 d | 0.99 ± 0.25 c | 2.67 ± 0.16a | 5.61 ± 0.54 b |
| 19th − Nov | 13/11ºC | 4.94 ± 0.99 cd | 1.96 ± 0.29 cd | 6.90 ± 1.27 cd | 1.28 ± 0.08 b | 2.50 ± 0.18a | 5.38 ± 0.74 b |
| 3rd − Dec | 20/15ºC | 5.05 ± 0.94 cd | 2.04 ± 0.52 cd | 7.09 ± 1.44 cd | 1.34 ± 0.31 b | 2.51 ± 0.27a | 5.34 ± 0.25 b |
| | *F* | 11.85 | 11.45 | 11.99 | 7.99 | 1.17 | 11.96 |
| | *p* | 0.0000 * | 0.0000 * | 0.0000 * | 0.000 * | 0.3494 ns | 0.0000 * |

Data are expressed as mean ± standard deviation, n = 5. Different letters represent significant differences ($p \leq 0.05$, Newman−Keuls test). *F*= F−test, p values followed by an asterisk (*) indicate statistical significance whereas ns indicates non−significance among the treatments.

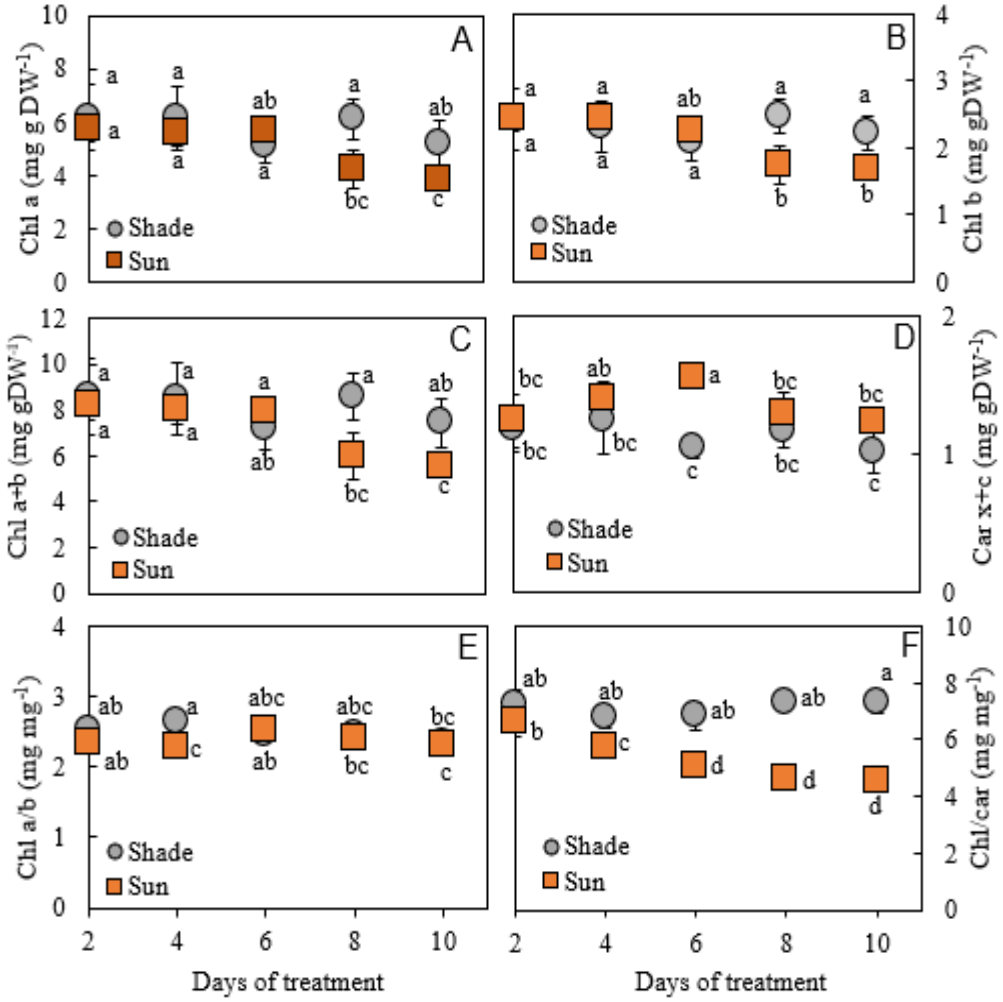


Fig. 7. Variation in the contents of photosynthetic pigments in response to excess excitation energy; (A) chlorophyll a (chl a), (B) chlorophyll b (chl b), (C) total chlorophyll (chl a + b), (D) total carotenoids (x + c), (E) chlorophyll a to b ratio (chl a / b) and (F) chlorophyll to carotenoid ratio (chl / car) in the leaves of coffee plants exposed to strong illumination (PPFD, 1810 µmol $m^{-2}$ $s^{-1}$) compared to shaded counterparts (PPFD, 250 µmol $m^{-2}$ $s^{-1}$) over a period of 10 days. Different letters represent significant differences amongst shade and sun leaves on different days of sampling whereas ns denotes non−significance amongst the treatment means, $p \leq 0.05$ (Newman−Keuls test) Error bars denote ± standard deviation of the mean, n = 5.

Exposure to excess excitation energy caused immediate fluctuations in the status of the photosynthetic pigments with the impact increasing with time of exposure (Fig. 7). The concentrations of the chlorophylls *a* and *b* as well as total chlorophylls were repressed when coffee plants were exposed to high illumination under direct sunlight (Fig. 7A, B and C). At the eighth day of sunlight exposure, the concentrations of the chlorophylls were significantly least in the sun grown plants than the shaded counterparts. On the hand, total carotenoids were stimulated by exposure to high light intensity with their concentration reaching maximum at day 6 of sunlight exposure and thereby significantly dropping at day 8 and day 10 of sunlight exposure to levels not significantly different from plants maintained under low light conditions. The ratio of chlorophyll *a* to *b* although dropped immediately on exposure to high light intensity conditions, it was reinstated to similar values to that of the low light acclimated counterparts by day 6. On the hand the ratio of total chlorophylls to carotenoids plummeted continuously with the day of exposure to high sunlight intensity and hence the least ratio was recorded by day 10 of high light exposure.

### 3.3.2 Changes in the selected metabolites

HPLC metabolite profiling revealed that the most abundant nonenzymatic antioxidant accumulating in coffee leaves was the hydroxycinnamic acid ester 5–CQA, followed by caffeine and trigonelline respectively and mangiferin being the least. Variation in the concentration of the nonenzymatic antioxidants in response to the circadian clock generally was not significant despite indicating some tendencies (Fig. 8). 5–caffeoylquinic acid increased by over 30.0% to 5.1% on a dry matter basis by midday compared to the least concentration of 3.9% in the morning. As a result, the total content of the metabolites was highest by 12:00pm. In addition, generally coffee leaves tended to contain more antioxidants during day than at night. The ratio of caffeoylquinic acid to caffeine was also highest during day and was least just before dawn and during the morning hours with a rapid change noticed by midday.

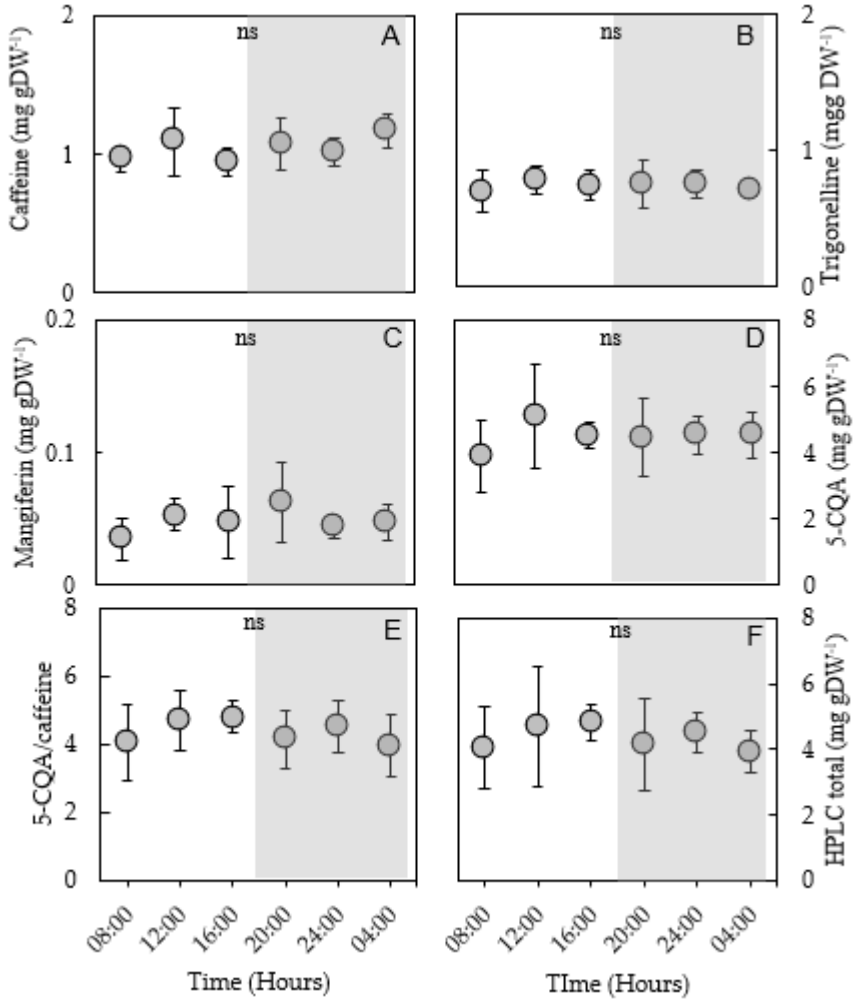


Fig. 8. Diel fluctuations in the concentration of selected metabolite compounds in the coffee leaves. Samples were collected every after 4 hours starting at 08:00 hrs in the morning. Gray portion represents night−time. Different letters represent significant differences between shade and sun leaves; ns denotes non−significance in the treatment means, $p \leq 0.05$ (Newman−Keuls test). The results are represented as mean ± SD, n = 5.

Both summer and winter conditions prompted higher accumulation of the metabolites compared to autumn conditions (Table 7). The 2 alkaloids, caffeine and trigonelline were significantly highest when coffee plants were growing under high temperatures in the summer thereafter maintaining non−significant variations in autumn and winter. Similarly, 5–caffeoylquinic acid was highest during summer and winter with significantly lowest concentrations noted in October during the autumn seasons when the ambient temperatures were moderate (Table 7). Mangiferin on the other hand, only significantly accumulated at the onset of winter season reaching a concentration of 1 mg $gDW^{-1}$ while generally the xanthonoid polyphenol remained unchanged throughout the seasons (Table 7). The concentration of the total antioxidant compounds was highest in summer with increase at the highest ambient temperature conditions compared to values recorded in the same seasons at lower temperatures (Table 7). Significantly least concentration of the nonenzymatic antioxidants was recorded at the beginning of October corresponding to the least values in the concentration of 5–caffeoylquinic acids. The ratio of 5–caffeoylquinic acid to caffeine generally exhibited a similar trend to that shown by the variation in the compounds although with slight differences. 5–caffeoylquinic acid/ caffeine ratio was highest during winter conditions when the temperatures were least. This ratio was also highest during summer whilst autumn conditions caused the least values.

Exposing the plants to high light intensity caused varied changes to the contents of the nonenzymatic antioxidant compounds in coffee leaves compared to those maintained under low light conditions (Fig. 9). The 2 alkaloids, caffeine and trigonelline although not significantly affected by excess excitation energy, showed sustained repressed levels under sunlight conditions compared to the plants growing under low light conditions (Fig. 9A and B respectively). On the contrary, 5–caffeoylquinic acid rapidly accumulated when the low light acclimated plants were transferred to excess direct sun light conditions and by day 8 of exposure, the content of 5–caffeoylquinic acid was significantly higher in sunlit plants than shaded ones (Fig. 9C). A similar trend was noticed in the content of mangiferin although the variation was not significantly between the 2 group (Fig. 9D). The rapid surge of 5–caffeoylquinic acid in the sunlit plants caused a higher 5–CQA/caffeine ratio while this ratio remained low and with no significant variation on all the sampling days (Fig. 9E). The total of antioxidant compound content showed a similar trend in variation with significantly highest content observed by day 8 of sunlight exposure (Fig. 9F).

Table 7. Seasonal changes in the contents of selected metabolites; ; caffeine, trigonelline, 5–caffeoylquinic acid (5–CQA), mangiferin, total metabolite content and the ratio of 5–caffeoylquinic acid to caffeine (5–CQA/CAF) in the leaves of coffee plants in response to changes in ambient environmental conditions over a period of 4 months and during a 24 h period from summer (August) to winter (December ).

| | | Seasonal fluctuations | | | | | |
|---|---|---|---|---|---|---|---|
| **Sampling date** | **Temperature (day/night)** | **Caffeine** | **Trigonelline** | **5–CQA** | **Mangiferin** | **Total** | **5–CQA / CAF** |
| | | | | **(mg gDW$^{-1}$)** | | | |
| 20th−Aug | 32/23ºC | 0.82 ± 0.14 b | 0.50 ± 0.07 b | 4.14 ± 0.74 ab | 0.06 ± 0.03 ab | 5.51 ± 0.83 bc | 5.10 ± 1.01 ab |
| 3rd − Sept | 34/24ºC | 1.25 ± 0.08 a | 0.70 ± 0.18 a | 5.32 ± 1.23 a | 0.06 ± 0.04 ab | 7.33 ± 1.28 a | 4.23 ± 0.90 bc |
| 24th − Sept | 23/18ºC | 1.00 ± 0.10 b | 0.54 ± 0.11 b | 3.32 ± 0.60 bc | 0.05 ± 0.01 ab | 4.90 ± 0.73 bc | 3.34 ± 0.64 c |
| 08th − Oct | 26/12ºC | 0.87 ± 0.08 b | 0.49 ± 0.02 b | 2.54 ± 0.18 c | 0.04 ± 0.02 b | 3.94 ± 0.26 c | 2.92 ± 0.14 c |
| 22nd − Oct | 20/12ºC | 1.01 ± 0.11 b | 0.52 ± 0.08 b | 3.61 ± 0.59 bc | 0.06 ± 0.02 ab | 5.21 ± 0.75 bc | 3.54 ± 0.26 c |
| 5th − Nov | 21/12ºC | 0.77 ± 0.12 b | 0.39 ± 0.07 b | 4.72 ± 1.00 ab | 0.10 ± 0.04 a | 5.97 ± 1.12 b | 6.16 ± 1.02 a |
| 19th − Nov | 13/11ºC | 0.75 ± 0.14 b | 0.39 ± 0.11 b | 4.17 ± 0.51 ab | 0.06 ± 0.02 ab | 5.37 ± 0.60 bc | 5.72 ± 1.03 a |
| 3rd − Dec | 20/15ºC | 0.92 ± 0.24 b | 0.44 ± 0.06 b | 3.83 ± 0.73 bc | 0.05 ± 0.04 ab | 5.24 ± 1.05 bc | 4.24 ± 0.44 bc |
| | *F* | 7.32 | 5.45 | 6.28 | 2.15 | 5.98 | 11.62 |
| | *p* | 0.0000 | 0.0003 * | 0.0001 * | 0.0677 * | 0.0002 * | 0.0000 * |

Data are expressed as mean ± standard deviation, n = 5. Different letters represent significant differences ($p \leq 0.05$, Newman−Keuls test). *F*= F−test, p values followed by an asterisk (*) indicate statistical significance whereas ns indicates non−significance among the treatments.

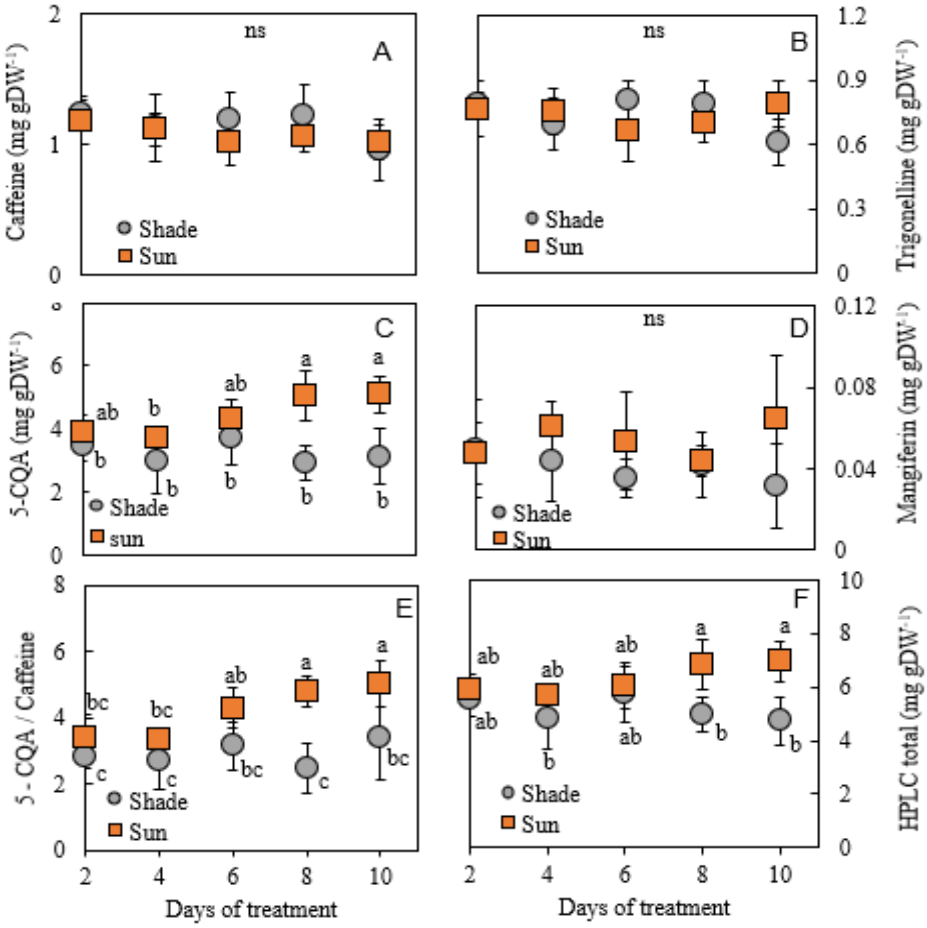


Fig. 9. Variation in the contents of selected metabolites in response to excess excitation energy; (A) caffeine, (B) trigonelline, (C) 5 – caffeoylquinic acid (5–CQA), (D) mangiferin, (E) 5 – caffeoylquinic acid – caffeine ratio and (F) total of the above metabolites in the leaves of coffee plants exposed to strong illumination (PPFD, 1810 µmol $m^{-2}$ $s^{-1}$) compared to shaded counterparts (PPFD, 250 µmol $m^{-2}$ $s^{-1}$) over a period of 10 days. Different letters represent significant differences amongst shade and sun leaves on different days of sampling whereas ns denotes non−significance amongst the treatment means, $p \leq 0.05$ (Newman−Keuls test) Error bars denote ± standard deviation of the mean, (n = 5).

### 3.3.3 Relationships amongst pigments and nonenzymatic antioxidant compounds

Principal component analysis (PCA) generally revealed strong positive correlations within the pigments and amongst the nonenzymatic antioxidant compounds while the two groups (pigments and nonenzymatic antioxidants) generally correlated negatively (Fig. 10, Table 8, 9 and 10). In response to diel fluctuations, although the variation in the variables did not indicate clear clustering as shown by the score plot (Fig. 10A), the loading plot still indicated the general trend in−terms of strong correlations within the pigments and within the nonenzymatic antioxidant compounds. Nevertheless, chl/car variation was independent of the two (Fig. 10B and Table 8).

On the hand, PCA for seasonal fluctuations generally clustered the experimental treatments in to 3 groups with summer on the left, winter on the right while samples collected during autumn conditions were positioned in the middle of the score plot although with some leaf samples overlapping (Fig. 10C). Moreover, this clustering was associated with corresponding variation in the content of the photosynthetic pigments and nonenzymatic antioxidants compounds especially phenolic compounds as well as 5–CQA/CAF ratio on the left and right of the loading plot respectively (Fig. 10D). Caffeine and trigonelline variation indicated strong positive correlations ($p=0.01$) with chl a, chl b, total chl a + b, as well as chl/car ratio while these pigments strongly negatively correlated ($p=0.01$) with mangiferin, 5–CQA and 5–CAQ/CAF ratio (Table 9).

In response to excess excitation energy, PCA categorised the leaf samples in to two groups situated on the left and the right side of the score plot (Fig. 10E) with the magnitude increasing with increasing day of growth under sunlight conditions. The corresponding loading plot revealed that 5–CQA, mangiferin, as well as 5–CQA/CAF ratio and the total antioxidant content showed strong positive correlations similar to that shown amongst chl a, chl b and chl a + b (Fig. 10F). The two groups of the variables however, showed significant negative correlations ($p=0.01$) and the magnitude was highest between chl/car and 5–CQA and between chl/car and 5–CQA/CAF ratios (Table 10).

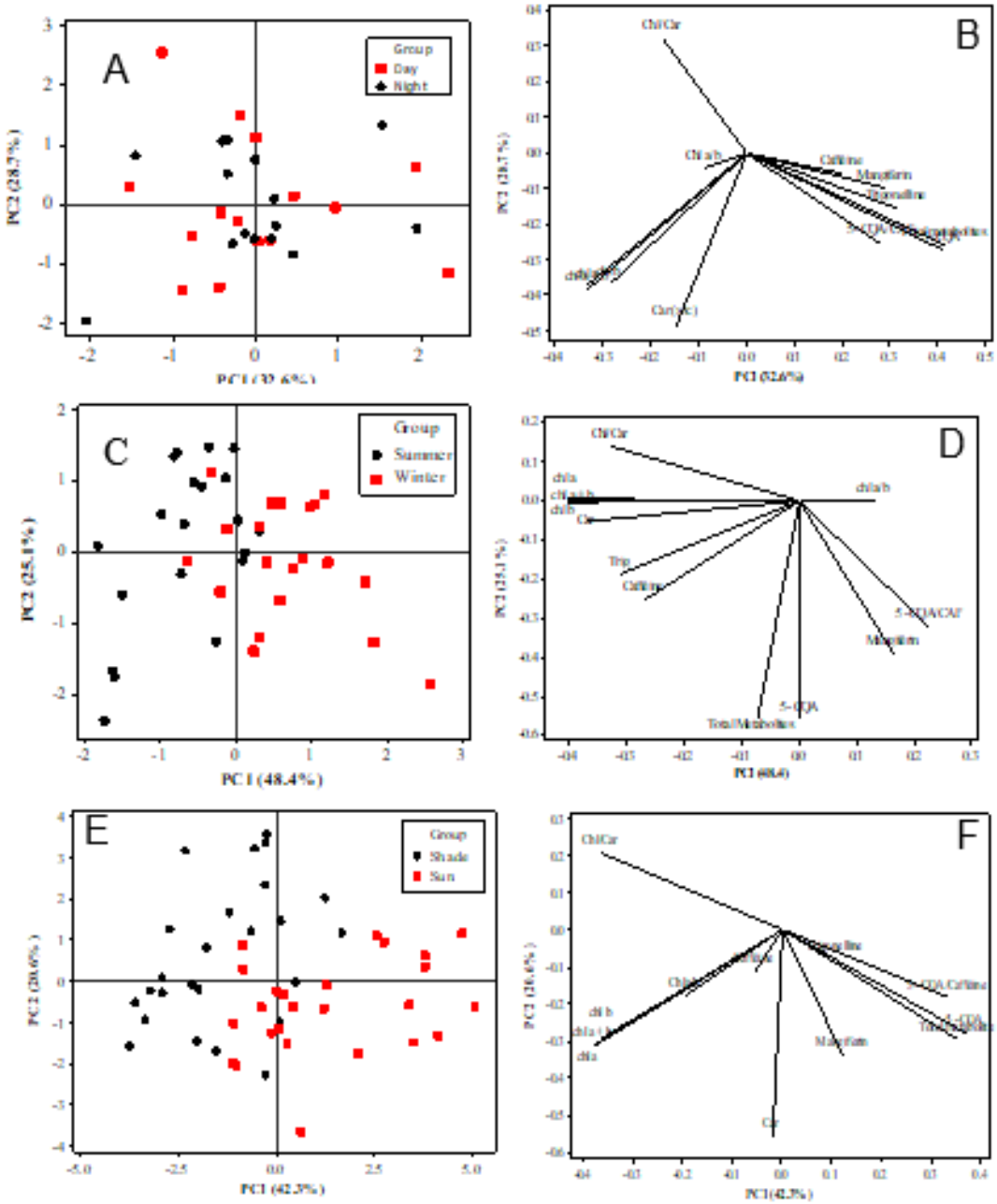


Fig. 10. Score plots and loading plots of diel, seasonal and in responses to excess excitation energy (A, C and E) and (B, D and F) of the variables: caffeine, 5 – CQA, trigonelline, mangiferin, 5–CQA/CAF, total nonenzymatic acid content, chl a, chl b, total chl a + b, total carotenoids, chl a/b and chl/car.

## 3.4 Discussion

Arabica coffee evolved as an understorey tropical species in the highlands of tropical Africa under moderate environmental conditions such as maximum annual average temperature of about 20°C (Damatta and Ramalho, 2006). However, the plasticity of its photosynthetic apparatus has enabled its cultivation in a range of geographical areas with extreme environmental conditions (Chaves *et al.,* 2008). Nevertheless, adaptation to such environments relies on physiological and metabolic adjustments that occur on daily or seasonal basis (Ramalho *et al.,* 2014).

### 3.4.1 Circadian oscillations

The circadian clock regulates several physiological and metabolic plant processes through modulating the relative expression of associated genes (Johnson *et al.,* 2003; Shin *et al.*, 2017). Light generally switches on genes that regulate photosynthetic activity in chloroplasts as the main activity during daytime (Sulpice *et al.,* 2014; Moraes *et al.,* 2019). In the chloroplasts, photosynthesis is carried out by the two types of chlorophyll molecules *a* and *b* that are normally localized in the photosystems in the thylakoid membrane (Porra *et al.,* 1989). Chlorophylls molecules execute the role of light capture and its subsequent utilization for starch metabolism in a process that result into photo–degradation of the pigments hence resulting into low chlorophyll concentration during the day (Rontani *et al.,* 1996; Lee *et al.,* 2014). However, leaves contain protective carotenoids which dissipate excess excitation during daylight thereby protecting the chlorophylls. This protection mechanism is achieved through de–epoxidation in the xanthophyll cycle through which violaxanthin is converted to xanthophyll (Demmig−Adams and Adams, 2004; Verhoeven, 2014). In the current study, slightly higher concentrations of carotenoids and lower concentrations were observed during the day than at night and hence causing significantly lower chlorophylls to carotenoid ratio by the end of the day. This ratio is an indication of the status of photosynthetic apparatus with lower values signifying exhaustion or reduced photosynthetic efficiency (Porra *et al.,* 1989). In addition, day light tended to cause somewhat rapid accumulation of antioxidants especially phenolic compounds like 5–CQA although with little effect on alkaloids especially caffeine. Therefore, daylit leaves tended to have higher ratios of 5–CQA/caffeine than their night counterparts. Higher ratios of chlorogenic acids like 5–CQA to caffeine is associated with higher ROS scavenging ability due to higher antioxidant capacity of phenolic compounds compared to alkaloids (Ky *et al.*, 2001).

### 3.4.2 Summer fluctuations

In many production areas, temperatures as high as 30$^{o}$C are not uncommon especially during summer (Damatta and Ramalho, 2006). Although high temperatures may translate in to rapid metabolism, prolonged exposure may result into uncoupling of several physiological and metabolic processes culminating into depressed growth (Awasthi *et al.,* 2015). In fact, it was earlier demonstrated that temperature above 24$^{o}$C may lead mesophyll resistance resulting in to diminished leaf conductance and photosynthetic productivity (Kumar and Tieszen, 1980). Moreover, extremely high temperatures in in coffee plants were associated with low photosynthetic rates especially in young leaves (Marias *et al.,* 2017; Rodrigues *et al.*, 2018). This is also caused by low photosynthetic efficiency which results in to production of ROS whose homeostatic regulation is crucial for survival under abiotic stress (Demmig−Adams and Adams, 2004). Summer conditions were associated with high pigments concentration which could have been an adaptive measure to support high metabolic rates (Table 6). Coffee plants exhibit some degree of plasticity and have been reported to adapt to relatively unfavourable environmental conditions (Damatta and Ramalho 2006; Ramalho *et al.,* 2014). Higher temperatures in the summer also elicited high amounts the nonenzymatic antioxidant compounds especially 5–CQA resulting in to higher 5–CQA/caffeine ratio hence enhancing their ROS scavenging capacity (Ky *et al.*, 2001).

### 3.4.3 Winter fluctuations

The month of October was associated with a remarkable drop in the ambient temperatures resulting into a typical autumn cool season through to mid−November after which temperature dropped further signifying the start of winter (Table 6 and 7). During cold temperature condition such as in winter coffee plants produce excessive amounts ROS due to leakage of electrons to oxygen molecule in the chloroplast and mitochondrial electron transport system (Mittler, 2002). During such abiotic stress conditions, the heightened increase in the production of these molecules increases the probability of erroneous energy transfers within the photosynthetic machinery leading to breaching of the pigments such as those in $P_{680}$ and D1 protein sub unit (Damatta and Ramalho, 2006). In the current study, this was evidenced by rapid disintegration of the pigments in the winter months which were associated with low positive temperatures (Table 6). Furthermore, low concentration of photosynthetic pigments leads to increased production of ROS due to reduction in the efficiency of the photosynthetic machinery as observed by a decrease in the chlorophyll to carotenoid ratio (Porra *et al.,* 1989)

similar to the observation in the current study (Table 7). Similarly, the content of antioxidant compounds especially 5–CQA which had reduced during autumn once again increased at the onset of colder conditions during winter as an adaptative measure against excessive ROS accumulation.

Table 8. Correlation between the content of pigments and selected metabolites during diel fluctuations

| | Caffeine | 5 − CQA | Trigonelline | Mangiferin | 5 − CQA/Caf | Total metabolites | Chl a | Chl b | Chl a+b | Carotenoids | Chl a/b | Chl/Car |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Caffeine | | | | | | | | | | | | |
| 5 − Caffeoylquinic acid | .491** | | | | | | | | | | | |
| Trigonelline | .161 | .535** | | | | | | | | | | |
| Mangiferin | .331 | .507** | .216 | | | | | | | | | |
| 5 − CQA/Caf | −.281 | .686** | .441* | .296 | | | | | | | | |
| Total metabolites | .585** | .990** | .587** | .521** | .597** | | | | | | | |
| Chl a | −.106 | −.153 | −.216 | −.118 | −.065 | −.170 | | | | | | |
| Chl b | .045 | −.048 | −.102 | −.306 | −.107 | −.050 | .813** | | | | | |
| Chl a+b | −.068 | −.131 | −.193 | −.182 | −.082 | −.144 | .985** | .902** | | | | |
| Carotenoids | −.110 | .141 | .063 | −.035 | .262 | .110 | .743** | .707** | .762** | | | |
| Chl a/b | −.213 | −.142 | −.199 | .322 | .076 | −.166 | .369* | −.239 | .202 | .106 | | |
| Chl/Car | .050 | −.415* | −.350 | −.224 | −.520** | −.387* | −.034 | −.044 | −.037 | −.668** | .018 | |

**. Correlation is significant at the 0.01 level (2−tailed).

*. Correlation is significant at the 0.05 level (2−tailed).

.

Table 9. Correlation between the content of pigments and selected metabolites during seasonal fluctuations

| | Trigonelline | 5 − CQA | Caffeine | Mangiferin | 5 − CQA/Caffeine | Total metabolite | Chl a | Chl b | Chl a+b | Car | Chl a/b | Chl/Car |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trigonelline | | | | | | | | | | | | |
| 5 − CQA | .215 | | | | | | | | | | | |
| Caffeine | .665** | .378* | | | | | | | | | | |
| Mangiferin | .044 | .526** | .096 | | | | | | | | | |
| 5 − CQA/Caffeine | −.343* | .622** | −.470** | .448** | | | | | | | | |
| Total metabolite | .406** | .973** | .569** | .506** | .440** | | | | | | | |
| Chl a | .652** | .015 | .505** | −.396* | −.414** | .156 | | | | | | |
| Chl b | .665** | .022 | .529** | −.397* | −.420** | .167 | .982** | | | | | |
| Chl a + b | .658** | .018 | .514** | −.398* | −.417** | .161 | .998** | .991** | | | | |
| Car | .587** | .111 | .467** | −.372* | −.280 | .227 | .903** | .931** | .914** | | | |
| Chl a/b | −.215 | .018 | −.206 | .193 | .164 | −.036 | −.174 | −.347* | −.225 | −.385* | | |
| Chl/Car | .546** | −.229 | .386* | −.379* | −.561** | −.088 | .806** | .753** | .793** | .491** | .042 | |

**. Correlation is significant at the 0.01 level (2−tailed).

*. Correlation is significant at the 0.05 level (2−tailed).

Table 10. Correlation between the content of pigments and selected metabolites during changes in excitation energy

| | Caffeine | 5 – CQA | Trigonelline | Mangiferin | 5 –CQA/CAF | HPLC Total | Chl a | Chl b | Chl a+b | Car | Chl a/b | Chl/Car |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Caffeine | | | | | | | | | | | | |
| 5 – CQA | −.034 | | | | | | | | | | | |
| Trigonelline | .425** | .277 | | | | | | | | | | |
| Mangiferin | .367** | .347* | .228 | | | | | | | | | |
| 5 – CQA/CAF | −.553** | .837** | −.031 | .113 | | | | | | | | |
| HPLC Total | .205 | .966** | .454** | .435** | .680** | | | | | | | |
| Chl a | .119 | −.466** | −.146 | −.046 | −.438** | −.431** | | | | | | |
| Chl b | .117 | −.461** | −.105 | .007 | −.434** | −.422** | .949** | | | | | |
| Chl a+b | .120 | −.470** | −.136 | −.032 | −.442** | −.433** | .996** | .972** | | | | |
| Car | −.065 | .267 | −.133 | .315* | .274 | .228 | .448** | .422** | .446** | | | |
| Chl a /b | .042 | −.201 | −.147 | −.165 | −.189 | −.199 | .530** | .239 | .457** | .251 | | |
| Chl/Car | .135 | −.726** | −.027 | −.347* | −.681** | −.664** | .582** | .589** | .590** | −.452** | .214 | |

**. Correlation is significant at the 0.01 level (2−tailed).

*. Correlation is significant at the 0.05 level (2−tailed).

### 3.4.4 Excess excitation energy

The photosynthetic apparatus in the leaves of arabica coffee plants is adapted to operate efficiently between photosynthetically active radiation (PAR) 300–700 µmol$^{-2}$ s$^{-1}$ (Clifford, 2012; Ramalho *et al.,* 2014). High light intensity causes excess excitation energy resulting into inability of the photosynthetic apparatus to utilize all the incident PAR (Muller, 2001). In the current study, sudden exposure of low light acclimated coffee plants to PAR 1800µmol m$^{-2}$ s$^{-1}$ was accompanied with progressive reduction in the concentration of chlorophyll molecules (Fig. 7). It has been suggested that this is an adaptive measure to reduce the amount of absorbed light energy hence reducing the likelihood of excess energy which would otherwise lead to accumulation of ROS and cause oxidative stress (Ruban, 2016). Nevertheless, the excess excitation energy in the current study caused damage to the photosynthetic apparatus which was also associated with increased production of the protective carotenoid molecules (Fig. 7) which is another photoprotective mechanism. In addition, higher amounts of antioxidant compounds were observed in sunlit plants compared to shaded counterparts (Fig. 9) for the same purpose (Das and Roychoudhury, 2014).

### 3.4.5 Photoprotective role of non–enzymatic antioxidants

Coffee plants are increasingly cultivated under adverse climatic conditions characterised by harsh environmental conditions caused by climate change as well expansion of cultivation to new production areas (Damatta and Ramalho, 2006; Davis *et al.,* 2012, 2019). In the current study, under extreme environmental conditions such as high and low temperatures and/or high light intensity, the photosynthetic apparatus of coffee plants was damaged (Fig. 6 and 7, and Table 6). This was accompanied by increased content of antioxidant compounds especially phenolics such as 5–CQA and to a less extent mangiferin. These compounds are known ROS scavengers especially in the young leaves of coffee plants during cold stress and excessive sunlight conditions (Campa *et al.,* 2017; Rodrigues *et al.,* 2018). Similar studies have observed high amounts of such compounds in addition to others like flavonoids and vitamins during both high light and cold stress conditions (Campa *et al.,* 2017; Ramalho *et al.,* 2018). The reliance on such compounds to scavenge ROS is attributed to a poorly developed enzymatic antioxidant defence system in the young leaves of coffee plants but also as a result of cold–induced suppression of enzyme activity (Campa *et al.,* 2017; Ramalho *et al.,* 2018).

These secondary metabolites neutralize ROS using their inherent glycosylic linkages and hydroxyl components (Das and Roychoudhury, 2014). Accumulation of these protective

molecules is likely regulated by ROS especially $H_2O_2$ whose role in systemic signaling is increasingly becoming appreciated (Sies, 2017). The results in the current study therefore suggest that coffee plants can adapt to extreme temperature and high light stress conditions through adjustment in the pigment system and antioxidant defense mechanism. Moreover, it is demonstrated (Fig. 10) that although oxidative stress conditions result into breakdown of the chlorophyll pigments, such conditions result into accumulation of protective compounds responsible for homeostatic control of ROS.

### 3.5 Conclusion

Changes in the ambient environmental dynamics were associated with fluctuations in the pigments and the antioxidant compounds assessed in the current study. During a 24–hr period, circadian rhythm was associated with light dependent changes in the concentration of chlorophylls and carotenoids although non–significantly. The latter tended to increase under daylight conditions hence causing significantly highest ratio chlorophylls of chlorophylls to carotenoids by the end of the day. In addition, daylight conditions were associated with slightly higher amounts of antioxidant compounds especially 5–CQA which peaked at midday. On the hand, both summer and winter conditions elicited higher amounts of the protective antioxidant compounds following a remarkable damage to the photosynthetic apparatus especially during winter. Similarly, exposure of coffee plants to excess excitation energy caused a decline in the contents of the photosynthetic pigments and damage to the photosynthetic apparatus as indicated by low chlorophyll to carotenoid ratio. This was accompanied by rapid accumulation of antioxidant compounds especially phenolics compared to alkaloids. Therefore, the current results suggest that antioxidant compounds especially 5–CQA and/or mangiferin are rapidly produced during abiotic stress conditions where they detoxify ROS to restore homeostatic conditions.

## Chapter four

## Foliar nitrogen supply enhances the recovery of photosynthetic performance in cold−stressed coffee seedlings

### 4.1 Introduction

In the previous chapter, changes in the ambient environmental conditions especially during winter caused a significant decline in the efficiency of the photosynthetic apparatus therefore posing several limitations to photosynthetic productivity of the plants. This chapter therefore evaluated the recovery of the photosynthetic apparatus of the cold−stressed plants through foliar application of nitrogen in urea form and its concomitant effect on the content of the selected metabolites.

Coffee (*Coffea* spp.) evolved as an understorey shrub species in the tropical rainforest of the mainland Africa and the island of Madagascar under moderate light and temperature conditions (Charrier and Berthaud, 1985). Due to its economic value, however, coffee cultivation has expanded to various geographical zones with more severe seasonal climatic conditions and fluctuations such as low night temperatures and high light intensity during winter seasons in Brazil which is the largest coffee producing country globally (DaMatta and Ramalho, 2006). The ability of the coffee plants to adapt to such adverse conditions is owed to the plasticity of its photosynthetic apparatus and a number of photoprotection mechanisms (Chaves *et al.,* 2008; Matos *et al.,* 2009; Ramalho *et al.,* 2014). Nevertheless, the typical low non−freezing temperatures (between 18 and 4°C) during winter conditions even in *C. arabica*, which is a seemingly more cold−tolerant species, result into diminished vegetative growth as a result of low photosynthetic productivity instigated by both biochemical and diffusivity limitations (DaMatta *et al.,* 1997; Ramalho *et al.,* 2003; Ramalho *et al.,* 2014).

Low non−freezing temperatures can cause significant degradation of chlorophyll pigments and breakdown of the photosynthetic apparatus (Adams *et al.,* 2004). This is instigated by low photosynthetic efficiency with leads to overproduction of reactive oxygen species (Mittler, 2002; Asada, 2006). Reactive oxygen species (ROS) in chloroplasts are produced as a result of photoinhibition caused by excess excitation energy in the photosynthetic apparatus (Asada 1999; DaMatta, 2004; Demming−Adams and Adams, 2004; Rio *et al.,* 2006). Plants are equipped with an elaborated ROS defence mechanism which is comprised of both enzymatic and nonenzymatic antioxidant systems (Logan *et al.,* 2006). Antioxidant enzymes such as

superoxide dismutase catalyse the breakdown of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide ($H_2O_2$). Consequently, the $H_2O_2$ is neutralized by catalase and ascorbate peroxidase (Asada, 1999). In addition, a number of low molecular weight compounds including both lipophilic and hydrophilic compounds have been characterised as antioxidant scavengers (Das and Roychoudhury, 2014). Recently, the role of alkaloids and phenolic compounds in defence against ROS in coffee plants in increasingly becoming appreciated (Ramalho *et al.,* 2018). Profound systematic accumulations of high amounts of alkaloids such as caffeine and trigonelline together with phenolic compounds such as chlorogenic acids and mangiferin in all the cells of the young leaf blades in sun−grown plants compared to their shade counterparts have been reported (Campa *et al.,* 2012; 2017). The surge in these secondary metabolites provides the evidence for their role in defence against ROS that are produced in conditions of excess excitation energy to which either the coffee plants is not usually adapted or instigated by abiotic stress conditions. Moreover, because of a repressed enzymatic antioxidant system during cold stress conditions, coffee plants rely on the nonenzymatic antioxidant scavengers for restoration of ROS to homeostatic conditions (Ramalho *et al.,* 2018).

The inherent inability of the rapid remobilization of nitrogen on resumption of favourable temperatures further causes oxidative stresses due to a mismatch between biochemical and diffusivity aspects of photosynthesis (DaMatta *et al.,* 1999; Pompelli *et al.,* 2010). Nevertheless, adequate nitrogen supply such as irrigating with 250 mL containing 23 mM N every after 2 weeks for 45 days has been reported to improve photosynthesis and overall metabolism of coffee plants during winter conditions and also in the growing season during summer (Ramalho *et al.,* 2000; DaMatta *et al.,* 2002; Carelli *et al.,* 2006; Pompelli *et al.,* 2010; Bote *et al.,* 2018). In addition, nitrogen (nutrition) has also been reported to facilitate the recovery of plants such as rice after exposure to low temperatures (Liu *et al.,* 2019). The use of foliar fertilizers such as urea as a corrective relief for physiological stress is commended for its efficiency and instantaneous assimilation into plant tissues compared to conventional nitrogen application in the soil normally used in coffee production (Gray and Akin, 1984; Carelli *et al.,* 2006; Bhuyan *et al.,* 2012; Oad *et al.,* 2018). Soil fertilization generally requires high application rates that result into NOx diminution by soil bacteria which lead to greenhouse gas production (Shepherd *et al.,* 1991). On the other hand, the application rates during foliar fertilization are normally lower with no risk of NOx diminution while inducing quick responses in plants (Fageria *et al.,* 2009). Nevertheless, foliar fertilization comes together with challenges that might arise when toxic concentration levels of foliar sprays are applied causing burns and cell mortality

(Krogmeier *et al.,* 1989). Moreover, the effect of different concentrations of foliarly applied nitrogen in form of urea on the recovery of the photosynthetic apparatus with concomitant effects on the net $CO_2$ assimilation and related parameters and the content of selected secondary metabolites such as caffeine, trigonelline, 5−CQA and mangiferin in coffee plants is rather not known. We anticipated that an appropriate concentration level of nitrogen applied as foliar urea sprays would improve the biochemical composition resulting into the recovery of the photosynthetic efficiency of the previously cold−stressed coffee plants.

This hypothesis was tested by measuring the changes in gas exchange and associated chlorophyll fluorescence parameters of the coffee plants treated with serial concentrations of nitrogen applied as foliar urea sprays. In addition, the contents of photosynthetic pigments, and selected leaf alkaloid and phenolic compounds in coffee plants were analysed.

## 4.2 Materials and methods

### 4.2.1 Plant material and growing conditions

Plant materials and growing conditions have been described in the previous chapter (see section 2. 2).

The coffee seedlings were cultivated until the onset of winter season (December 2018) under naturally fluctuating environmental conditions mostly temperature (see chapter 3, Fig. 5). The average minimum winter temperatures caused visual signs of cold stress including stunted growth and yellowing of the leaves (Fig. 11).

### 4.2.2 Experimental treatments

For experimentation, uniformly visually cold−stressed seedlings were transferred into the growth chamber and grown under 250 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD) provided by white luminescent lamps at a 12 hours photoperiod, temperature of 25/20°C, (day and night, respectively) and a 70% relative humidity. Five treatments were thereafter established by adjusting the concentration of nitrogen applied as foliar sprays in form of urea. The treatments included: Control (0), 5, 10 20, and 40 mM nitrogen. Isopropyl alcohol (Kao Global Chemicals, Tokyo, Japan) solution (1%) was used as a surfactant in all the treatments. The sprays were administered just before darkness, one time per week for 90 days on the whole shoot until runoff on the leaves occurred. The sprays were applied on all the experimental units at once using a volume of 20 mL of the urea solution per treatment. All the seedlings were

continuously irrigated with the previously described Hoagland solution but without nitrogen throughout the experimental term.

### 4.2.3 Gas exchange and chlorophyll fluorescence measurements

Photosynthetic responses and chlorophyll fluorescence parameters were measured on fully expanded leaves of the first pair using Li 6400 XT open gas exchange system (LICOR Biosciences, Lincoln, NE, USA). The system was checked for leaks and calibrated each time before the measurements were taken. Gas exchange and light−adapted state fluorescence measurements were done at the 6$^{th}$ hour of illumination at a 400 ppm $CO_2$ concentration and a photosynthetic photon flux density (PPFD) of 500 µmol $m^{-2}$ $s^{-1}$ (10% blue) as according to Pompelli *et al*. (2010). The dark−adapted state fluorescence parameters were measured 6 h after the onset of darkness using the same system. Nonphotochemical quenching, proportion of open photosystem II (PSII) centres and the maximum quantum efficiency of the photosystem II photochemistry for both the dark−adapted and light−adapted states were calculated according to Murchie and Lawson (2013).

### 4.2.4 Biochemical analysis

*4.2.4.1 Sample preparation*

The leaves were sampled and processed as previously described in the previous chapter (subsection 3.2.3).

*4.2.4.2 Total nitrogen concentrations*

The concentration of nitrogen in the leaf samples was measured using an organic elemental analyser (CN corder JM1000CN Tokyo, Japan).

*4.2.4.3 Photosynthetic pigments extraction and quantification*

Chlorophylls and carotenoids were assayed and determined as previously described (subsection 3.2.4).

*4.2.4.4 HPLC quantification of the selected metabolites*

Selected alkaloids and phenolic were assayed and determined as previously described (subsection 2.2.4.4).

Fig. 11. Coffee seedlings exhibiting visual signs such as yellowing of the leaves induced by cold stress conditions

### 4.3 Statistical analyses

Experimental data was analysed using a one–way analysis of variance (one–way ANOVA) by comparing variation in the parameters among the treatments. Comparison for statistical differences were made using the Newman–Keuls test at $p \leq 0.05$. Normality tests were done using the Kolmogorov–Smirnov test and data transformed accordingly to attain a normal distribution whenever necessary. All the statistical analyses were done using Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). Data are expressed as means ± SD, $n \geq 3$.

### 4.4 Results

#### 4.4.1 Total nitrogen concentrations

Total nitrogen content in the leaves increased considerably with increasing concentration of the urea foliar sprays (Table 11). Compared to control treatment plants, the concentration of nitrogen in the leaves increased with increasing concentration of nitrogen in the foliar sprays for 5, 10, and 20mM treatments. However, despite showing no statistical significance, a further increase of 100% in the concentration of foliar nitrogen supply to 40 mM caused a 3.5% decline in the concentration of leaf nitrogen compared to the preceding (20 mM) treatment.

#### 4.4.2 Photosynthetic pigments

Variation in the concentration of chlorophylls and carotenoids is indicated in Table 11. The concentrations of the respective chlorophyll components *a* and *b* and their corresponding total (*a*+*b*) were least in the leaves of control treatment plants. These increased with increasing concentration of the nitrogen supply in the treatments. This increasing trend climaxed in the 20 mM treatment plants with a concentration while further increase in the concentration level of the foliar sprays to 40 mM caused a consistent decline of 16.5, 13.7 and 15.9% in the concentrations of chlorophyll *a*, *b* and their corresponding total (*a*+*b*).

Table 11. Effect of foliar nitrogen supply on the concentrations of leaf total nitrogen (N) and photosynthetic pigments; chlorophylls a and b (Chl a and Chl b respectively), total chlorophyll (Chl a + b), total carotenoids (Car x+c), ratios of chlorophyll a to b (Chl a/b), chlorophyll to carotenoids (Chl/Car), chlorophyll a to nitrogen (Chl a/N), chlorophyll b to N (Chl b/N), total chlorophyll to N Chl/N) and that of total carotenoids to N (Car/N) in the leaves of coffee seedlings recovering from cold−stress treated with different concentrations of foliar nitrogen in form of urea weekly for 3 months in a growth chamber.

| | Foliar nitrogen supply [mM] | | | | |
|---|---|---|---|---|---|
| Parameter | 0 | 5 | 10 | 20 | 40 |
| Total N (mg $gDW^{-1}$) | 19.3 ± 4.3c | 25.3 ± 3.0b | 29.4 ± 2.6ab | 34.4 ± 1.0a | 33.2 ± 3.8a |
| Chl *a* (mg $gDW^{-1}$) | 3.77 ± 0.59b | 5.30 ± 1.00ab | 6.58 ± 1.60a | 6.84 ± 2.07a | 5.71 ± 0.74ab |
| Chl *b* (mg $gDW^{-1}$) | 1.47 ± 0.19b | 1.92 ± 0.38ab | 2.30 ± 0.44a | 2.34 ± 0.59a | 2.02 ± 0.19ab |
| Chl *a* + *b* (mg $gDW^{-1}$) | 5.25 ± 0.78b | 7.23 ± 1.38ab | 8.88 ± 2.04a | 9.18 ± 2.66a | 7.72 ± 0.93ab |
| Car *x* + *c* (mg $gDW^{-1}$) | 1.03 ± 0.23a | 1.13 ± 0.18a | 1.52 ± 0.32a | 1.59 ± 0.37a | 1.42 ± 0.14a |
| Chl *a*/*b* (mg $gDW^{-1}$) | 2.55 ± 0.08b | 2.76 ± 0.11a | 2.84 ± 0.19a | 2.90 ± 0.14a | 2.82 ± 0.10a |
| Chl/Car (mg $gDW^{-1}$) | 5.20 ± 0.81b | 6.35 ± 0.35a | 5.81 ± 0.16ab | 5.72 ± 0.56ab | 5.43 ± 0.18b |
| Chl *a*/N(mg $gDW^{-1}$) | 0.20 ± 0.06a | 0.21 ± 0.04a | 0.22 ± 0.05a | 0.20 ± 0.06a | 0.17 ± 0.03a |
| Chl *b*/N (mg $gDW^{-1}$) | 0.08 ± 0.02a | 0.08 ± 0.01a | 0.08 ± 0.01a | 0.07 ± 0.02a | 0.06 ± 0.01a |
| Chl/N (mg $gDW^{-1}$) | 0.28 ± 0.08a | 0.29 ± 0.06a | 0.30 ± 0.06a | 0.27 ± 0.08a | 0.23 ± 0.04a |
| Car/N(mg $gDW^{-1}$) | 0.06 ± 0.02a | 0.05 ± 0.01a | 0.05 ± 0.01a | 0.05 ± 0.01a | 0.04 ± 0.01a |

Different letters in a row denote significant differences among the treatment means ($p<0.05$, Newman–Keuls' test). Data are expressed as means ± SD ($n = 4$).

Although not significantly different, the concentration of the total carotenoids determined as a sum of xanthophylls and beta carotene (x+c), also showed a similar trend to that of chlorophylls in variation amongst the treatments. Total carotenoids were least in the control treatment plants and thereafter increasing in the 5, 10, and 20 mM treatment plants. Similarly, a further increase to 40 mM of foliar sprays instigated a decline in the concentration of total carotenoids.

The ratio of chlorophylls a to b was least in the control plants and increased with increasing nitrogen concentration in the 5, 10, and 20 mM treatment plants, respectively. The ratio of the chlorophyll components indicated a slight decline despite an increase in the concentration of foliarly applied nitrogen in the 40 mM the treatment. On the other hand, although the ratio of total chlorophylls to total carotenoids was least in the control plants, it was highest in the 5 mM and thereafter consistently reducing despite increase in the concentration of nitrogen in the urea treatment (10, 20, and 40 mM). Nevertheless, the ratios of the concentration of the pigments to that of nitrogen in the leaves was generally constant amongst that treatments despite slight declines in the respective ratios in the 40 mM treatment plants compared to the rest of the plants.

Taken together, the current results generally indicated that foliar application of nitrogen up to 20 mM improved the nitrogen status in the leaves of the coffee plants compared to control counterparts. This was accompanied by an increase in the accumulation of the photosynthetic pigments especially chlorophylls. On the other, a further increase in the concentration of nitrogen in the urea treatments caused low concentrations of both leaf nitrogen and photosynthetic pigment.

### 4.4.3 Improved gas exchange and chlorophyll fluorescence

The net $CO_2$ assimilation rate ($P_N$) was least in the control treatment plants at 5.9 µmol($CO_2$) $m^{-2}$ $s^{-1}$, gradually increasing with increase in the concentration level of the foliarly applied nitrogen in the treatments and peaking in the plants treated with 10 mM nitrogen at 8.3 µmol($CO_2$) $m^{-2}$ $s^{-1}$ (Fig. 12A). Further increase in the concentration of nitrogen in the treatments was however associated with a steady decline in $P_N$ from to 6.7 and 7.2 µmol($CO_2$) $m^{-2}$ $s^{-1}$ in the 20, and 40 mM nitrogen treated plants respectively. Similarly, although stomatal conductance ($g_s$) was not statistically different amongst the treatments (Fig. 12B), gs was least in the control and 40 mM plant at 51.4 and 50.3 µmol($H_2O$) $m^{-2}$ $s^{-1}$ and highest in the 10 and 20 mM plants at 62.6 and 63.3 µmol($H_2O$) $m^{-2}$ $s^{-1}$ while plants under 5 mM indicated gs at 54.3 µmol($H_2O$) $m^{-2}$ $s^{-1}$.

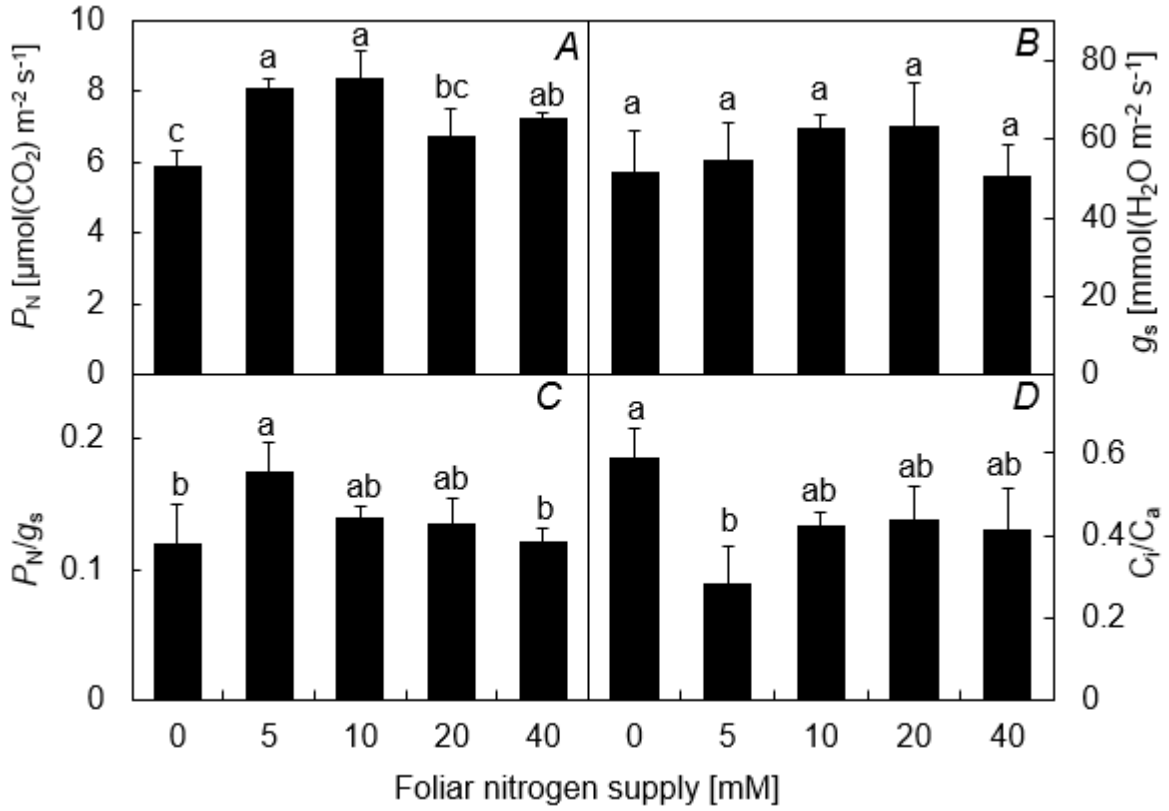


Fig. 12. Effects of foliar nitrogen supply on gaseous exchange parameters; net photosynthetic rate ($P_N$) (*A*), stomatal conductance ($g_s$) (*B*), intrinsic water−use efficiency ($P_N/g_s$) (*C*) and the ratio of internal to ambient $CO_2$ concentration ($C_i/C_a$) (*D*). Nitrogen was applied as weekly foliar sprays in the form of urea on previously cold stressed plants for 3 months in a growth chamber (25/20ºC, Day/night respectively). Gas exchange measurements were done at 6th hour of lighting. Different letters denote significant differences in the treatment means at $p<0.05$, Newman–Keuls' test (Foliar nitrogen effect). Error bars represent standard deviation of the mean ($n = 3$).

Intrinsic water−use efficiency ($WUE_i$) estimated as a ratio of net $CO_2$ assimilation rate to stomatal conductance ($P_N/g_s$) was also low in the control and 40 mM plants at 0.12 for the tow treatments. Plants under 5 mM had significantly higher $WUE_i$ at 0.17 whereas 10− and 20−mM plants indicated moderate values at 0.14 and 0.13, respectively (Fig. 12C). Contrastingly, the ratio of internal to ambient $CO_2$ concentration ($C_i/C_a$) was significantly higher for the control plants at 0.59 whereas nitrogen treatments had relatively lower $C_i/C_a$ at 0.28, 0.42, 0.44 and 0.41 for plants under 5, 10, 20, and 40 mM, respectively (Fig. 12D). The current results suggest therefore, that nitrogen supply especially at lower application levels improved the rates of gas exchanges in the leaves of the coffee plants. This was accompanied by higher rate of $CO_2$ assimilation and hence resulting into low $C_i/C_a$ in the treated compared to control plants.

The improved gas exchange in the treated plants was also accompanied with improved PSII photochemistry (Table 12). Despite showing no significant differences amongst the treatments, both the photosystem II photochemical efficiency ($\Phi_{PSII}$) and the electron transport rate (ETR) were highest in plants under 10 mM treatment. On the other hand, both control and 40 mM plants had the least values of $\Phi_{PSII}$ and ETR while plants under 5, and 20 mM treatments had moderate $\Phi_{PSII}$ values treatments. Similarly, the ETR values for the two treatments (5 and 20 mM) was moderate when compared to control and 40 mM plants.

Allocation of absorbed photosynthetically active radiation (PAR) to photochemistry indicated a consistent decline in the proportion of photochemical quenching ($q_P$) with the increase in the concentration level of the nitrogen treatments (Table 12). As a consequence, control and 5 mM plants had the highest $q_P$, and reducing in the 10 mM plants whereas 20, and 40 mM had the least $q_P$ values. On the other hand, although the non−photochemical quenching (NPQ) declined in the 5, and 10 mM plants compared to the control plants, a slight increment in NPQ was noticed in the 20, and 40 mM plants (Table 12). The proportion of PAR neither used in photochemistry nor dissipated as heat also known as fluorescence quenching ($q_N$) varied inversely to NPQ (Table 12). Therefore, $q_N$ was significantly higher in 5, 10, 20 mM plants and lower in the control and 40 mM plants at 1.97 and 1.98, respectively. Similarly, higher proportions of PSII reaction centres ($q_L$) also tended to remain open in plants treated with low concentration levels of nitrogen in the sprays (Table 12). On the contrary, both control plants and those treated with high concentration levels of nitrogen, however, sustained less open PSII photocenters.

Table 12 Effect of foliar nitrogen supply on the photosystem II photochemistry; quantum yield of photosystem II photochemistry ($\Phi_{PSII}$), electron transfer rate (ETR), photochemical quenching ($q_P$), fluorescence quenching ($q_N$), non−photochemical quenching (NPQ) and the proportion of PSII open centres ($q_L$) in the leaves of coffee seedlings recovering from cold stress treated with foliar nitrogen weekly for 3 months in a growth chamber.

| Parameter | Foliar nitrogen supply (mM) | | | | |
|---|---|---|---|---|---|
| | 0 | 5 | 10 | 20 | 40 |
| $\Phi_{PSII}$ | $0.10 \pm 0.01^a$ | $0.11 \pm 0.01^a$ | $0.12 \pm 0.00^a$ | $0.11 \pm 0.02^a$ | $0.11 \pm 0.01^a$ |
| ETR [µmol $m^{-2}$ $s^{-1}$] | $22.8 \pm 1.93^a$ | $24.3 \pm 3.10^a$ | $25.5 \pm 0.95^a$ | $23.2 \pm 3.94^a$ | $20.2 \pm 2.35^a$ |
| $q_P$ | $0.23 \pm 0.02^a$ | $0.23 \pm 0.02^a$ | $0.19 \pm 0.02^a$ | $0.17 \pm 0.04^a$ | $0.17 \pm 0.04^a$ |
| NPQ | $1.39 \pm 0.32^a$ | $1.06 \pm 0.17^a$ | $0.85 \pm 0.21^a$ | $0.87 \pm 0.15^a$ | $1.30 \pm 0.21^a$ |
| $q_N$ | $1.97 \pm 0.05^b$ | $2.25 \pm 0.07^a$ | $2.45 \pm 0.12^a$ | $2.30 \pm 0.18^a$ | $1.98 \pm 0.13^b$ |
| $q_L$ | $0.37 \pm 0.03^a$ | $0.40 \pm 0.05^a$ | $0.42 \pm 0.01^a$ | $0.38 \pm 0.05^a$ | $0.35 \pm 0.02^a$ |

Different letters in a row denote significant differences among the treatment means ($p<0.05$, Newman–Keuls' test). Data are expressed as means ± SD ($n = 3$).

The maximum quantum efficiency of photosystem II for the dark−adapted state expressed as $F_v/F_m$ and $F_v/F_o$ (Fig. 13A and B) and that during the light−adapted state, $F_v'/Fm'$ and $F_v'/F_o'$ (Fig. 13C and D), where highest for the low concentration nitrogen treated plants. Moreover, despite varying non−significantly amongst the treatments, $F_v/F_m$ was least for both control and 40 mM plants at 0.75 while the $F_v/F_m$ values for the other treatments was 0.77 (Fig. 13A). Similarly, $F_v/F_o$ was least for control and 40 mM plants at 3.0 for the two treatments while low nitrogen treatments exhibited 3.23, 3.44 and 3.26 $F_v/F_o$ values for 5, 10, and 20 mM plants (Fig. 13B). The improvements in the maximum quantum efficiency of PSII as a result of adequate nitrogen supply was more visible during the light−adapted chlorophyll state measurements with both control and 40 mM plants indicating significantly lower $F_v'/F_m'$ values at 0.49 while 5, 10 and 20 indicated 0.56, 0.59 and 0.56 $F_v'/F_m'$ values, respectively (Fig. 13C). In addition, $F_v'/F_o'$ were also significantly lower in the control and 40 mM plants at 0.97 and 0.98 while plants under 5, 10 and 20 mM showed 1.25, 1.45 and 1.30 values, respectively (Fig. 13D). The current results suggest therefore, that foliar nitrogen at lower concentration levels improved the photochemical efficiency of PSII compared to control plants whereas at the highest concentration (40 mM), a decline in the PSII efficiency was noticed.

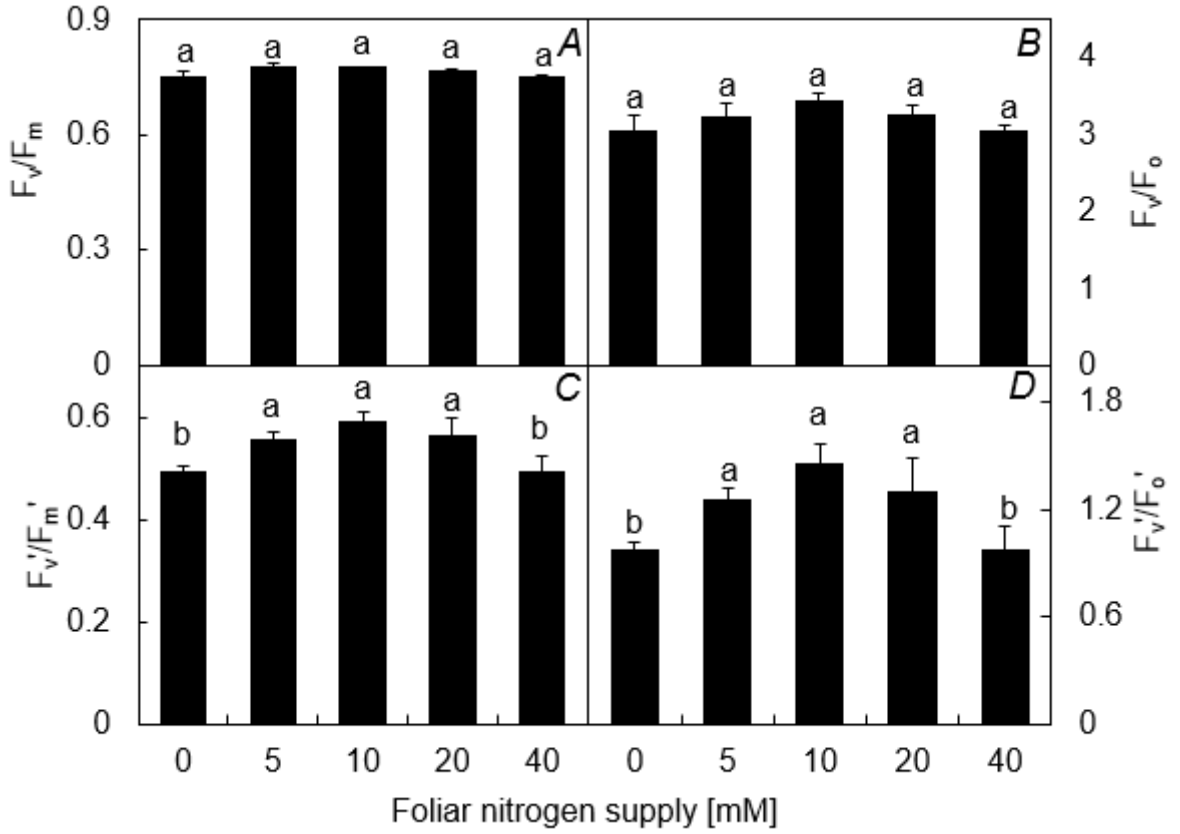


Fig. 13. Effect of foliar nitrogen supply on the maximum efficiency of PSII; indicated as ratios of variable to maximum ($F_v/F_m$, $F_v'/F_m'$) (A,C), and variable to initial ($F_v/F_o$, $F_v'/F_o'$) (B,D) photosystem II maximum efficiency during the dark adapted ($F_v/F_m$, $F_v/F_o$) (A,B) and the light adapted ($F_v'/F_m'$, $F_v'/F_o'$) (C,D) chlorophyll states in the leaves of coffee plants. Nitrogen was applied as weekly foliar sprays in the form of urea on previously cold stressed plants for 3 months in a growth chamber (25/20$^{o}$C, Day/night respectively). Light−adapted chlorophyll state was done at 6$^{th}$ hour of lighting whereas for dark−adapted state, the plants measured after 6 hours in total darkness. Different letters denote significant differences in the treatment means at $p<0.05$, Newman–Keuls' test (Foliar nitrogen effect). Error bars represent standard deviation of the mean (n = 3).

Table 13. Effects of foliar nitrogen supply on the concentrations of selected metabolites; caffeine, 5–caffeoylquinic acid, trigonelline mangiferin, ratio of 5–caffeoylquinic acid to caffeine and the total of these metabolites in the leaves of coffee seedlings recovering from cold stress treated with foliar nitrogen weekly for 3 months in a growth chamber.

| | Foliar nitrogen concentration (mM) | | | | |
|---|---|---|---|---|---|
| Parameter | 0 | 5 | 10 | 20 | 40 |
| Caffeine (mg $gDW^{-1}$) | $0.99 \pm 0.17^{a}$ | $0.69 \pm 0.10^{a}$ | $0.93 \pm 0.11^{a}$ | $0.94 \pm 0.08^{a}$ | $0.73 \pm 0.19^{a}$ |
| Trigonelline (mg $gDW^{-1}$) | $0.61 \pm 0.16^{a}$ | $0.41 \pm 0.02^{ab}$ | $0.43 \pm 0.08^{ab}$ | $0.45 \pm 0.03^{ab}$ | $0.33 \pm 0.07^{b}$ |
| 5–Caffeoylquinic acid (mg $gDW^{-1}$) | $5.89 \pm 1.06^{a}$ | $3.45 \pm 0.77^{b}$ | $4.07 \pm 0.71^{ab}$ | $4.26 \pm 0.16^{ab}$ | $3.19 \pm 1.21^{b}$ |
| Mangiferin (mg $gDW^{-1}$) | $0.14 \pm 0.04^{a}$ | $0.08 \pm 0.00^{a}$ | $0.10 \pm 0.02^{a}$ | $0.09 \pm 0.03^{a}$ | $0.08 \pm 0.04^{a}$ |
| Total content (mg $gDW^{-1}$) | $7.63 \pm 0.93^{a}$ | $4.63 \pm 0.82^{b}$ | $5.54 \pm 0.79^{b}$ | $5.74 \pm 0.24^{b}$ | $4.33 \pm 1.46^{b}$ |
| 5–Caffeoylquinic acid/Caffeine | $6.15 \pm 1.74^{a}$ | $5.06 \pm 0.99^{a}$ | $4.34 \pm 0.29^{a}$ | $4.53 \pm 0.22^{a}$ | $4.29 \pm 0.85^{a}$ |

Different letters in a row denote significant differences among the treatment means ($p<0.05$, Newman–Keuls' test). Data are expressed as mean ± SD ($n = 3$).

### 4.4.4 Content of the selected metabolites

Variation in the content of the selected metabolites amongst the treatments is indicated in Table 13. Caffeine neither showed no significant differences nor peculiar trend amongst the treatments. Nevertheless, plants under control, 10, and 20 mM treatments containing the highest content of caffeine while plants under 5, and 40 mM treatments contained the least caffeine content, respectively. On the other hand, trigonelline was significantly higher in control plants and lower in 40 mM plants. Plants under 5, 10, and 20 mM treatments contained moderate contents of trigonelline, respectively. Similarly, 5–CQA, was significantly higher in control plants and lower in plants under 40 mM while plants under 5, 10, and 20 mM treatments had moderate contents of the hydroxycinnamic acid ester, respectively. On the other hand, although mangiferin was not significantly different amongst the treatments, control plants had the highest content of the xanthonoid phenolic compound at 0.14 mg $gDW^{-1}$ while the content of mangiferin in all the nitrogen treatments was well below 0.10 mg $gDW^{-1}$.

Consequently, the total contents of these antioxidant compounds were significantly higher in control plants and lower in plants under 40 mM while 5, 10 and 20 mM contained median values, respectively. Similarly, the ratio of 5–CQA to caffeine despite showing no significant differences in variation amongst the treatments, was highest in plants under control and lower in the nitrogen treated plants for the 5, 10, 20, and 40 mM treatments, respectively.

## 4. 5 Discussion

The coffee plants used for this experiment had developed visual symptoms of cold stress conditions as a result of low temperatures from October as indicated in the previous chapter (Fig. 5). Characteristically, the coffee plants had pale yellow leaves and stunted growth which is normally associated with low concentrations of chlorophyll pigments (Fig. 11). Foliar nitrogen sprays elevated the leaf nitrogen status to levels above 23 mg $DW^{-1}$ (Table 11), a threshold under which nitrogen deficiency symptoms are reported to occur in coffee plants (Pompelli *et al.,* 2010). The control treatment plants on the other hand, were still N deficient at the end of the experiment and consequently accumulated less amounts of chlorophylls and carotenoid pigments compared to the nitrogen supply treatments whose pigment concentration increased by 75, and 54%, respectively with increasing nitrogen concentration up to 20 mM (Table 11). Low nitrogen nutrition generally results into low contents of both chlorophylls and carotenoids, with the pigments increasing by over 135 and 125%, respectively, when coffee plants were fertilized with 23 mM N twice every after 2 weeks (Pompelli *et al.,* 2010).

Nevertheless, in this study, high nitrogen concentration in the 40 mM treatment was associated with a remarked reduction in the concentration of the pigments. This is plausibly due to the burning effect associated with high concentration of foliar urea sprays. The negative effects of high concentration urea application on plant metabolism has been reported to result from accumulation of urea in plant tissues to toxic levels rather its conversion to toxic substrates such as ammonia (Krogmeier *et al.,* 1989). Therefore, at 40 mM, urea application was above the threshold and therefore its toxicity supressed plant metabolism although no burns were visually observed. Nonetheless, nitrogen supply in an appropriate concentration from foliar urea can be readily assimilated through combining with carbonyl chains form carbohydrates to form proteins and other important molecules such as chlorophylls and carotenoids (Carelli *et al*, 2006; Pompelli *et al.,* 2010) similar to observations made in the current study.

The low contents of photosynthetic pigments in the control plants were associated with low rates of gas exchange (Fig. 12A–D). The seedlings used in the current study had been raised under a shaded vinyl house with irradiance levels similar to those used in the growth chamber (250 µmol $m^{-2}$ $s^{-1}$ PPFD). The current results agree with Praxedes *et al.* (2006) and Pompelli *et al.* (2010) who reported low net $CO_2$ assimilation in plants with low concentration nitrogen and photosynthetic pigments. Chlorophylls play a vital role in both absorption and conversion of sunlight energy into chemical energy. In the current study, a consistent increase in the chlorophyll concentration in the nitrogen treated plants was accompanied with high rates of $CO_2$ assimilation (Fig. 12A). Stomatal conductance also tended to be highest in plants with high $CO_2$ assimilation rates suggesting a positive effect of the improved water status in the coffee plants as a result of foliar nitrogen treatment on photosynthesis in general (Gimenez *et al.,* 2005). Moreover, compared to control plants, nitrogen treatment improved $WUE_i$ indicating a higher biomass gain per unit water loss that occurs through transpiration (Hartfield and Dold, 2019). Control plants also indicated the highest $C_i/C_a$ ratio compared to nitrogen treated plants (Fig. 12D). This was possibly due to the inability of the mesophyll cells to assimilate the absorbed $CO_2$ in the intercellular air spaces (Farquhar *et al.,* 1980). This phenomenon could have been induced by several biochemical limitations such as low photosynthetic enzymatic activities that were greatly improved by foliar nitrogen concentrations of up to 20 mM (Sun *et al.,* 2016). On the other hand, plants under 40 mM had low rates of gas exchange (Fig. 12A–D), empathizing the negative effects caused by high nitrogen concentration in this treatment. Similar results were obtained in coffee plants under conventional nitrogen supply (Bote *et al.,* 2018) and in apple trees where plants grown under

adequate nitrogen supply maintained higher photosynthetic rates even at low temperatures compared to those growing under soil nitrogen deficient conditions (Greer, 2008).

The trend in the $CO_2$ assimilation rate was further supported by corresponding variations in the PSII photochemistry (Table 12). Both $\Phi_{PSII}$ and ETR showed similar quadratic variation amongst the treatments being lower in the both control and 40 mM plants whereas low concentration nitrogen treated plants had moderate values. The current results suggest therefore, the PSII photochemical efficiency during steady−state photosynthesis tended to be downregulated in the photosynthetically depressed plants as a mechanism of photoprotection which would otherwise result into photoinhibition due to an inefficient photosynthetic apparatus (Maxwell and Johnson, 2000; Murchie and Lawson, 2013). Moreover, control plants had both the highest qP and NPQ with the two parameters reducing with increasing concentrations in the nitrogen treatments (Table 12). Nonetheless, a slight increase in the NPQ in plants under 40 mM indicated plausible damages to the photosynthetic apparatus resulting from the toxicity of the urea application in high concentrations. Moreover, plants under control and 40 mM treatments tended to close larger proportions of the PSII traps which otherwise could have caused photoinhibition due to biochemical limitations for high photosynthetic rates (Table 12). On the contrary, plants under moderate nitrogen concentration treatments had higher $q_L$ hence facilitating the flow of electrons from PSI to PSII, which resulted into higher photosynthetic rates (Murchie and Lawson, 2013).

The maximum quantum efficiency of PSII under both dark and light conditions was also lowest in the control and 40 mM treated plants (Fig. 13A–D). Both $F_v/F_m$ and $F_v/F_o$ for both the dark and the light−adapted states ($F_v'/F_m'$ and $F_v'/F_o'$) are indicators of the degree of photoinhibition in PSII (Adams *et al.,* 1995; Maxwell and Johnson, 2000; Demmig-Adams and Adams, 2004). The current results agree with Pompelli *et al.* (2010) who also reported lower $F_v/F_m$ ratio in low N coffee plants especially under high light conditions. Such low $F_v/F_m$ ratios have also been reported in coffee plants after exposure to cold stress conditions (Batista−Santos *et al.,* 2011; Guo and Cao, 2004). This decline in the maximum quantum efficiency is attributed to downregulation of PSII due to photoprotective energy dissipation which is associated with nocturnal retention and diurnal build−up of zeaxanthin in the xanthophyll cycle (Ramalho *et al.,* 2003; Partelli *et al.,* 2009; Pompelli *et al.,* 2010; Batista−Santos *et al.,* 2011). This defence mechanism is however associated with inactive reaction centres and competes for energy with PSII photochemistry hence reducing its photochemical efficiency (Partelli *et al.,* 2009).

Despite evolving as an understorey tropical species, coffee cultivation has expanded across the globe including areas with temperatures as low as 5°C during winter conditions (Ramalho *et al.,* 2014). Such stressful conditions are characterised with diminished photosynthetic rates (Partelli *et al.,* 2009) which are partly caused by a rapid breakdown of the photosynthetic pigments and reduced efficiency of the photosystem. This results into production of reactive oxygen species as a result of the inability of the plants to effectively utilize the excess excitation energy under stressful conditions (Fortunato *et al.,* 2010).

Coffee plants have an extensive mechanism of photoprotection which can be enhanced by adequate nutrient supply, particularly nitrogen (Pompelli *et al.,* 2010). Previous reports have indicated that this defence mechanism involves the development of an elaborated antioxidant system comprising of both enzymatic and nonenzymatic antioxidant components (Pompelli *et al.,* 2010; Ramalho *et al.,* 2014; Campa *et al.,* 2017; Ramalho *et al.,* 2018). The current results have shown that in absence of an efficient photosynthetic system such as in control treatment plants, coffee plants accumulate relatively higher amounts of the secondary metabolites except caffeine (Table 13). Coffee plants contain two main alkaloids compounds whose role in antioxidant defence are still well unknown (Ashihara, 2006). Accumulation of caffeine has been more associated with defence against herbivory, whereas trigonelline accumulates as a consequence of detoxification of nicotinic acid, a reservoir for biosynthesis of nicotinamide adenine dinucleotide (Shimizu and Mazzafera, 2000). High contents of trigonelline (up to 0.61 mg $gDW^{-1}$) in the control plants compared to other treatments were observed in the current study. This is in consistence with Garg (2006) who recently indicated that trigonelline may have antioxidant roles in plant cells.

The main hydroxycinnamic acid ester, 5−CQA (Campa *et al.,* 2012) and the xanthonoid, mangiferin were also highest in the control plants and decreasing in the nitrogen supply treatments (Table 13). The two phenolic compounds have been found to increase directly in response to oxidative stresses induced by high light intensities and cold stress conditions to which the coffee plant is not adapted to (Ramalho *et al.,* 2014; Campa *et al.,* 2017). The current results indicated a higher ratio of 5−CQA to caffeine in the photosynthetically limited plants compared to those treated with foliar sprays of nitrogen (Table 13). Chlorogenic acids such as 5−CQA have a higher metabolic plasticity compared to caffeine and act as storage forms of cinnamic acid derivatives which are used for lignification during cell wall development (Aerts and Baumann, 1994). Therefore, the decline in the 5−CQA concentration could be attributed to a higher need for cell wall development than defence against ROS in plants whose

photosynthetic apparatus had been recovered by foliar nitrogen treatment. Yildirim *et al.* (2007) also observed a decline in ascorbic acid, a well−known antioxidant compound in nitrogen treated broccoli plants compared to those under control conditions.

Nonenzymatic antioxidant compounds such as phenolics, carotenoids, ascorbic acid, tocopherols and alkaloids contain electrophilic carboxylic linkages and hydroxyl components which neutralise the excess ROS generated by oxidative stress conditions (Das and Roychoudhury, 2014). The presence of high contents of the secondary metabolites in the control treatment could therefore be due to the inability of the coffee plants to recover their photosynthetic apparatus in absence of foliar nitrogen sprays as indicated by contracted photosynthetic efficiency. On the other hand, the low photosynthetic rates in the high concentration foliar treatment especially 40 mM did not result into high antioxidant content possibly due to accumulation of urea to toxic levels thereby supressing pigments and secondary metabolism in the leaves of the coffee plants (Krogmeier *et al.,* 1989).

### 4.6 Conclusion

Foliar supply of different nitrogen concentrations (0, 5, 10, 20 and 40 mM) variably affected the recovery of both photosynthetic apparatus and its efficiency in the leaves of coffee seedlings with 10 mM causing the most significant effect. This recovery was associated with improved leaf nitrogen status and concentration of the photosynthetic pigments. On the contrary, nitrogen nutrition in the foliar urea sprays was associated with low concentrations of both alkaloids and phenolic compounds such as trigonelline and 5−CQA, respectively. Therefore, foliar urea supply containing 10 mM nitrogen was appropriate in recuperating the photosynthetic apparatus and hence enhanced the recovery of the photosynthetic performance of the previously cold−stressed coffee seedlings.

## Chapter five

# Effect of exogenous application of phytohormones and nanoparticles on the nonenzymatic antioxidant system and photosynthetic physiology of coffee plants under cold stress conditions

## 5.1 Introduction

In the previously, despite foliar nitrogen application improving the photosynthetic performance of previously cold-stressed seedlings, it was associated with a decline in the content of some components of the nonenzymatic antioxidant system. This chapter therefore evaluated the effects of several elicitor compounds on reinforcement of the nonenzymatic antioxidant system of coffee plants with concomitant effects on the photosynthetic physiology during cold stress conditions.

The genus *Coffea* comprises of over 126 species, all of which evolved as understorey species in the tropical rainforests of continental Africa, Madagascar and the Mascarene islands (Davis *et al.* 2006). Out of these, two species (*C. canephora* Pierre ex A. Froehner and *C. arabica* L.) have been commercialized and subsequently cultivated in many countries, such as Brazil, which is the leading producer of coffee globally (ICO, 2019). In many of the regions where the plants have been introduced, low, non−freezing temperatures during the winter season are common. Coffee species are very sensitive to chilling, which not only affects vegetative growth, but causes substantial yield losses (DaMatta and Ramalho, 2006).

Low temperatures (below 18°C) cause a marked decrease in the photosynthetic rate, which is induced by both biochemical and diffusive limitations (DaMatta *et al.,* 1997; DaMatta and Ramalho, 2006; Partelli *et al.,* 2009). The low stomatal conductance and the corresponding low internal to ambient carbon dioxide concentration ratio have been reported to cause low photosynthetic rates resulting from low carboxylation and/or the mesophyll efficiency of $CO_2$ fixation capacity. In addition to the repression of several carboxylation enzymes, low temperatures are associated with a rapid breakdown of photosynthetic pigments, such as chlorophylls, hence causing a positive feedback loop, which further reduces the photosynthetic rates. The resultant low $CO_2$ fixation capacity causes higher degrees of photoinhibition resulting from excessive excitation energy, whose chances of occurrence are higher when the photosynthetic apparatus is stressed (Verhoeven *et al.,* 1997). The subsequent photooxidative stress is associated with an overproduction of reactive oxygen species (ROS), as well as

charged states of chlorophylls, namely $^{3}$Chl* and $^{1}$Chl in the chloroplasts (Asada, 2006). However, leaves contain carotenoids (molecules), which dissipate excess excitation energy away from the PSII through de−epoxidation of the xanthophyll cycle in which violaxanthin is converted to xanthophyll, hence avoiding the generation of ROS (Verhoeven *et al.,* 1997; Adams *et al.,* 2004; Adams and Demming-Adams, 2006). Nevertheless, this generation of ROS is not only restricted to chloroplasts, but rather occurs in the mitochondria and the peroxisomes due to presence of several oxidative and electron transport reactions in these organelles (Mittler, 2002; Mittler *et al.,* 2004). Although ROS are produced under normal cellular metabolism as signaling molecules, their exacerbated production under stress conditions causes the oxidation and disintegration of lipids, proteins, pigments and DNA, as well as the inactivation of the enzymes of the photosystems and might culminate in cell death (Asada, 1999; Mittler, 2017).

Plants are endowed with an elaborate antioxidant mechanism, which is comprised of both enzymatic and nonenzymatic antioxidants systems. During oxidative stress conditions, plants upregulate the activities of several enzymes, such as superoxide dismutase, catalase and peroxidases, which scavenge the ROS (Logan *et al.,* 2006). In addition, plants rely on the nonenzymatic, antioxidant molecules which can be either lipophilic or hydrophilic for the effective scavenging of the reactive radicals. Recently, it has been reported that the employment of either nonenzymatic or enzymatic antioxidative systems depends upon the abiotic stress factor to which the plants are exposed. That is, the superimposition of both cold and drought stress simultaneously reinforced the two antioxidative systems, while the enzymatic antioxidative systems was more reinforced during single exposure to drought than to cold stress. On the other hand, exposure to cold stress resulted in the accumulation of antioxidant compounds with less effect on the activities of the antioxidant enzymes (Ramalho *et al.,* 2018). Therefore, during cold stress conditions, while the nonenzymatic antioxidant system is more repressed, coffee plants rely on an alternative antioxidant system, which is made up of antioxidant compounds. It has been reported that young coffee leaves accumulate high amounts of phenolic compounds, such as chlorogenic acids, mangiferin and other phenolic compounds for ROS scavenging, rather than on enzymatic antioxidants (Campa et al. 2017). The accumulation of phenolic compounds, such as anthocyanins, is also associated with an increased adaptation to abiotic stress conditions (Chalker-Scott, 1999; Zhu *et al.,* 2017). Moreover, coffee plants also contain high amounts of alkaloids, mainly caffeine and trigonelline, with potential antioxidant properties (Koshiro *et al.,* 2006; Garg, 2006). The

antioxidant potency of these compounds and their related secondary metabolites is attributed to the presence of hydroxyl components and glycosylic linkages which scavenge the ROS (Das and Roychoudhury, 2014). The accumulation of these compounds, in particular polyphenols, is associated with increased ROS scavenging capacity, which is normally assessed as vitamin E equivalent and expressed as trolox (tocopherol analogue) equivalent antioxidant capacity (Chen *et al.,* 2018).

The exogenous foliar application of plant growth hormones is recently being explored as a possible means of mitigating, as well as conferring tolerance, to abiotic stress conditions (Sawan *et al.,* 2000; Zhang *et al.,* 2015; Wang and Zhang, 2017; Li *et al.,* 2017; Li *et al.,* 2018; Campos *et al.,* 2019;). Substances such as cytokinins are well known phytohormones that promote cell division and differentiation (Barciszewski *et al.,* 1999). Despite their role in mediating several biological processes during normal cellular metabolism and development, as well as the prevention of oxidation during abiotic stress conditions, several reports have indicated a decline in the endogenous levels of cytokinins during oxidative stress conditions (Todorova *et al.,* 2005; Zwack and Rashotte, 2015). The exogenous supplementation of such phytohormones might be useful in maintaining normal cellular metabolism during abiotic stress conditions.

**5.2 Kinetin**

Amongst the cytokinins, kinetin ($N^6$−furfuryladenine), a compound first isolated from autoclaved herring sperm DNA (Miller *et al.,* 1956), recently received tremendous attention for its oxidative stress mitigation and growth promotion effects (Ahanger *et al.,* 2018; Kaya *et al.,* 2018; Singh *et al.,* 2019). Moreover, due to its abundance amongst the cytokinins, its commercial applications, particularly in agricultural science, has been implored (Hamayun *et al.,* 2015). The exogenous foliar application of kinetin with concentrations ranging from 0.01 mM to 2 mM has been reported to increase the growth rate, yield, crop quality and tolerance to abiotic stress conditions, such as salinity, heavy metal stress and drought, among others in several crops (Sawan *et al.,* 2000; Kaya *et al.,* 2010; Hamayun *et al.,* 2015; Ahanger *et al.,* 2018; Kaya *et al.,* 2018). Although the mechanism of kinetin action in mitigating oxidative stresses is not yet clearly understood, it has been suggested that kinetin upregulates both the enzymatic and the nonenzymatic antioxidative systems, hence reinforcing the ROS scavenging abilities of plants (Ahanger *et al.,* 2018). The exogenous foliar application of kinetin activates and stimulates the biosynthesis of secondary metabolites through the upregulation of the

corresponding transcripts or by increasing the activities of the biosynthetic enzymes in a number of plants (Hamayun *et al.,* 2015; Jalaluddin *et al.,* 2015; Weremczuk-Jezyna *et al.,* 2018). Therefore, due to the repressed enzyme activity during cold stress conditions, amplification of the nonenzymatic antioxidative system through kinetin application might offer a necessary tolerance mechanism to enhance the growth of plants, such as coffee.

Of the two commercially important species of coffee, *C. arabica* is the most significant, contributing over 60% on the global market. This species is relatively more tolerant to cold stress conditions, due to its ability to maintain a higher photochemical efficiency of PSII, as well as the triggering of several biochemical adjustments, unlike its counterpart *C. canephora*. Consequently, *C. arabica* is more grown in highland areas, where chilling temperatures are common during winter conditions. Moreover, the negative impacts of cold stress on the growth and productivity of coffee crops is likely to be exacerbated by current trends in climatic changes. In this study, we evaluated the potential of the exogenous foliar application of kinetin on the reinforcement of the nonenzymatic oxidative system of young *C. arabica* plants towards the improvement of photosynthetic physiology and the tolerance of coffee plants to cold stress conditions.

### 5.2.1 Materials and methods

#### *5.2.1.1 Plant material and growing conditions*

Plant materials and growing conditions have been described in the previous chapter (see section 2. 2).

#### *5.2.1.2 Kinetin and cold stress treatments*

After acclimation, the plants were randomly assigned to two temperature conditions and two kinetin treatments in a 2 × 2 factorial experimental design. The four treatments were as follows: (1) control, for which the plants were maintained at optimum temperatures (day/night, 25/20 °C, respectively), and sprayed with distilled water; (2) Kinetin, sprayed with 0.35 mM kinetin and maintained at optimum temperature; (3) Cold, sprayed with distilled water and subjected to cold stress; (4) Cold + Kin, sprayed with 0.35 mM kinetin and subjected to cold stress.

Basing on related works (Sawan *et al.,* 2000; Jalaluddin *et al.,* 2015; Ahanger *et al.,* 2018; Moura *et al.,* 2018), it was presumed that the concentration of kinetin used in the current study would have significant physiological effects on the coffee plants. Foliar sprays were

administered three times every after 3 days, using a hand sprayer immediately after the growth chamber lights were routinely turned off. In all the treatments, 0.1% isopropyl alcohol (Kao Global Chemicals, Tokyo, Japan) surfactant was used. Cold stress conditions were started 24 h after the last foliar sprays by reducing the growth chamber temperature from 25/20°C to 12/12°C, day/night, respectively, for 5 days, while maintaining the other ambient conditions constant.

*5.2.1.3 Photosynthetic Measurements*

Gaseous and chlorophyll *a* fluorescence measurements were done as previously described in the previous chapter (subsection 4.2.3).

Chlorophyll *a* fluorescence parameters were thereafter calculated according to Murchie and Lawson (2013), whereas the instantaneous and the intrinsic water−use efficiency, as well as the mesophyll efficiency, were calculated as the ratios of the net $CO_2$ assimilation to the transpiration rate, ($P_N$/E); the net $CO_2$ assimilation rate to stomatal conductance, ($P_N/g_s$), and the intracellular $CO_2$ concentration to stomatal conductance ($C_i/g_s$), respectively.

*5.2.1.4 Electrolyte Leakage Index Determination*

Membrane damage in the coffee leaves was assessed by electrolyte leakage index (ELI), using an electrical conductivity meter (Laqua F−74, Horiba, Japan), according to Zhu *et al.* (2004). Fresh leaf tissues were washed thoroughly with distilled water to remove surface contamination, cut in to 1 cm strips and placed in 15 mL stoppered tubes containing 10 ml of ultrapure water. The samples were then shaken at 100 rpm on a shaker at room temperature (25 °C) for 24 hrs. The electrical conductivity (EC) of the bathing solution was recorded as EC1. Thereafter, the tubes with their contents were placed into boiling water for 15 minutes, and then cooled to room temperature, after which their new EC was recorded as EC2. The electrolyte leakage index was calculated and was expressed as a percentage (Eqn. 5).

$$ELI = \frac{EC1}{EC2} x\ 100$$

Equation 5. Electrolyte leakage index determination

*5.2.1.5 sample preparation for biochemical Assays*

For biochemical analysis, the leaves were sampled and processed as previously described (subsection 3.2.3).

*5.2.1.6 Photosynthetic pigments*

Chlorophylls and carotenoids pigments were assayed and determined as previously described in the previous chapter (subsection 3.2.4).

*5.2.1.7 Extraction and HPLC analysis of selected antioxidant compounds*

Selected alkaloids and phenolic compounds were assayed and determined as previously described in the previous chapter (subsection 2.2.4.4).

*5.2.1.8 Total anthocyanin content (TAC) determination*

Total anthocyanin content in the leaves was assayed according to Neff and Chory (1998), with a few modifications. For each sample, 100 mg of the freeze−dried powder was incubated in 20 mL methanol, which was previously acidified with 1% HCl in a dark refrigerator at 4 °C for 24 h. The samples were then diluted with 10 mL of ultrapure water, and thereafter, the anthocyanins were separated from chlorophylls, using 20 mL of chloroform (triChloromethane). Spectrophotometric measurements were made at $A_{520.0}$, $A_{530.0}$, $A_{657.0}$ and $A_{700.0}$. Total anthocyanin content was then calculated as in Oki *et al.,* (2017) and expressed as both peonidin 3−(6−caffeoylsophoroside)−5 glucoside (YGM−5b) and cyanidin 3−glucoside (Cy3G) mg equivalents per unit dry mass.

*5.2.1.9 Total phenolic Content (TPC) Determination*

The content of the total phenolic compounds was assayed and determined as previously described in the previous chapter (subsection 2.2.4.6).

*5.2.1.10 Radical Scavenging Capacity*

Total radical scavenging capacities of the coffee leaves was assayed using 2,2−diphenyl−1−Picryl−Hydrazyl (DPPH) Radical, 2,2−Azino bis (3−Ethyl Benzothiazoline−6−Sulphonic Acid (ABTS) Radical and the Ferric Ion Reducing Antioxidant Power (FRAP) Assays as previously describe in the previous chapter (subsection 2.2.4.7).

**5.2.2 Statistical Analysis**

Experimental data were statistically examined using two−way analysis of variance (two−way ANOVA), following a completely randomized design for both kinetin and cold stress effects. The parameter differences were compared for statistical significance amongst the treatments using the Newman–Keuls test at $p \leq 0.05$. Assumptions of normality were tested using the

Kolmogorov–Smirnov test, and the data transformed accordingly to attain a normal distribution whenever necessary. All the statistical analyses were done using the Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). Data are expressed as means ± SD, $n = 3$.

### 5.2.3 Results

#### *5.2.3.1 Gas Exchange*

Although the net $CO_2$ assimilation rate was not significantly different amongst the treatments, possibly due to relatively large differences between the individual measurements for each coffee plant, exposure to cold stress conditions generally caused low diffusive gas exchanges while increasing both the intrinsic and the instantaneous water use efficiencies (Table 14). The high values of water−use efficiencies were as a result of significant reductions in stomatal conductance and transpiration rates, whose decline is normally associated with low photosynthetic rates. The cold−induced decline in stomatal conductance and the transpiration rate was, however, more apparent in non−kinetin−treated plants (79.6% and 77.3%) than in the kinetin−treated plants (23.4% and –2.27%), respectively. Moreover, under cold stress conditions, kinetin treatment caused an improvement in the net $CO_2$ assimilation rate by 63.8%.

This was accompanied by higher stomatal conductance (121.7%), transpiration rate (111.8%) and intracellular $CO_2$ concentration (99.4%), as well as a higher internal−to−ambient $CO_2$ concentration ratio (87.8%) in the kinetin compared to non−treated kinetin counterparts under cold stress conditions. Similarly, the mesophyll efficiency for carbon fixation was 18.7% higher in kinetin−treated plants under cold stress conditions than in the non−kinetin−treated plants. This means that the exogenous foliar application of kinetin tended to reduce the negative effects induced by cold stress conditions in coffee plants, by maintaining relatively higher gas exchange rates in the treated plants compared to the non−treated plants.

#### *5.2.3.2 Chlorophyll Fluorescence*

Similar to gas exchange, cold stress conditions reduced the photosystem II photochemical efficiency more in non−kinetin−treated plants than in the kinetin−treated plants (Table 15). This was evinced by a larger reduction in the photosystem II operating efficiency ($\Phi_{PSII}$) in the non−kinetin−treated plants (40.0%), compared to (30.8%) in kinetin−treated plants on exposure to cold stress conditions. This was also accompanied by similar variation in the electron transfer rate, which diminished by 38.0% and 30.3% in the two groups, respectively. Despite the proportion of light energy allocated to photochemistry (qP), and that dissipated as

heat (photochemical and nonphotochemical quenching, respectively), not showing significant variation amongst the treatment, the latter (NPQ) showed a remarkable reduction with cold stress conditions more in the non−kinetin−treated plants 46.5%, compared to 36.6% in the kinetin plants, respectively. Similarly, fluorescence quenching ($q_N$) was lowest under cold stress conditions, which was also associated with the cold−stress−induced closure of photosystem reaction centers ($q_L$), especially in the non−kinetin−treated plants (67.0%), compared to the kinetin−treated plants, which maintained 0.35% of the PSII reaction centers open during cold stress conditions. The current results suggest that this exogenous kinetin treatment caused no significant improvement in the chlorophyll fluorescence parameters in the leaves of coffee plants under cold stress conditions after 5 days. Nevertheless, long−term assessments exposure might result in improvements of chlorophyll fluorescence in the kinetin−treated plants compared to their non−treated counterparts under cold stress conditions.

Table 14. Effect of exogenous kinetin and cold stress conditions on gas exchange parameters; net $CO_2$ assimilation rate ($P_N$), stomatal conductance ($g_s$), intrinsic water−use efficiency ($P_N/g_s$), instantaneous water−use efficiency ($P_N/E$), transpiration rate (Trmmol or E), intracellular $CO_2$ concentration ($C_i$), internal to ambient $CO_2$ concentration ratio ($C_i/C_a$) and mesophyll efficiency in the leaves of coffee seedlings under optimum and cold stress conditions, 25/20 °C and 12/12 °C, respectively.

| Parameter | Optimum Conditions | | Cold Stress Conditions | |
|---|---|---|---|---|
| | Control | Kinetin | Cold | Cold + Kinetin |
| $P_N$ (µmol $CO_2$ $m^{-2}$ $s^{-1}$) | 2.84 ± 1.61 Aa | 1.83 ± 0.11 Aa | 1.55 ± 0.55 Aa | 2.54 ± 0.17 Aa |
| $g_s$ (mmol $m^{-2}$ $s^{-1}$) | 31.9 ± 16.1 Aa | 17.6 ± 3.2 Aa | 8.12 ± 4.7 Ba | 18.0 ± 1.8 Aa |
| $P_N/g_s$ (µmol $mmol^{-1}$) | 0.10 ± 0.04 Ba | 0.11 ± 0.02 Aa | 0.21 ± 0.05 Aa | 0.14 ± 0.02 Ab |
| $P_N/E$ (µmol $mmol^{-1}$) | 4.13 ± 1.86 Ba | 3.99 ± 0.88 Aa | 10.4 ± 2.62 Aa | 7.03 ± 0.79 Ab |
| Trmmol (mmol $H_2O$ $m^{-2}$ $s^{-1}$) | 0.75 ± 0.38 Aa | 0.47 ± 0.08 Aa | 0.17 ± 0.10 Bb | 0.36 ± 0.04 Aa |
| $c_i$ (µL $L^{-1}$) | 333 ± 125 Aa | 215 ± 37.8 Aa | 81.2 ± 54.9 Ba | 161.9 ± 29.7 Aa |
| $c_i/c_a$ | 0.83 ± 0.31 Aa | 0.54 ± 0.09 Aa | 0.05 ± 0.003 Bb | 0.41 ± 0.07 Aa |
| $c_i/g_s$ | 7.91 ± 1.03 Ab | 12.23 ± 0.15 Aa | 7.54 ± 3.00 Aa | 8.95 ± 1.04 Ba |

Within a row, different capital letters, ($^{A,B}$) denote the statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote the statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman–Keuls test). Data are expressed as means ± S.D ($n = 3$).

Table 15. Effect of exogenous kinetin and cold stress conditions on the photochemistry of the photosystem II; PSII operating efficiency ($\Phi_{PSII}$), electron transfer rate (ETR), photochemical quenching ($q_P$), non−photochemical quenching (NPQ), fluorescence quenching ($q_N$), and the fraction of open PSII centres ($q_L$) in the leaves of coffee seedlings under optimum and cold stress conditions, 25/20°C and 12/12°C, respectively.

| Parameter | Optimum Conditions | | Cold Stress Conditions | |
|---|---|---|---|---|
| | Control | Kinetin | Cold | Cold + Kinetin |
| $\Phi_{PSII}$ | 0.15 ± 0.03 Aa | 0.13 ± 0.02 Aa | 0.09 ± 0.02 Aa | 0.09 ± 0.01 Aa |
| ETR (μmol $m^{-2}$ $s^{-1}$) | 31.8 ± 6.0 Aa | 28.7 ± 3.6 Aa | 19.4 ± 4.1 Ba | 20.0 ± 2.9 Aa |
| qP | 0.28 ± 0.04 Aa | 0.24 ± 0.05 Aa | 0.21 ± 0.02 Aa | 0.23 ± 0.04 Ba |
| qN | 2.08 ± 0.01 Aa | 2.30 ± 0.33 Aa | 1.72 ± 0.15 Aa | 1.69 ± 0.14 Ba |
| NPQ | 1.27 ± 0.45 Aa | 1.12 ± 0.50 Aa | 0.68 ± 0.48 Aa | 0.71 ± 0.41 Aa |
| qL | 0.50 ± 0.08 Aa | 0.47 ± 0.04 Aa | 0.33 ± 0.05 Ba | 0.35 ± 0.04 Ba |

Within a row, different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas treatment means followed by the same small letter, ($^{a}$) are not statistically significantly different between the kinetin treatments ($p < 0.05$, Newman–Keuls test). Data are expressed as means ± S.D ($n = 3$).

*5.2.3.3 PSII quantum Efficiency*

The quantum efficiency of PSII evaluated as the ratio of variable to maximum and variable to initial fluorescence yield indicated increased sensitivity to photoinhibition in cold stressed leaves (Table 16). Although no improvement in the PSII quantum efficiency was observed in kinetin−treated plants under cold stress conditions, the exogenous foliar application of kinetin slightly improved the effective quantum efficiency during the light−adapted state, as indicated by higher amplitudes of both $F_v'/F_m'$ and $F_v'/F_o'$ in kinetin treatment plants; 0.56 and 1.30, compared to 0.52 and 1.08, respectively, in control treatment plants under optimum conditions. Therefore, the current results indicate that the expected positive effects of the application under the conditions on PSII quantum efficiency might be realized in a longer term, rather than over a short period of time.

*5.2.3.4 Electrolyte Leakage Index*

Membrane disintegration as measured by the electrolyte index indicated that exposure to cold stress conditions caused injury to the cell membrane (Fig. 14). Under both optimum and cold stress conditions, the exogenous foliar application of kinetin increased the leakage of cellular electrolytes by 39.0% and 33.4%, compared to control and cold−treated plants, respectively. In fact, the electrolyte index was the highest at 15.7% in the kinetin−treated plants under the stress conditions compared to 8.24%, 11.4% and 11.7% in control, kinetin and cold treated plants, respectively. Contrary to the expected, the current results indicate that the exogenous application of kinetin tended to increase the content of ROS, which was accompanied by a higher electrolyte leakage index. Nevertheless, this could be a short−term effect, and therefore long−term exposure might yield contrasting results.

Table 16. Effect of exogenous and cold stress conditions kinetin on maximum and effective quantum efficiency of PSII; expressed as ratios of variable to maximum and variable to initial PSII quantum efficiency in the light and in the dark−adapted states in the leaves of coffee seedlings under optimum and cold stress conditions.

| Parameter | Optimum Conditions | | Cold Stress Conditions | |
|---|---|---|---|---|
| | Control | Kinetin | Cold | Cold + Kinetin |
| Fv'/Fm' | 0.52 ± 0.02 Aa | 0.56 ± 0.06 Aa | 0.42 ± 0.05 Ba | 0.40 ± 0.05 Ba |
| Fv/Fm | 0.75 ± 0.04 Aa | 0.75 ± 0.02 Aa | 0.57 ± 0.14 Aa | 0.58 ± 0.05 Aa |
| Fv'/Fo' | 1.08 ± 0.10 Aa | 1.30 ± 0.33 Aa | 0.72 ± 0.15 Aa | 0.69 ± 0.14 Ba |
| Fv/Fo | 3.02 ± 0.71 Aa | 3.00 ± 0.25 Aa | 1.46 ± 0.75 Ba | 1.43 ± 0.31 Ba |

Within a row, different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas treatment means followed by the same small letter, ($^{a}$) are not statistically significantly different between the kinetin treatments ($p < 0.05$, Newman Keuls' test). Data are expressed as means ± S.D ($n = 3$).

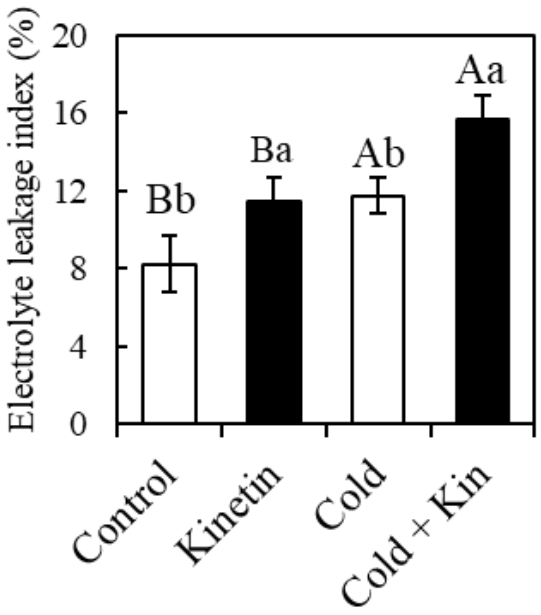


Fig. 14. Effect of exogenous kinetin on the electrolyte leakage index in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25/20°C, whereas cold stress conditions, 12/12 °C. Kin, kinetin. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman–Keuls test). Means are a replicate of three independent samples ($n = 3$). Error bars represent standard deviation of the mean.

*5.2.3.5 Photosynthetic Pigments*

The content of individual chlorophyll types *a* and *b* and their total (*a* + *b*) decreased on exposure to cold stress conditions, as well as on the exogenous foliar application of kinetin by 14.1%, 16.8% and 21.2% for Chl *a* and 17.7%, 21.3% and 24.4% for Chl *b* and 15.0%, 17.9% and 22.1% for total chlorophylls in kinetin, cold and cold + kin treatments, respectively compared to control−treated plants (Fig. 15A, B and C, respectively). On the contrary, the content of carotenoids was stimulated by the foliar application of kinetin under optimum conditions, hence reaching a maximum content of 1.65 mg $gDW^{-1}$ compared to 1.28 mg $gDW^{-1}$ in control−treated plants (Fig. 15D). Nevertheless, this promotive effect was not observed under cold stress conditions, since the content of carotenoids was non−significantly different between cold and cold + kin treated plants at 1.43 and 1.35 mg $gDW^{-1}$, respectively. Although the ratio of chlorophyll *a* to *b* was not significantly affected by either cold stress imposition or kinetin treatments (Fig. 15E), that of chlorophylls to carotenoids (Chl/Car) was significantly reduced by 33.2, 26.7 and 26.4 in kinetin, cold and cold + kin treated, compared to control−treated plants (Fig. 15F). It is suggested therefore that, despite increasing the content of carotenoids during optimum conditions, the protective role of kinetin on chlorophyll pigments could not be realised after 5 days of cold stress conditions, although contrasting results may be obtained in long−term studies.

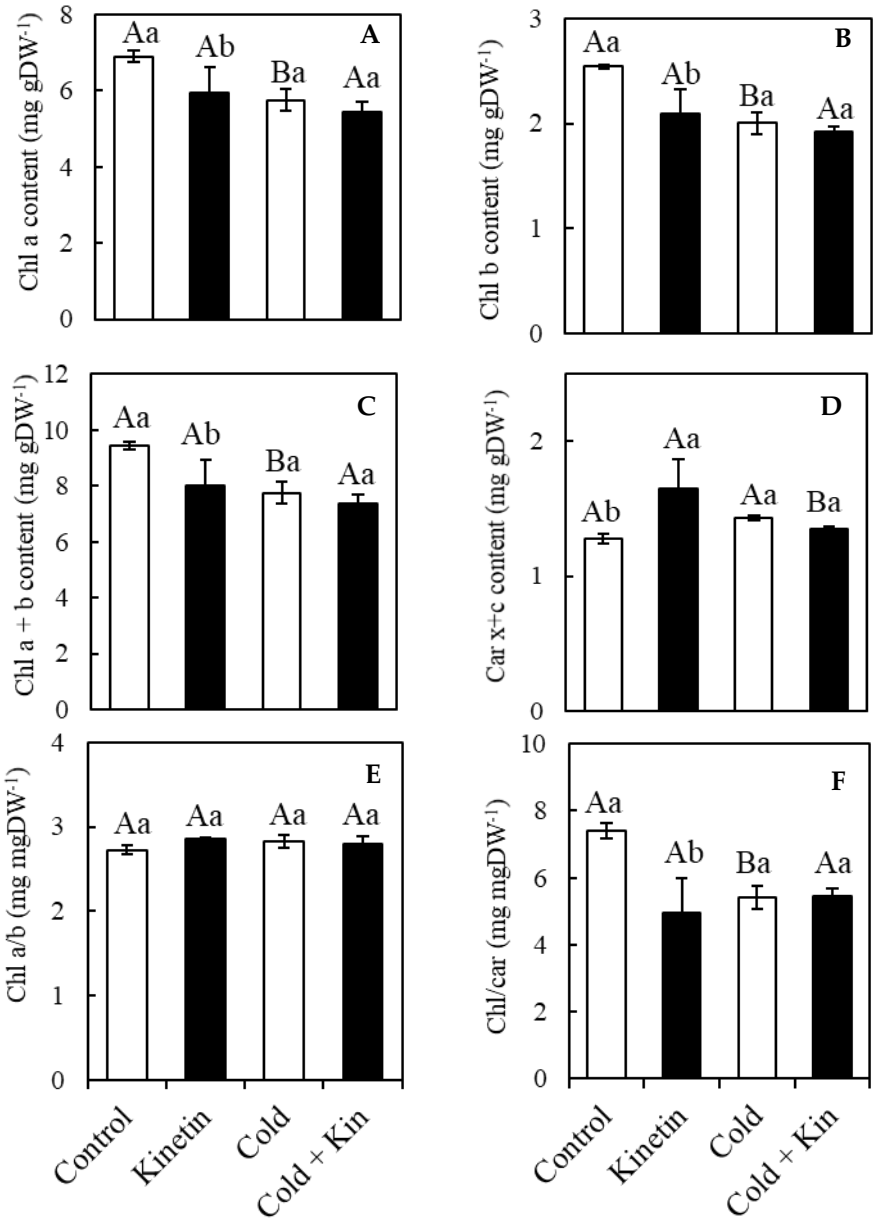


Fig. 15. Effect of exogenous kinetin and cold stress on the contents photosynthetic pigments; chlorophyll a (A), chlorophyll b (B), total chlorophylls (C), total carotenoids (D) as well as the ratios of chlorophyll a/b (E) and total chlorophyll /total carotenoid (F). Kin, kinetin. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman Keuls' test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

*5.2.3.6 Content of selected metabolite compounds*

Under both optimal and cold stress conditions, kinetin treatment caused a consistent upsurge in the content of the small weight compounds determined by HPLC (Fig. 16). On average, the content of caffeine, trigonelline, 5−caffeoylquinic acid and mangiferin were increased by 64.3%, 50.0%, 96.0% and 71.4%, respectively, under optimum conditions, hence causing an increase of 185% in the total HPLC−determined compounds as a result of kinetin treatment (Fig. 16 A, B, C D and F, respectively).

Similarly, under cold conditions, kinetin elicited an increase of 20.5%, 23.7%, 27.1% and 22.2% in the contents of caffeine, trigonelline, 5−CQA and mangiferin, respectively, and an increase of 25.8% in the total content of the HPLC compounds (Fig. 16A–F, respectively). Although the contents of both the alkaloids and phenolics were upregulated by kinetin treatment, the magnitude of increment was much more for the latter than for the former. As a result, the ratio of 5−CQA/Caffeine was increased by 20.6% and 5.3% under optimum and cold stress conditions, respectively (Fig. 16E). Nonetheless, in the absence of kinetin, cold stress conditions also tended to increase the content of the antioxidants in the non−kinetin−treated plants. Compared to optimum conditions, exposure to cold stress conditions increased the contents of caffeine, trigonelline, 5−CQA and mangiferin by 33.3%, 35.7%, 56.7% and 28.5%, respectively, causing a total upsurge of 116.3% in the HPLC antioxidants (Fig. 16A–F, respectively). Similar to kinetin treatment, cold treatment elicited more 5−CQA than caffeine, resulting in an increment of 20.3% compared to optimum conditions (Fig. 16E). Nevertheless, the current results indicate that the exogenous foliar application of kinetin stimulated the metabolism of both alkaloid and phenolic compounds that were determined by the HPLC, hence causing their accumulation in the treated plants.

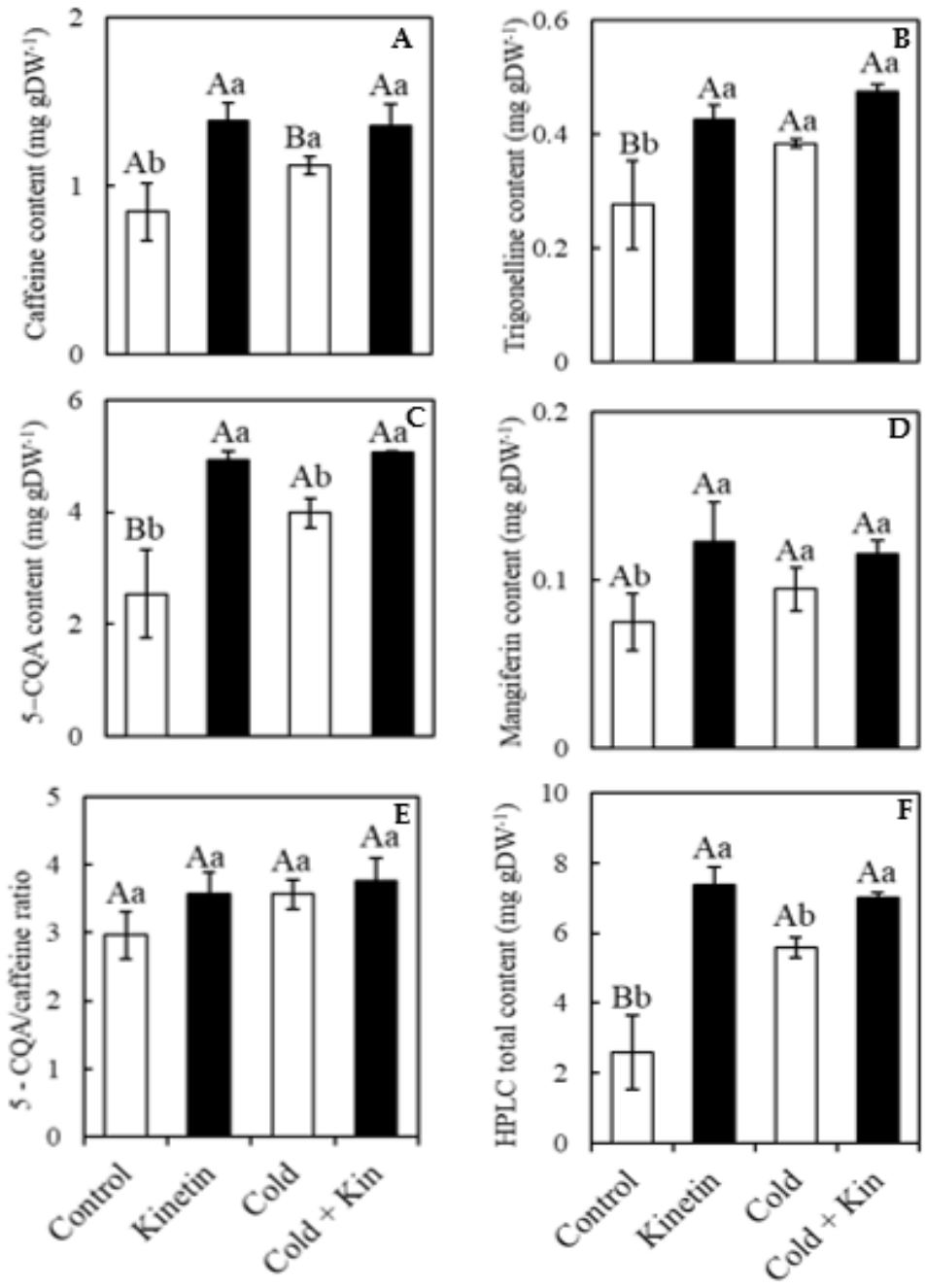


Fig. 16. Effect of exogenous kinetin and cold stress conditions on the content of the selected metabolites compounds in coffee; Caffeine (**A**) trigonelline (**B**); 5−caffeoylquinic acid (**C**); mangiferin (**D**); 5−caffeoylquinic acid/caffeine ratio (**E**) and HPLC total content (**F**). Kin, kinetin. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman–Keuls test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

5.2.3.7 Total Anthocyanin Content (TAC)

Accumulation of anthocyanins in coffee leaves was expressed on the basis of both peonidin−3−(6−caffeoyl−sophoride)−glucoside and cyanidin−3−glucoside equivalents (Fig. 17A and B, respectively). Neither kinetin nor cold treatments significantly affected TAC in coffee leaves (Fig. 17). Nevertheless, TAC was consistently the highest in kinetin treatment under both cold stress and optimum temperature conditions at 1.74 and 1.61 mg YGM−5b eq $gDW^{-1}$ and 0.84 and 0.76 mg Cy3G eq $gDW^{-1}$, respectively (Fig. 17A and B). On the other hand, TAC was lowest in control treatment plants at 1.15 YGM−5b eq $gDW^{-1}$ and 0.56 mg Cy3G eq $gDW^{-1}$, while cold stress conditions had less effect on the content of the anthocyanins with cold−treated plants, containing 1.39 mg YGM−5b eq $gDW^{-1}$ and/or 0.66 mg Cy3G eq $gDW^{-1}$. Despite varying non−significantly amongst the treatments, the results show that kinetin treatment tended to increase the accumulation of anthocyanin under both optimum and cold stress conditions.

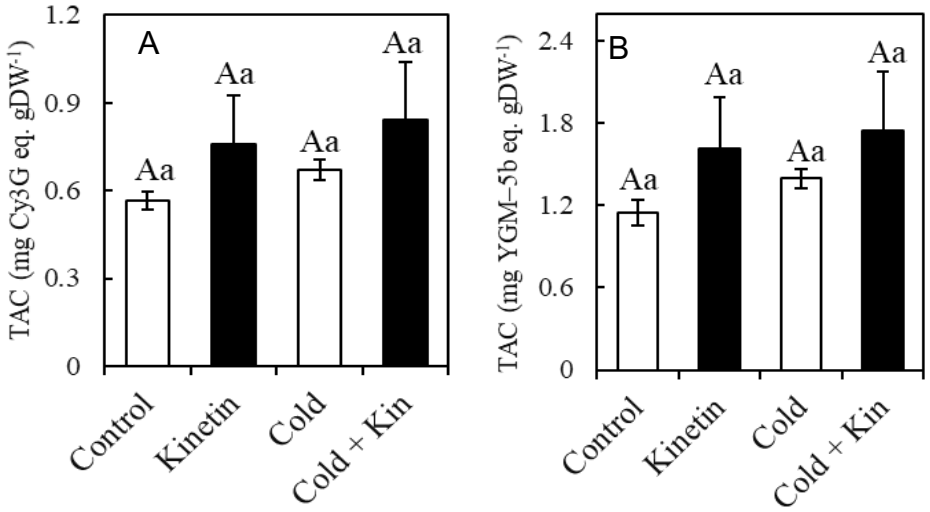


Fig. 17. Effect of exogenous kinetin on total anthocyanin content (TAC) expressed on the basis of both peonidin−3−(6−caffeoyl−sophoride)−glucoside (**A**) and cyanidin 3 glucoside (**B**) in the leaves of the coffee plants under optimum and cold stress conditions. Kin, kinetin. Treatment means followed by the same capital letter, ($^{A}$) are not statistically significantly different between the temperature treatments, whereas treatment means followed by the same small letter, ($^{a}$) are not statistically significantly different between the kinetin treatments ($p < 0.05$, Newman–Keuls test). Means are a replicate of three independent samples (n = 3). Error bars represent standard deviation of the mean.

*5.2.3.7 Total phenolic content (TPC)*

TPC showed a typical trend in variation amongst the treatments, being at the highest in kinetin−treated plants at 55.5 and 54.9 mg $gDW^{-1}$ GAE under optimum and conditions, respectively (Fig. 18). Contrastingly, control treatment plants accumulated the least TPC (38.9 mg $gDW^{-1}$ GAE), while exposure to cold stress conditions slightly increased the TPC to 42.4 mg $gDW^{-1}$ GAE (Fig. 18). These results indicate that the exogenous foliar application of kinetin elicited an accumulation of phenolic compounds in the leaves of the coffee plants.

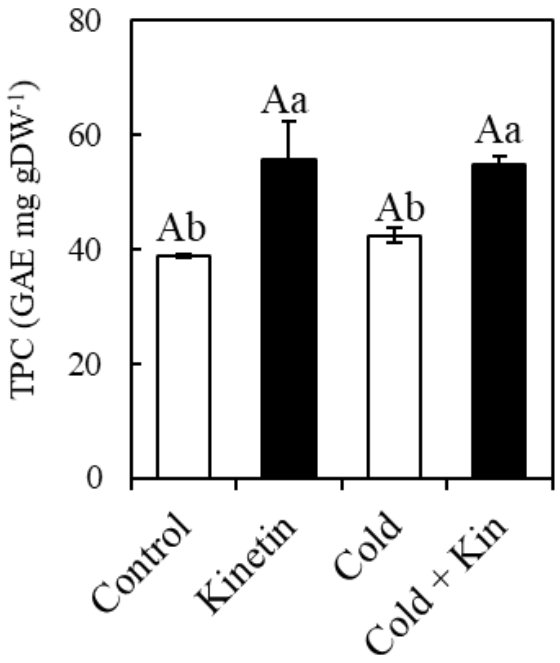


Fig. 18. Effect of exogenous kinetin and cold stress conditions on total phenolic content (TPC) expressed on the basis of gallic acid equivalent (GAE) in the leaves of coffee plants under optimum and cold stress conditions. Kin, kinetin. Different capital letters denote the statistically significant differences between the parameter means within temperature treatments, whereas different small letters denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman–Keuls test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

*5.2.3.8 Radical scavenging capacity*

Like the variations in the content of the analyzed compounds, the radical scavenging capacity as expressed by the TEAC values was consistently highest in kinetin−treated plants under the two temperature conditions (Table 17). The FRAP assay indicated that the radical scavenging capacity was significantly highest in kinetin−treated plants under optimum conditions, followed by those under cold stress conditions at 1138.6 and 938.7 µmol Trolox $gDW^{-1}$, respectively. Similarly, both DPPH and ABTS TEAC values were high at 317.9 and 277.2 µmol Trolox $gDW^{-1}$ in kinetin treatment and, 270.1 and 281.6 µmol Trolox $gDW^{-1}$ for cold + kin treatment plants, respectively. On the other hand, control and cold−treated plants had low radical scavenging capacity values with the assays, indicating TEAC values of 752.5, 262.6 and 211.3 µmol Trolox $gDW^{-1}$ in control plants, and 542.1, 236.9 and 190.2 µmol Trolox $gDW^{-1}$ in cold−stressed plants for FRAP, ABTS and DPPH assays, respectively (Table 17). Consequently, the DPPH $IC_{50}$ was at the least in the kinetin−treated plants at 114.4 and 102.8 µgDW $mL^{-1}$ under both optimum and cold stress conditions, respectively, while non−kinetin−treated plants indicated the highest values at 125.8 and 154.0µgDW $mL^{-1}$ for the two respective temperature conditions (Table 17). It is suggested therefore that this kinetin treatment increased the ability of coffee leaves to scavenge the ROS which are more detrimental during oxidative stresses, typical of cold conditions.

Table 17. Effect of exogenous kinetin and cold stress conditions on the ROS scavenging capacities of coffee leaves under optimum and cold stress conditions

| Parameter | Optimum Conditions | | Cold Stress Conditions | |
|---|---|---|---|---|
| | Control | Kinetin | Cold | Cold + Kinetin |
| DPPH $IC_{50}$ (µgDW $mL^{-1}$) | 125.8 ± 21.5 Ba | 114.4 ± 12.7Aa | 154.0 ± 8.5 Aa | 102.8 ± 7.2 Ab |
| DPPH TEAC (µmol Trolox $gDW^{-1}$) | 211.3 ± 0.1 Ab | 317.9 ± 30.5Aa | 190.2 ± 10.6 Ab | 270.1 ± 35. 2Ba |
| ABTS TEAC (µmol Trolox $gDW^{-1}$) | 262.2 ± 3.0 Aa | 277.2 ± 19.4Aa | 236.9 ± 23.3 Ab | 281.6 ± 7.15 Aa |
| FRAP TEAC (µmol Trolox $gDW^{-1}$) | 752.5 ± 73.8 Ab | 1138.6 ± 169.6Aa | 542.1 ± 48.4 Bb | 938.7 ± 78.6 Ba |

Within a row, different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote the statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman–Keuls test). Data are expressed as means ± S.D ($n = 3$).

### 5.2.4 Discussion

Temperatures below 18°C are one of the abiotic stresses limiting the productivity and growth of coffee plants (Ramalho *et al.,* 2014). Abiotic stresses including cold stress reduce the $CO_2$ fixation capacity, which is normally associated with a low net $CO_2$ assimilation rate, stomatal conductance and transpiration rate in plants (Demmig−Adams and Adams, 2004). Subjecting coffee plants to 12°C in the current study induced cold stress conditions which were associated with low gas exchanges, hence inducing diffusive limitations to photosynthetic productivity in coffee plants (Table 14). Similar effects of cold stress conditions on the photosynthetic physiology of coffee plants have been reported (Pompelli *et al.,* 2010; Ramalho *et al.,* 2014). These limitations are accompanied by a reduction in the PSII operating efficiency, resulting from the breakdown of the photosynthetic apparatus under cold stress conditions (Havaux *et al.,* 2000). This is in agreement with the observations made in the current study, where cold−stressed plants had the least values of $\Phi_{PSII}$, which was associated with low electron transport rates (Table 15). The reduction in the $CO_2$ fixation capacity, together with low PSII operating efficiency, result in an over production of reactive oxygen from any given incident light (Lima *et al.,* 2002). This normally results in a phenomenon known as photoinhibition, when excess amounts of ROS are produced in the antenna pigments of the chloroplasts by the Mehler reaction (Asada, 1999; Kovtun *et al.,* 2000). An overproduction of ROS in the photosynthetic apparatus typical under oxidative stress conditions causes a severe reduction in both the effective and maximum quantum yield of PSII (Verhoeven *et al.,* 1997). This agrees with the results of the current study, where cold−stressed plants had the low values of quantum yield in the dark and light−adapted chlorophyll states (Table 16). Nevertheless, the exogenous application of kinetin tended to modulate the dampening effects of cold stress on the PSII operating efficiency, hence resulting in improved gas exchange, which was accompanied with higher $\Phi_{PSII}$, ETR and an improved quantum efficiency in kinetin−treated plants compared to the non−treated plants under cold stress conditions (Tables 14, 15 and 16). Our results agree with a number of studies which reported the positive effects of exogenous kinetin treatment on the photosynthetic machinery (Ahanger *et al.,* 2018; Moura *et al.,* 2018). Although the protective roles of kinetin are concentration dependent, similar to the concentration used in the current study, Moura *et al.* (2018) reported that 0.35 mM (75 mg $L^{-1}$) effectively improved the carboxylation efficiency, with corresponding effects on the photosynthetic apparatus. It has been reported that kinetin induces stomatal opening, hence encouraging gas exchange with associated net $CO_2$ assimilation under stress conditions (Pharmawati *et al.,* 1998). This is

associated with both the protective effects of kinetin on the functioning of the photosynthetic light reaction and the functional enzymes of PSII and PSI, such as the protein gradient regulation−5 (PGR−5) protein, as well as maintaining a pH gradient for a smooth electron flow from PSII to PSI (Tikkanen *et al.,* 2015).

Our results further indicated that exposure to cold stress conditions had a negative impact on the membrane stability in the leaves of the coffee plants (Fig. 14). This could be attributed to an increased production of ROS in the chloroplasts, as well as other organelles with an electron chain system, such as the mitochondria and peroxisomes (Rio *et al.,* 2002). ROS are normally produced by plants under optimal conditions, in this case acting as signaling and transduction molecules for normal cellular metabolism (Mittler, 2002; Mittler, 2017). However, their amplified production during oxidative stress conditions results in a disruption of the normal cellular homeostasis, leading to membrane lipid peroxidation characterized by the leakage of cellular electrolytes, protein oxidation and enzyme inhibition, as well as the disintegration of deoxyribonucleic acid and ribonucleic acid (Mittler, 2002; Gill and Tuteja, 2010). Cold stress conditions also resulted in the breakdown of chlorophyll molecules (Fig. 15). The accumulation of high amounts of ROS in the photosynthetic machinery particularly breaks down the D1 protein subunit of the PSII reaction center, leading to the oxidation and bleaching of chlorophyll molecules (Asada, 2006; Roach and Krieger-Liszkay, 2014). Exogenous kinetin applications in several concentrations have been reported to stabilize cellular membranes in several crops exposed to a heavy metal stress (Wang *et al.,* 2015; Gadallah, 1995), waterlogged conditions (Gadallah, 1995), UV−B stress (Singh *et al.,* 2019) and salinity stress (Ahanger *et al.,* 2018), which is associated with high amounts of photosynthetic pigments resulting from kinetin−mediated enhancement in the expression of genes that encode several proteins involved in pigment biosynthesis (Gangwar *et al.,* 2014). In the current study, besides increasing carotenoid content under optimum conditions, kinetin application elicited contrasting results to the prior mentioned studies. Kinetin application tended to increase membrane damage, as well as the disintegration of chlorophyll molecules (Fig. 14 and 15, respectively). This could be related to the kinetin mechanism of action after exogenous application, through which it increases nicotinamide adenine dinucleotide activity, leading to the generation of $H_2O_2$, which acts as a signaling molecule for antioxidant defense (Gangwar *et al.,* 2014). Although the short−term increase in the content of the ROS is associated with increased cellular damage, it might substantially contribute to the overall enhancement of the antioxidant defense system, with improved tolerance to abiotic stress conditions in the long

run. On the other hand, the up−regulatory effect of kinetin on carotenoids metabolism also coincides with the metabolism of polysaccharides and proteins of the photosynthetic apparatus, and this in fact causes the stimulation of the entire biogenesis of chloroplasts (Choudhury and Choe, 1996; Bris, 2017).

The kinetin–hydrogen peroxide−mediated reinforcement of the antioxidant system is supported by high amounts of antioxidant compounds analyzed in the current study (Fig. 16 – 18). The current results are in agreement with several reports were exogenous kinetin treatment increased the tolerance of plants to abiotic stress conditions through the increased activities of antioxidant enzymes (Wang *et al.,* 2015; Ahanger *et al.,* 2018; Kaya *et al.,* 2018; Singh *et al.,* 2019), as well as the increased contents of several antioxidant compounds, such as flavonoids, gibberellins, salicylic acid, jasmonic acid and abscisic acid in salinity−stressed soybean plants (Hamayun *et al.,* 2015), proline in drought−stressed rice (Jalaluddin *et al.,* 2015), salvianolic acid and rosmarinic acid in *Dracocephalum forrestii* (Weremczuk-Jezyna *et al.,* 2018). Although the accumulation of several secondary metabolites in the leaves of coffee plants under oxidative stress conditions is a well−known phenomenon (Ramalho *et al.,* 2018; Campa *et al.,* 2017), this study is the first to report a profound increment in the contents of these compounds in response to exogenous kinetin treatment under optimal or cold stress conditions. These secondary metabolites play a number of roles in the plant defense mechanisms against oxidative stress due to the presence of hydroxyl components and glycosylic linkages which scavenge the ROS, hence maintaining cellular homeostasis during oxidative stress conditions (Das and Roychoudhury, 2014; Thakur and Nayyar, 2013; Bennet *et al.,* 1994; Edreva *et al.,* 2008). Kinetin increased the content of both alkaloids, such as trigonelline and caffeine, and phenolic compounds, including 5−caffeoylquinic acid, the main chlorogenic acid in coffee plants (Campa *et al.,* 2012), mangiferin and anthocyanins. Unlike alkaloids such as caffeine, whose role in the detoxification of ROS is less reported (Ashihara, 2006), the accumulation of phenolic compounds has been associated with an increased tolerance to several abiotic stress conditions (Ramalho *et al.,* 2018; Campa et al., 2017; Chalker-Scott, 1999; Zhu *et al.,* 2017). Nevertheless, trigonelline (1−methylpyridinium−3−carboxylate), another alkaloid compound, is a reservoir for NAD biosynthesis, and therefore its accumulation may contribute to the cellular energy metabolism which might lead to enhanced abiotic stress tolerance (Ashihara, 2006). During cold stress conditions when the activities of the enzymatic antioxidants are repressed by low temperatures, coffee plants were found to rely on the accumulation of mainly

phenolic compounds as alternative defense mechanisms against oxidative stress *(Ramalho et al.,* 2018).

Accumulation of these powerful antioxidants is more important in the young leaves of coffee plants, whose enzymatic antioxidant system is normally underdeveloped at this stage, and therefore unable to neutralize the rapid accumulation of ROS from intense oxidative stresses (Campa *et al.,* 2017). Therefore, by increasing the content of these compounds, exogenous kinetin application improves the nonenzymatic antioxidant system of the young coffee plants. The current study agrees with a number of reports, where kinetin enhanced the production of phenolic and alkaloid compounds in the explants of different species under several abiotic stress conditions (Steinhart *et al.,* 1964; Angelova *et al.,* 2001; Siahpoush, 2011).

Although the mechanism through which exogenous kinetin influences the metabolic pathways of these compounds is not yet known, it has been suggested that kinetin upregulates the associated transcription factors (Barciszewski *et al.,* 1999), as well as directly enhancing the activities of phenolic and alkaloid biosynthesis enzymes (Steinhart *et al.,* 1964; Angelova *et al.,* 2001). This could be due to kinetin−induced increase in the concentration of $H_2O_2$ molecules, which although produced in the chloroplasts, they rapidly diffuse into the nucleus, where they act as signaling molecules for the metabolism of phenolic and alkaloid compounds, hence contributing to antioxidant defense against the overproduction of ROS (Maruta *et al.,* 2012). In addition, it has been reported that kinetin scavenges free oxygen radicals by directly neutralizing ROS using the hydrogen from the α−carbon of the amine bond of $N^6$−furfuryladenine. Kinetin also reacts with copper, forming a Cu (II)−kinetin complex, which encourages a faster dismutation of the radical oxygen species in solution (Barciszewski *et al.,* 1999), hence contributing to the antioxidative capacity of plants. Taken together, the exogenous foliar application of kinetin increased the ROS scavenging capacities in the treated plants (Table 17). This increase was more ostensibly indicated by the FRAP assay, whereas DPPH and ABTS, despite showing increment tendencies with kinetin treatment, were not affected significantly amongst the treatments. Although the assays employ different principles in the determination of radical scavenging values, they normally report consistent values (Chen *et al.,* 2018; Alvarez-Jubete *et al,* 2010), as was observed in the current study.

Results of the current study indicated that exposure to cold stress conditions impacted the physiology and metabolic processes of coffee plants. The ROS generated as a result of oxidative stress associated with cold stress conditions caused the disintegration of the cell

membranes and photosynthetic pigments. This was accompanied by diminished photosynthetic efficiency of the PSII with concomitant reductions in the gas exchange and photosynthetic activity in the leaves of coffee plants. On the other hand, the exogenous foliar application of kinetin improved the antioxidative capacity of the coffee plants by upregulating the metabolism of the nonenzymatic antioxidant compounds. This was associated with increased reactive species scavenging capacity in the kinetin−treated plants. Exogenous kinetin application slightly increased the photochemical and mesophyll efficiency for $CO_2$ fixation in addition to maintaining somewhat higher gas exchanges, even under cold stress conditions. Therefore, on the basis of the results presented in the current study, it is suggested that kinetin has a potential to modulate the growth of the coffee plants under cold stress conditions, and therefore more studies should be conducted to explore its efficacy under field conditions. Exploring cold mitigation or tolerance enhancement is not only useful in the face of unpredictable climatic changes, but also is likely to expand coffee cultivation to new production areas, whose environmental conditions are currently unsuitable for coffee production.

**5. 3 Salicylic acid**

Salicylic acid is a plant phenolic compound normally present in plant cells where it regulates a number of physiological processes that affect growth and development of plants (Rivas−San Vicente and Plasencia, 2011). Among the many biological process mediated by salicylic acid, its role in signalling for modulation of responses to abiotic and biotic stresses is becoming more of interest to science (Shabanian *et al.,* 2019). Increased concentration of cellular salicylic acid is normally associated with improved tolerance or alleviation of abiotic stresses including salinity (Miura and Tada, 2014), heavy metal stress (Kovács *et al.,* 2014; Singh *et al.,* 2017; Lu *et al.,* 2018), high light stress (Wang *et al.,* 2014), low temperature (Min *et al.,* 2018; Shin *et al.,* 2018; Wang *et al.,* 2019) drought (Tang *et al.,* 2017) and pesticide toxicity (Wang and Zhang, 2017) among others in several plants.

Cold stress resulting from low positive temperatures is recently becoming a global threat to production of *C. arabica* L in many coffee producing regions (Ramalho *et al.,* 2014). Moreover, this trend in the drop in ambient temperature during winter season which is projected to increase further given the current trends in global climatic changes. Cold stress conditions result into several physiological and biochemical limitations for coffee production (Damatta and Ramalho, 2006). Under cold stress conditions, there is a high likelihood of excess excitation energy resulting from incident photosynthetically active radiation (PAR) as a result

of cold–induced diminished efficiency in electron transport system (Muller, 2001). Low temperatures are also associated with repressed enzymatic activity including those involved in the carboxylation cycle (Partelli *et al.,* 2009). These and other physiological or biological limitations result into generation reactive oxygen species which breakdown several molecules including chlorophylls and D1 protein subunit of PSII (Asada, 1999). Nevertheless, plants have developed several mechanisms through which they homeostatically control the accumulation of ROS. These mechanisms involve employment of either the enzymatic or non–enzymatic antioxidant defence system or both (Asada 1999; Logan *et al.,* 2006).

Recently, utilization of salicylic acid in priming as an agronomic practice towards enhanced tolerance to abiotic stress conditions is becoming common (Miura and Tada, 2014). Exogenous foliar application of salicylic acid enhanced protection against chlorophyll degradation hence maintaining photosystem II photochemistry with concomitant improvement in the photosynthetic efficiency and net carbon dioxide assimilation (Li *et al.,* 2014). In particular, exogenous salicylic acid application protected the photosystem II complex against oxidative stress induced photodamage through enhancing the transcription of the psbA gene that encodes the D1 protein subunit of PSII (Wang *et al.,* 2014). This mechanism of protection is further attributed to the role of salicylic acid in maintenance of cellular redox homeostasis through upregulation of several transcripts that increase the activities of antioxidant enzymes in addition to other cold responsive genes (Sun *et al.,* 2012; Li *et al.,* 2013; Wang *et al.,* 2019).

Plants employ different tolerance mechanisms against oxidative stress conditions depending on the stressor. Although many plants rely on the enzymatic antioxidant system, it has recently been demonstrated that coffee plants produce a number of protective antioxidant compounds during cold stress conditions due to cold–repressed enzymatic activities (Ramalho *et al.,* 2018). These compounds such as ascorbic acid, tocopherols, phenolics and alkaloids contain electrophilic glycosylic linkages and hydroxyl components that neutralize reactive oxygen species (Das and Roychoudhury, 2014). Since the enzymatic antioxidant defence system in coffee leaves develops with age, young leaves of coffee plants rely on the non–enzymatic antioxidant system for ROS scavenging (Campa *et al.,* 2017). Therefore, reinforcement of the non–enzymatic antioxidant system might increase tolerance of coffee plants to cold stress conditions. Although, exogenous application of salicylic acid has been reported to modulate oxidative stress through increased activities of several antioxidant enzymes, its mechanism of stress tolerance conferment in crops such as *C. arabica* is far from being understood.

This study therefore investigated the potential of exogenous application of salicylic acid in reinforcement of the non–enzymatic antioxidant compounds with concomitant modulation of cold stress induced decline in photosynthetic efficiency. Response to exogenous salicylic acid foliar sprays of HPLC profiles of antioxidant compounds caffeine, trigonelline, 5–caffeoylquinic acid and mangiferin was assessed under optimum and during cold stress conditions. In addition, the protective effects of exogenous salicylic acid on membrane damage and the contents of nitrogen and photosynthetic pigments in the leaves were evaluated. The result of this study might be helpful in alleviating the negative impacts of cold stresses on coffee production under the increasingly stressful environmental conditions.

### 5.3.1 Material and methods

#### *5.3.1.1 Plant material and growing conditions*

Plant materials and growing conditions have been described in the previous chapter (see section 2. 2).

#### *5.3.1.2 Salicylic acid and cold treatments*

After acclimation, the plants were randomly assigned to two temperature conditions and two salicylic acid (SA) treatments in a 2 x 2 factorial experimental design. The four treatments were as follows: (1) control, for which the plants were maintained at optimum temperatures (day/night, 25$^{o}$/20$^{o}$C respectively) and sprayed with distilled water plus surfactant; (2) SA, sprayed with 1.0 mM salicylic acid and maintained at optimum temperature; (3) Cold, sprayed with distilled water and subjected to cold stress; (4) SA + Cold, sprayed with 1.0 mM salicylic acid and subjected to cold stress. Foliar sprays were administered 3 times every after 3 days using a hand sprayer immediately after the growth chamber lights routinely turned off. In all the sprays, 0.1% isopropyl alcohol (Kao Global Chemicals, Tokyo, Japan) was used as the surfactant. Cold stress was instigated 24 h after the last foliar sprays by reducing the growth chamber temperature from 25$^{o}$/20$^{o}$C to 12$^{o}$/12$^{o}$C, day/night respectively for 5 days while maintaining the other ambient conditions constant.

#### *5.3.1.3 Photosynthetic measurements*

Gaseous and chlorophyll *a* fluorescence measurements were done as previously described in the previous chapter (Subsection 4.2.3) and the subsequent calculation made as previously described in subsection 5.2.1.3.

*5.3.1.4 Electrolyte leakage index analysis*

Electrolyte leakage index was assessed as previously described in the previous chapter (subsection 5.2.1.4).

*5.3.1.5 Sample preparation for biochemical assays*

For biochemical analysis, the leaves were sampled and processed as previously described (subsection 3.2.3).

*5.3.1.6 Nitrogen determination*

Nitrogen concentration in the samples was determined as described in the previous chapter (subsection 4.2.4.2).

*5.3.1.7 Photosynthetic pigments*

Chlorophylls and carotenoids pigments were assayed and determined as previously described in the previous chapter (subsection 3.2.4).

*5.3.1.8 HPLC analysis of selected metabolite compounds*

Selected alkaloids and phenolic compounds were assayed and determined as previously described in the previous chapter (subsection 2.2.4.4).

**5.3.2 Statistical analysis**

Experimental data were statistically examined using two−way analysis of variance (two−way ANOVA), following a completely randomized design for both kinetin and cold stress effects. The parameter differences were compared for statistical significance amongst the treatments using the Newman–Keuls test at $p \leq 0.05$. Assumptions of normality were tested using the Kolmogorov–Smirnov test, and the data transformed accordingly to attain a normal distribution whenever necessary. All the statistical analyses were done using the Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). Data are expressed as means ± SD, $n = 3$.

**5.3.3 Results**

*5.3.3.1 Electrolyte leakage index*

Membrane damage as measured by electrolyte leakage index was not significantly affected by exogenous salicylic acid application (Fig. 19). On the other hand, instigation of cold

temperatures significantly increased membrane damage resulting into over 15% increase in electrolyte leakage index. Moreover, under cold conditions (12/12$^{o}$C) salicylic acid slightly reduced electrolyte leakage index compared to non–salicylic acid treated plants (Fig. 19).

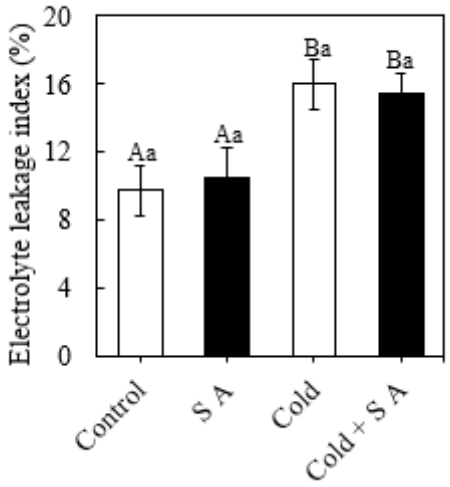


Fig. 19. Effect of exogenous salicylic acid on the electrolyte leakage index in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25/20 °C, whereas cold stress conditions, 12/12 °C. SA, salicylic acid. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman–Keuls test). Means are a replicate of three independent samples ($n = 3$). Error bars represent standard deviation of the mean.

*5.3.3.2 Nitrogen*

Salicylic acid effectively prohibited the loss of nitrogen from coffee leaves prevented under cold conditions as indicated by significant decline in the concentration of nitrogen in plants not treated with salicylic acid (Fig. 20).

*5*.3.3.*3* Photosynthetic pigments

Protection against nitrogen loss was accompanied by slight inhibition in the breakdown of photosynthetic pigments (Fig. 21). Salicylic acid application slowed down the disintegration of chlorophylls molecules especially chlorophyll a which was significantly least in non–salicylic acid treated plants under cold stress conditions (Fig. 21A). This resulted into maintenance of total chlorophyll content at levels not significantly different from those under optimum conditions (Fig. 21C). Salicylic acid also stimulated the accumulation of carotenoids especially under optimum conditions compared to cold stress conditions (Fig. 21D). However, the ratios of chlorophyll a to b and total chlorophylls to total carotenoids were not significantly affected by both cold stress instigation and salicylic acid treatment (Fig. 21A and F, respectively).

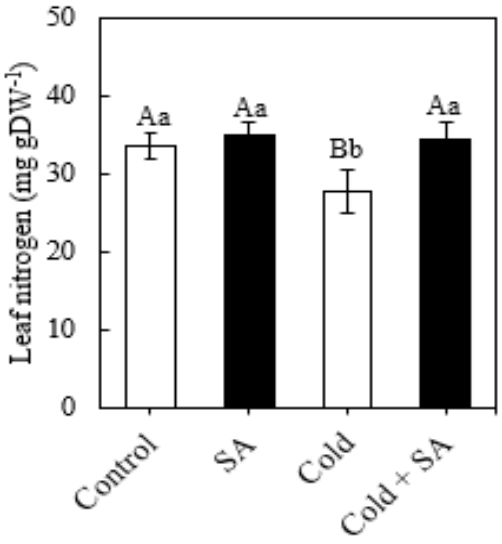


Fig. 20. Effect of exogenous salicylic acid on the concentration of nitrogen in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25/20 °C, whereas cold stress conditions, 12/12 °C. SA, salicylic acid. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman–Keuls test). Means are a replicate of three independent samples ($n = 3$). Error bars represent standard deviation of the mean.

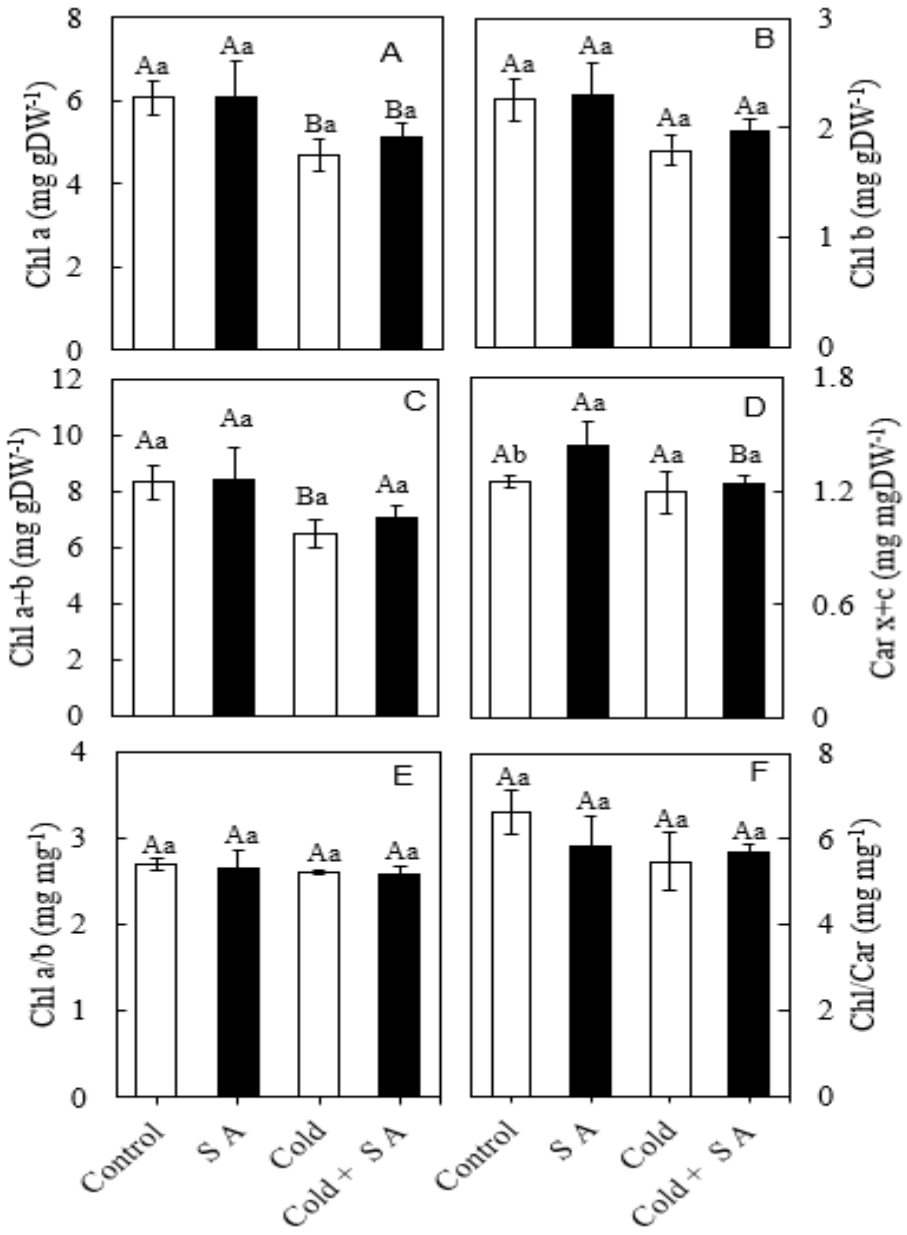


Fig. 21. Effect of exogenous salicylic acid and cold stress conditions on the concentration of photosynthetic pigments; chlorophyll a (A), chlorophyll b (B), total chlorophyll a+b (C), total carotenoids (D), chlorophyll a/b ratio (E) and chlorophyll/carotenoid ratio (F) in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25ºC/20ºC whereas cold stress conditions, 12ºC/12ºC.S A, Salicylic Acid. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman Keuls' test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

*5.3.3.4 Gas exchange*

Instigation of low temperatures caused a decline in the net photosynthesis ($P_N$), stomatal conductance ($g_s$) and transpiration rate (Trmmol) of coffee leaves (Fig. 22). On the other, although salicylic acid did not affect the above parameters significantly under optimum conditions, there was a noticeable decline in $P_N$, gs and Trmmol as a result of foliar salicylic acid application. Nevertheless, under cold stress conditions, salicylic acid tended to improve these gaseous exchange parameters (Fig. 22A, B and C). Intrinsic water use efficiency tended to show no significant changes on instigation of cold stress conditions or foliar application of salicylic acid.

*5.3.3.5 Chlorophyll florescence*

Exogenous salicylic acid tended to alleviate the reduction in the maximum and the effective quantum efficiency of photosystem II (Fig. 23). This was more pronounced during the light−adapted state compared to the dark−adapted state as both the $F_v'/F_o'$ and $F_v'/F_m'$ values were slightly higher in the cold + SA treatment compared to cold treatment without salicylic acid application. This was accompanied by slight improvements in fluorescence quenching and electron transfer rate despite these parameters not showing statistically significant differences amongst the treatments (Table 18). In addition, although cold stress instigation, caused significant declines in the actual efficiency of photosystem II ($\Phi_{PSII}$) and non–photochemical quenching (NPQ), application of salicylic acid had little effect on these chlorophyll fluorescence parameters (Table 18). Similarly, photochemical quenching ($q_P$) was neither affected by cold stress instigation nor salicylic acid treatment (Table 18).

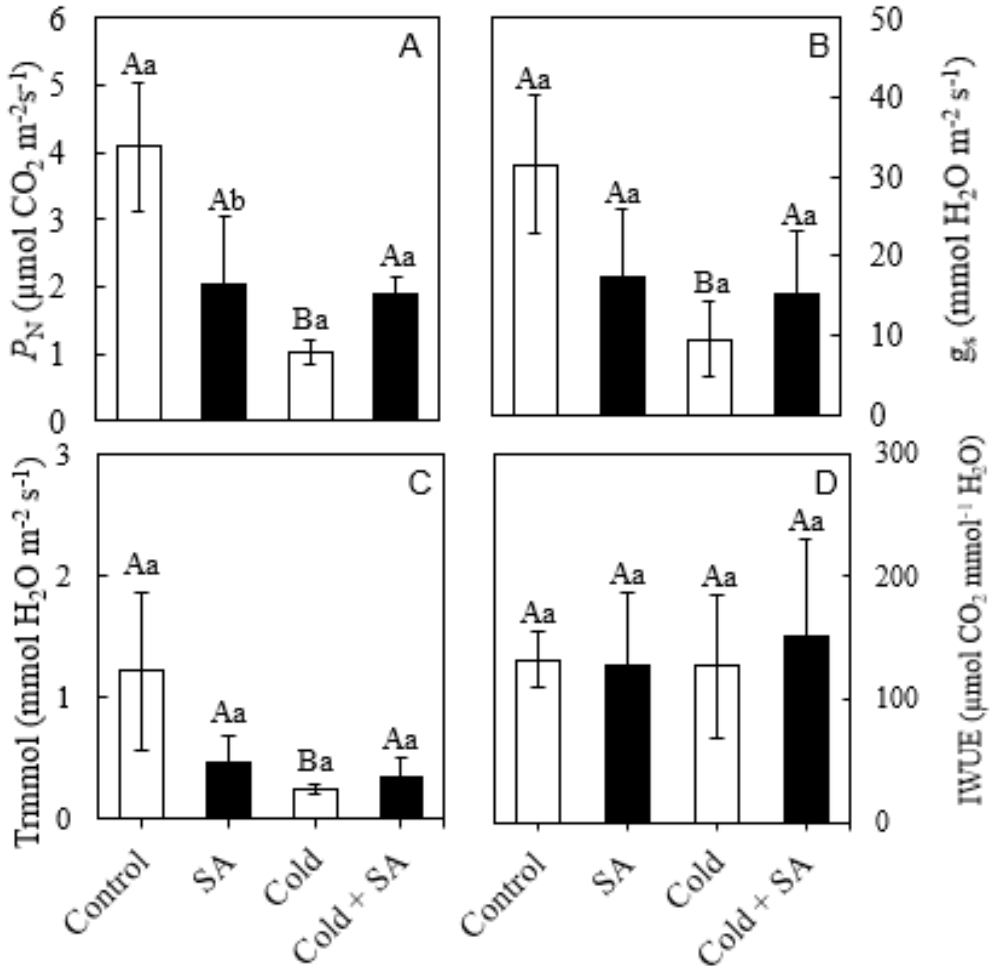


Fig. 22. Effect of exogenous salicylic acid and cold stress conditions on gas exchange parameters; net $CO_2$ assimilation (A), stomatal conductance (B), transpiration rate (C) and intrinsic water−use efficiency (D) in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25ºC/20ºC whereas cold stress conditions, 12ºC/12ºC.S A, Salicylic Acid. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman Keuls' test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

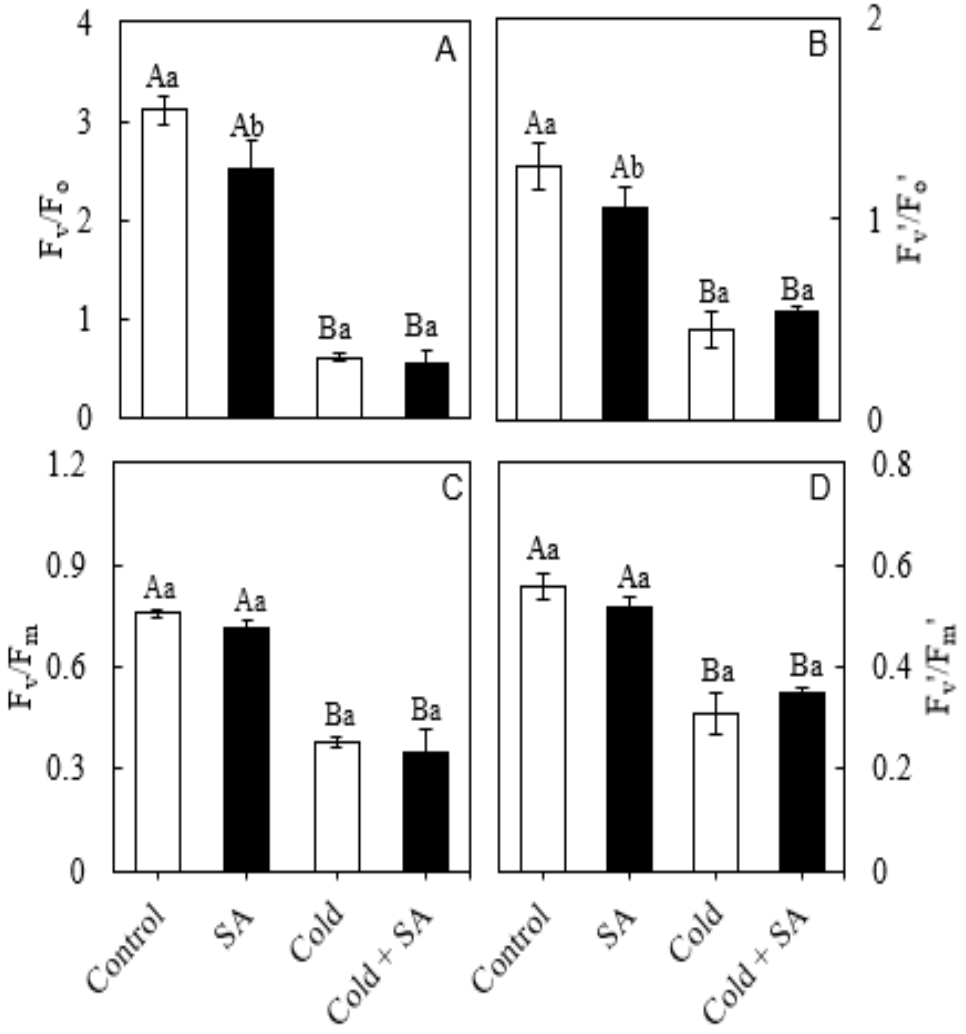


Fig. 23. Effect of exogenous salicylic acid and cold stress conditions on the maximum potential quantum efficiency of photosystem II during dark−adapted (A and C) and light−adapted states (B and D). SA, salicylic acid. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p$ < 0.05, Newman Keuls' test). Means are a replicate of three independent samples ($n$ = 3). Error bars represent the standard deviation of the mean.

Table 18. Effect of exogenous salicylic acid and cold stress conditions on the photosystem II photochemistry; electron transfer rate (ETR), photosystem II operating efficiency ($\Phi_{PSII)}$, fluorescence quenching ($q_N$), photochemical quenching ($q_P$) and non–photochemical quenching (NPQ) in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25ºC/20ºC whereas cold stress conditions, 12ºC/12ºC.S A, Salicylic Acid.

| Parameter | Optimum conditions | | Cold conditions | |
|---|---|---|---|---|
| | Control | SA | Cold | Cold + SA |
| ETR (µmol electrons $m^{-2}$ $s^{-1}$) | 28.7 ± 4.5 Aa | 24.9 ± 10.0 Aa | 15.0 ± 1.2 Aa | 15.1 ± 2.3 Aa |
| $\Phi_{PSII}$ | 0.13 ± 0.02 Aa | 0.11 ± 0.05 Aa | 0.07 ± 0.01A a | 0.07 ± 0.01 Aa |
| $q_N$ | 2.27 ± 0.12 Aa | 2.06 ± 0.10 Ab | 1.45 ± 0.09 Ba | 1.54 ± 0.03 Ba |
| $q_P$ | 0.24 ± 0.04 Aa | 0.22 ± 0.08 Aa | 0.22 ± 0.03 Aa | 0.20 ± 0.03 Aa |
| NPQ | 1.11 ± 0.11 Ab | 1.23 ± 0.20 Aa | 0.17 ± 0.03 Ba | 0.15 ± 0.04 Ba |

Within a row, different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote the statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman–Keuls test). Data are expressed as means ± S.D ($n = 3$).

*5.3.3.6 HPLC selected metabolite content*

Both cold induction and salicylic acid foliar treatment had no significant effect on the concentration of the antioxidant compounds determined by HPLC (Fig. 24). Nevertheless, the concentration of the total HPLC antioxidant compounds was highest in the salicylic acid treated coffee plants under optimum temperatures at 7.4 mg $gDW^{-1}$ and least in the control treatment plants at 6.74 mg $gDW^{-1}$. This elicitation in the total antioxidant compounds was as a result of salicylic acid induced increase in the content of 5–CQA from 4.8 mg $gDW^{-1}$ to 5.5 mg $gDW^{-1}$. Moreover, the increment in this phenolic compound was consistent under cold treatment with salicylic acid treatment having 5% more 5–CQA than their untreated counterparts (Fig. 24). On the other hand, although mangiferin accumulation was highest in salicylic acid and cold treatment plants at 0.14 mg $gDW^{-1}$, foliar treatment with salicylic acid under cold conditions resulted into the least content of this xanthonoid compound. The trend in variation in the content of the two alkaloid compounds assessed tended to be similar (Fig. 24). Both caffeine and trigonelline were highest in the leaves of control treated plants at 1.3 and 0.4 mg $gDW^{-1}$ respectively. Nevertheless, although the content of trigonelline was least in plants under cold treatment, that of caffeine was least under the same conditions in salicylic acid treated plants (Fig. 24). The antagonistic effect on the accumulation of 5–CQA and caffeine in the leaves of coffee plants under the different treatment conditions resulted into control plants having the lowest 5–CQA/Caffeine ratio. This ratio was nonetheless highest in salicylic acid treatment plants under cold conditions followed by those under optimum conditions. In addition, cold treated plants tended to have higher 5–CQA/Caffeine ratio compared to control plants.

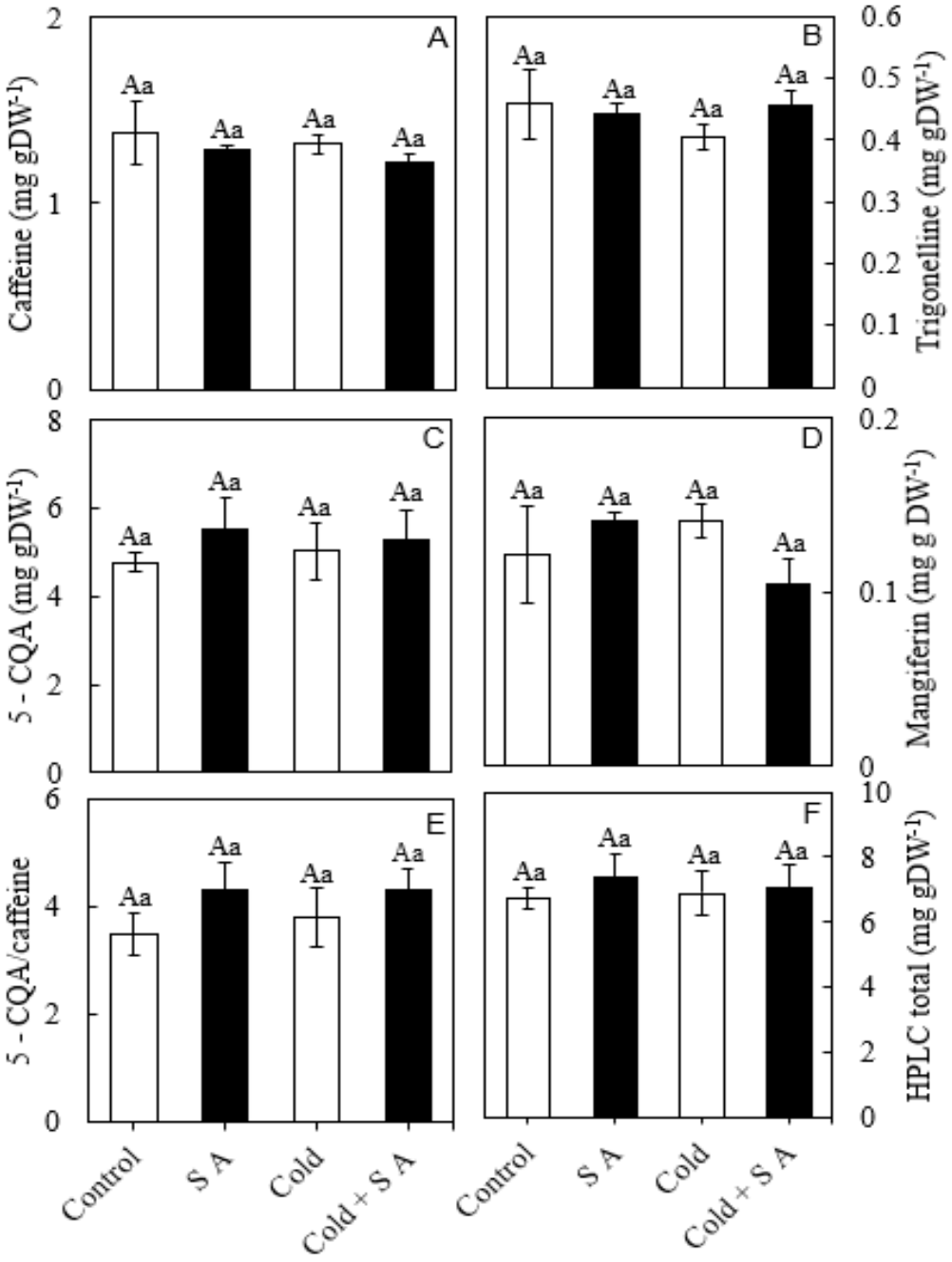


Fig. 24. Effect of exogenous salicylic acid and cold stress conditions on the concentrations of selected metabolites; caffeine (A), trigonelline (B), 5−caffeoylquinnic acid (C), mangiferin (D), ratio of caffeoylquinic acid to caffeine (E), and the HPLC total content (F) in the leaves of coffee plants . SA, salicylic acid. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman Keuls' test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

### 5.3.4 Discussion

Exogenous application of salicylic acid has been reported to alleviate the dampening of a number of physiological and metabolic plant process resulting from abiotic stress (Rivas−San Vicente and Plasencia, 2011). Abiotic stresses such as cold temperatures whose deleterious effects is increasingly being reported in *C. arabica* plants (Damatta and Ramalho, 2006) induce high accumulation of ROS species which disrupt cellular homeostasis leading to disintegration of many molecules including chlorophylls and several proteins (Mittler, 2002; Gill and Tuteja, 2010). Salicylic acid, a phenolic compound plays an important role in ROS scavenging hence reducing their over–accumulation to toxic levels (Miura and Tada, 2014; Singh *et al.,* 2017). In addition, its widely reported that salicylic acid is involved in signalling of several defence mechanisms which protect the cells against ROS (Herrera−Vásquez *et al.,* 2015; El−Esawi et *al.,* 2017). Moreover, high activities of antioxidant enzymes such as superoxide dismutase which scavenges the superoxide radical and catalase and peroxidases which detoxify the resultant $H_2O_2$ as a result of salicylic acid treatment under abiotic stresses have been reported in a number of plants (Kang and Saltveit, 2002; Wang *et al.,* 2014; Tang *et al.,* 2017). Salicylic acid application has also been reported to increase several defence compounds especially phenols (War *et al.,* 2011; Shin *et al.,* 2018).

In the current study, induction of cold temperatures resulted into significant membrane damages as assessed by the leakage of cellular electrolytes in both Cold and Cold + SA treatments plants (Fig. 19). Nevertheless, although non–significantly, exogenous application of salicylic acid tended to reduce the extent of membrane damage in the leaves of coffee plants by 3%. This could be as a result of accumulation of slightly less amounts of ROS in the salicylic acid treated plants under cold conditions hence causing less damage to cellular membrane and leakage of cellular components (Mittler, 2002; Shin *et al.,* 2008). Moreover, this protective effect of salicylic acid on cold stressed coffee plants was accompanied by significantly higher contents of nitrogen compared to cold treatment plants (Fig. 20). Application of exogenous salicylic acid has been reported to protect the degradation of proteins through enhancement of transcription of psbA gene which encodes the D1 protein subunit of PSII complex (Wang *et al.,* 2014). Similarly, although cold stress induction resulted into disintegration of chlorophylls pigments, exogenous application of salicylic acid slowed down this breakdown (Fig. 21). The current results are consistent with findings in similar work where salicylic acid treated plants contained higher amounts of chlorophyll pigments compared to non–treated plants under abiotic stress conditions (Wang *et al.,* 2014; Tang *et al.,* 2017). The results of the current study

indicated that exogenous salicylic acid under optimum conditions upregulated the metabolism of carotenoids molecules resulting into significantly highest content of these protective molecules. Our results agree with similar finding reported El–Tayeb *et al.* (2006). Carotenoids are auxiliary molecules that protect the photosynthetic apparatus against excess excitation through de–epoxidation of the xanthophyll cycle hence avoiding photo–bleaching of chlorophyll molecules (Verhoeven *et al.,*1997).

Light energy captured by chlorophyll molecules in the leaves is normally utilized through a series of photochemical redox reactions that result into formation of ROS. Photosynthesis a highly regulated plant process is known to be very sensitive to ROS whose accumulation is accelerated by abiotic stress conditions (Foyer, 2018). In the current study, instigation of cold stress conditions resulted into repression of several gas exchange parameters including net carbon dioxide assimilation ($P_N$), stomatal conductance ($g_s$) and transpiration rate (Trmmol) as indicated in Fig. 22. This could be as a result of higher contents of ROS as also indicated by higher electrolyte index and disintegration of pigment molecules compared to plants under optimum conditions. Plants normally respond to abiotic stress conditions by modulating stomatal closure hence affecting gaseous exchange parameters (Daszkowska-Golec and Szarejko, 2013; Kollist *et al.,* 2019). Exogenous application of salicylic acid under optimum conditions was associated with depressed $P_N$, gs and Trmmol (Fig. 22). Similar findings have been reported regarding salicylic acid induced reduction in $P_N$, gs, E and Ci under optimum conditions which could be attributed to salicylic mediated stomatal closure (Tang *et al.,* 2017). Conversely, under cold stress conditions, salicylic acid improved net $CO_2$ assimilation with associated gas exchange parameters (Fig. 22). Therefore, it is suggested that the beneficial roles of salicylic acid are more evident under abiotic stress compared to optimum conditions. It has further been suggested that the improved photosynthetic activity resulting from salicylic acid under abiotic stress conditions is due to improved $CO_2$ uptake activity in the chloroplasts rather than mere modulation of stomatal opening (Tang *et al.,* 2017).

Reduction in photosynthetic activity due to abiotic stresses such as cold is associated with photoinhibition of PSII as a result of both diffusive and biochemical stomatal limitations (Pompelli *et al.,* 2010). In the current study, instigation of cold stress temperatures resulted into a reduction in both actual PSII operating efficiency ($\Phi_{PSII}$) as well as the maximum potential efficiency of PSII (Table 18 and Fig. 23, respectively). This indicates the occurrence of higher degrees of photoinhibition in the cold stressed plants compared to those under optimum

conditions. Although non–significantly, foliar sprays of salicylic acid tended to reduce the degree of photoinhibition especially during the light–adapted state as indicated by higher ratios of both $F_v'/F_o'$ and $F_v'/F_m'$. It has been suggested therefore, that exogenous application of salicylic acid can regulate chloroplast energy state hence resulting in to less accumulation of ROS (Tang *et al.,* 2017). Moreover, salicylic acid treated plants tended to have higher values of fluorescence quenching ($q_N$) than their un–treated counterparts although non–significantly (Table 18). These results suggest therefore that salicylic acid might alleviate photoinhibition resulting from cold stress conditions.

Plants have evolved a number of mechanisms to deal with accumulation of ROS such those instigated by cold stress conditions (Asada, 1999; Gill and Tuteja, 2010). Coffee plants in particular, rely on non–enzymatic compounds including phenolic and/or alkaloids for ROS scavenging (Ramalho *et al.,* 2018). These compounds are well known ROS scavengers due to presence of hydroxyl component and glycosylic linkages (Das and Roychoudhury, 2014). Promotion of phenolic compounds with concomitant improvement in ROS scavenging ability has been reported (War *et al.,* 2011). In the current study, salicylic acid treatment tended to increase the content of phenolic compounds especially 5–CQA under both optimum and cold stress conditions while having less effects on the alkaloids (Fig.24). This resulted into higher 5–CQA ratio and total content of the protective compounds in salicylic acid treated plants. Higher ratio of 5–CQA/caffeine is associated with higher ROS scavenging capacity due to potency of phenolic compounds over alkaloids (Ky *et al.,* 2001). Although the mechanism of stimulation of antioxidant compounds like 5–CQA cannot be confirmed at this point, it is suggested that salicylic acid differentially affect the expression of antioxidant transcripts resulting into modification of secondary composition in the cells (El−Esawi *et al.,* 2017). By reinforcing the antioxidant system, salicylic acid application therefore restores homeostatic conditions and hence minimize the deleterious effects of over accumulation of ROS.

Instigation of cold temperatures reduced the operating efficiency of PSII with concomitant reduction in net $CO_2$ assimilation and stomatal conductance. This resulted into ROS bursts since any light incident on a stressed photosynthetic apparatus is likely to be in excess and hence results in photoinhibition. Accumulation of ROS caused disintegration of cellular membranes hence increasing the leakage of internal electrolytes, bleaching of chlorophylls as disintegration of protection molecules. On the other hand, exogenous spraying with 1.0 mM salicylic acid modulated the dampening effect of cold stress by suppressing the accumulation

of ROS in the leaves of the treated coffee plants. This was associated with a decline in the collapse of the cellular membrane resulting into slightly less electrolyte leakage in the cells of salicylic acid treated plants under cold stress conditions. Moreover, salicylic acid slowed down the breakdown of chlorophylls and the D1 protein subunit of PSII as indicated by higher leaf nitrogen content in the salicylic acid treated plants under cold stress conditions. This was accompanied by salicylic acid improvement in the photosystem II operating efficiency and net photosynthetic rate. The current results suggest that exogenous salicylic acid treatment might reverse the negative effects induced by low positive temperatures whose occurrence is on an increasing trend during coffee cultivation.

### 5.4 Melatonin

Melatonin (N–acetyl–5–methoxy tryptamine) is a pleiotropic ubiquitous hormone with multifunctional effects in both animals and plants (Arnao and Hernández−Ruiz, 2019). Although first discovered in the bovine pineal gland as a secretory molecule (Lerner *et al.,* 1958), the subsequent discovery of melatonin in several plant tissues suggested its involvement in modulation of several physiological, metabolic and biochemical processes necessary for plant growth and development (Sharif *et al.,* 2018; Hattori *et al.,* 1995; Lerner *et al.,* 1958). Besides, its regulatory role under normal cellular functioning (Arnao and Hernández−Ruiz, 2006; Hardeland, 2016), melatonin accumulation has been reported to increase as a result of exposure to abiotic stress conditions where it directly scavenges reactive oxygen species (Arnao and Hernández−Ruiz, 2013; Yu *et al.*, 2018). As a result, exogenous application of melatonin as a natural bio–stimulating treatment for field crops under abiotic stress conditions has recently received tremendous attention (Arnao and Hernández−Ruiz, 2014). Being an amphipathic molecule, exogenously applied melatonin readily diffuses across the cell membrane into the cytoplasm and into the subcellular compartments where it triggers several processes that confer tolerance to abiotic stress (Zhang *et al.,* 2015). Exogenous application of melatonin in various application levels modulates the negative effects induced by several abiotic stresses on plant metabolism and physiology (Kul *et al.,* 2016). Exogenously applied melatonin has been reported to increase tolerance of several plants to abiotic stress conditions such as salinity stress in; *Helianthus annus* (Arora and Bhatla, 2017), *Zea mays* (Chen *et al.,* 2018) *Cucumis sativa* (Wang *et al.,* 2016), heavy metal stress in Medicago sativa (Gu *et al.,* 2017), pepper plants (Sarafi *et al.,* 2017), drought in; kiwi seedlings (Liang *et al.,* 2019), coffee plants (Campos *et al.,* 2019), and extreme temperature stress in; Cucumis sativa

(Zhao *et al.,* 2017), Bermuda grass (Hu *et al.,* 2016), *Camelia sinensis* (Li *et al.*, 2017; Li *et al.*, 2018).

Melatonin mediated improvement in tolerance against abiotic stress conditions is accompanied with modulation of oxidative stress induced–decline in the photosynthetic activity which is also associated stabilization of cellular membranes and photosynthetic pigments (Kul *et al.,* 2016). This is accompanied with improved efficiency of photosystem II in the melatonin treated plants compared to the untreated plants under abiotic stress conditions (Wang *et al.,* 2016). In addition to prevention of reactive oxygen species burst under oxidative stress conditions, exogenous application of melatonin has also been reported to increase the activities of antioxidant enzymes such as SOD, APX and CAT through upregulation of the related transcripts (Arora and Bhatla, 2017; Chen *et al.,* 2018). This promotion of the enzymatic antioxidant system results into restoration of the redox homeostatic conditions hence resulting in to growth modulation under oxidative stress conditions (Sarafi *et al.*, 2017; Li *et al.*, 2018).

Having evolved as understory species in the tropical rain forests of sub−Saharan Africa under moderate temperatures and light conditions (Davis *et al.,* 2006), coffee species are very susceptible to conditions associated with suboptimal conditions such as low temperature (Damatta and Ramalho, 2006). Cold stress in coffee plants induces diffusive and biochemical limitations that result in to diminished photosynthetic activity (Damatta *et al.,* 1997; Pompelli *et al.*, 2010). The reduction in the photosynthetic capacity of the stressed coffee leaves results into photoinhibition which is associated with accumulation of ROS resulting from excess excitation energy reaching a stressed photosynthetic apparatus (Müller *et al.,* 2001). However, coffee plants exhibit some degree of tolerance to cold stress conditions largely because of the plasticity of their photosynthetic apparatus (Chaves *et al.,* 2008). During cold stress conditions, leaves accumulate high amounts of carotenoids molecules in the chloroplast which protect the leave against high light intensity through de–epoxidation of the xanthophyll cycle where violaxanthin is converted to which anthexanthin and zeaxanthin resulting into dissipation of the excess light energy (Adams *et al.*, 2004; Demmig-Adams and Adams, 2004; Verhoeven *et al.,* 1997). Plants are also endowed with both the enzymatic and nonenzymatic antioxidant systems which scavenge excess ROS to restore homeostatic conditions (Asada, 2006; Gill and Tuteja, 2010; Foyer, 2018). Due to suppression of enzymatic activities of SOD, APX and CAT during cold stress conditions, leaves of coffee plants rely on accumulation of non–enzymatic

compounds for ROS detoxification (Ramalho *et al.,* 2018). Employment of this non–enzymatic antioxidant system is more crucial in the young leaves due to a less developed enzymatic antioxidant system unlike in the older counterparts (Campa *et al.,* 2017). The non–enzymatic antioxidant compounds especially phenolic compounds such as chlorogenic acids and mangiferin are endowed with several hydroxyl components and electrophilic glycosylic linkages that neutralise the excess ROS during oxidative stress conditions hence restoring homeostatic conditions (Das and Roychoudhury, 2014). Coffee plants also accumulate high amounts of alkaloid compounds such as caffeine and trigonelline although their role in ROS detoxification is less understood. Reinforcement of this non–enzymatic antioxidant system might therefore confer tolerance to abiotic stress conditions due enhanced ROS scavenging capacity during oxidative stress conditions initiated by cold stress conditions. Exogenous application of melatonin has been reported upregulated the metabolism of some compounds such as polyamines and modulated abscisic acid and salicylic acid which play key roles in oxidative stress tolerance (Zhao *et al.,* 2017). Nevertheless, effect of melatonin on the phenolic and alkaloid compounds of coffee plants towards improved tolerance to cold stress conditions in coffee plants remains unknown.

In addition to being the most important coffee species economically, contributing to over 60% on the global trade (ICO, 2019), *C. arabica* has been reported to be more cold tolerant than its counterpart *C. canephora* (Damatta *et al.,* 1997). As a result cultivation of *C arabica* is more extensive in the highland areas under subtropical climate where chilling temperatures are common during winter conditions especially during winter (Ramalho *et al.,* 2014). Moreover, the negative impacts of cold stress on growth and productivity of coffee crops is already being exacerbated by the current trends in the climatic changes which have led to decline in the productivity of arabica coffee (Alemayehu and Merga, 2019). Therefore, its of profound importance to alleviate the effects of cold stress on coffee productivity. This study, therefore investigated the possible reinforcement of the nonenzymatic oxidative system through promotion of antioxidant compounds in the leaves of the coffee plants as a result of exogenous foliar melatonin application towards improvement of photosynthetic physiology and tolerance of coffee plants to cold stress conditions.

### 5.4.1 Material and methods

*5.4.1.1 Plant material and growing conditions*

Plant materials and growing conditions have been described in the previous chapter (see section 2.2).

*5.4.1.2 Melatonin and cold stress treatments*

After acclimation, the plants were randomly assigned to two temperature conditions and two melatonin treatments in a 2 x 2 factorial experimental design. The four treatments were as follows: (1) control, for which the plants were maintained at optimum temperatures (day/night, 25°/20°C respectively) and sprayed with distilled water plus surfactant; (2) melatonin, sprayed with 100 µM of melatonin solution and maintained at optimum temperature; (3) Cold, sprayed with distilled water and subjected to cold stress; (4) Cold + MT, sprayed with 100 µM melatonin solution and subjected to cold stress. Foliar sprays were administered 3 times every after 3 days using a hand sprayer immediately after the growth chamber lights routinely turned off. In all the sprays, 0.1% isopropyl alcohol (Kao Global Chemicals, Tokyo, Japan) was used as the surfactant. Cold stress was instigated 24 h after the last foliar sprays by reducing the growth chamber temperature from 25°/20°C to 12°/12°C, day/night respectively for 5 days while maintaining the other ambient conditions constant.

*5.4.1.3 Photosynthetic measurements*

Gaseous and chlorophyll *a* fluorescence measurements were done as previously described in the previous chapter (Subsection 4.2.3) and the subsequent calculation made as previously described in subsection 5.2.1.3.

*5.4.1.4 Electrolyte leakage index analysis*

Electrolyte leakage index was assessed as previously described in the previous chapter (subsection 5.2.1.4).

*5.4.1.5 Sample preparation for biochemical assays*

For biochemical analysis, the leaves were sampled and processed as previously described (subsection 3.2.3).

*5.4.1.6 Photosynthetic pigments*

Chlorophylls and carotenoids pigments were assayed and determined as previously described in the previous chapter (subsection 3.2.4).

*5.4.1.7 HPLC analysis of selected metabolite analysis compounds*

Selected alkaloids and phenolic compounds were assayed and determined as previously described in the previous chapter (subsection 2.2.4.4).

### *5.4.2 Statistical analysis*

Experimental data were statistically examined using one−way analysis of variance (One−way ANOVA) following a completely randomized design. The parameter differences were compared for statistical significance amongst the treatments using the Newman−Keuls test at $P \leq 0.05$ except where stated otherwise. Assumptions of normality were tested using the Kolmogorov−Smirnov test and data transformed accordingly to attain a normal distribution whenever necessary. All the statistical analyses were done using Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). Data are expressed as means ± SD, n = 3.

### 5.4.3 Results

*5.4.3.1 Electrolyte leakage index*

The extent of membrane damage as measured by electrolyte leakage index (ELI) was highest under cold stress conditions reaching a peak of 30% in the Mel + Cold treatment plants while absence of melatonin resulted into an ELI of 20% (Fig. 25 1). On the other hand, membrane damage was least under optimum conditions both coffee plants under both control and melatonin treatments showing an ELI of 12%.

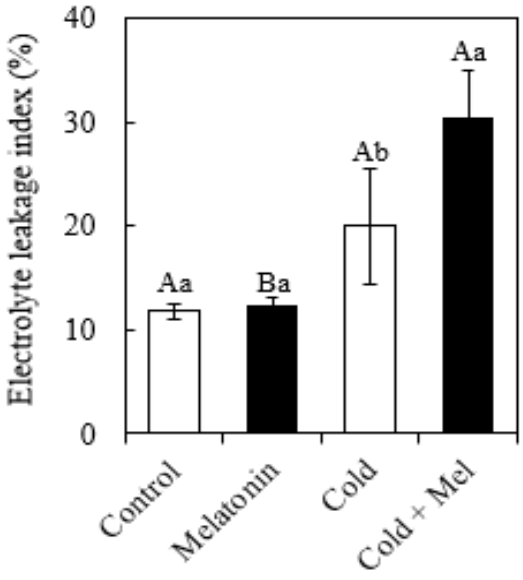


Fig. 25. Effect of exogenous melatonin on the electrolyte leakage index in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25/20 °C, whereas cold stress conditions, 12/12 °C. Mel, melatonin. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means between melatonin treatments ($p < 0.05$, Newman–Keuls test). Means are a replicate of three independent samples ($n = 3$). Error bars represent standard deviation of the mean.

*5.4.3.2 Photosynthetic pigments*

Instigation of cold stress conditions resulted into breakdown of both chlorophyll and carotenoid pigments (Fig. 26). The concentrations of both total chlorophylls and total carotenoids were significantly reduced from 8.2 and 1.4 to below 5.3 and 1.1 mg $gDW^{-1}$, respectively when cold stress conditions were introduced. Similarly, both chlorophyll a and b were lowest under cold stress conditions at an average of 3.8 and 1.3 mg $gDW^{-1}$ compared to an average of 6 and 2.2 mg $gDW^{-1}$, respectively. In this study, exogenous application of melatonin showed no protective effects against the disintegration of the pigments. In fact, despite showing non−significance, a slight reduction in the concentrations of chlorophylls was observed as a result of exogenous melatonin application under both control and cold stress conditions. As a result, the concentrations of both chlorophyll a and b was least at 3.7 and 1.2 mg $gDW^{-1}$, respectively, in the melatonin treated plants under cold stress conditions.

*5.4.3.3 Gas exchange*

Instigation of low temperatures caused a decline in the net photosynthesis ($P_N$), stomatal conductance ($g_s$) and transpiration rate (E) in the leaves of coffee plants (Fig. 27). On the other, the ratio of intercellular to ambient $CO_2$ concentrations ($C_i/C_a$) was highest under cold stress conditions. Under optimum conditions, exogenous application of melatonin significantly reduced the $P_N$, gs and E although these parameters remained higher than in plants under cold stress conditions (Fig. 27A−C, respectively) despite showing a slight increase in the same parameters under cold stress conditions. On the other hand, despite showing non−significance, a slight reduction in the $C_i/C_a$ was observed under cold stress conditions in the melatonin treated plants (Fig. 27D).

*5.4.3.4 Chlorophyll florescence*

Instigation of cold stress conditions significantly lowered the efficiency of photosystem II photochemistry (Fig. 28). Similarly, exogenous melatonin application under both optimum and cold stress conditions significantly reduced the during both the dark and light−adapted state. The same trend was exhibited by fluorescence parameters of PSII (Table 19). Cold stress conditions caused a significant reduction in the electron transfer rate (ETR), the operating efficiency of PSII ($\Phi_{PSII}$), fluorescence quenching ($q_N$) and the non−photochemical quenching (NPQ) in the leaves of coffee leaves compared to those under optimum conditions. Despite showing no significant changes in variation, both photochemical quenching ($q_P$) and the

proportion of PSII open centres ($q_L$) also showed a slight reduction after instigation of cold stress conditions. Exogenous application of melatonin under cold stress conditions showed no significant effects on ETR, ΦPSII, qP and $q_L$ despite causing slight improvements in these parameters . Nonetheless, $q_N$ and NPQ varied antagonistically with melatonin treatment under optimum conditions with the latter reducing by 30% and the former increasing by 73%, respectively.

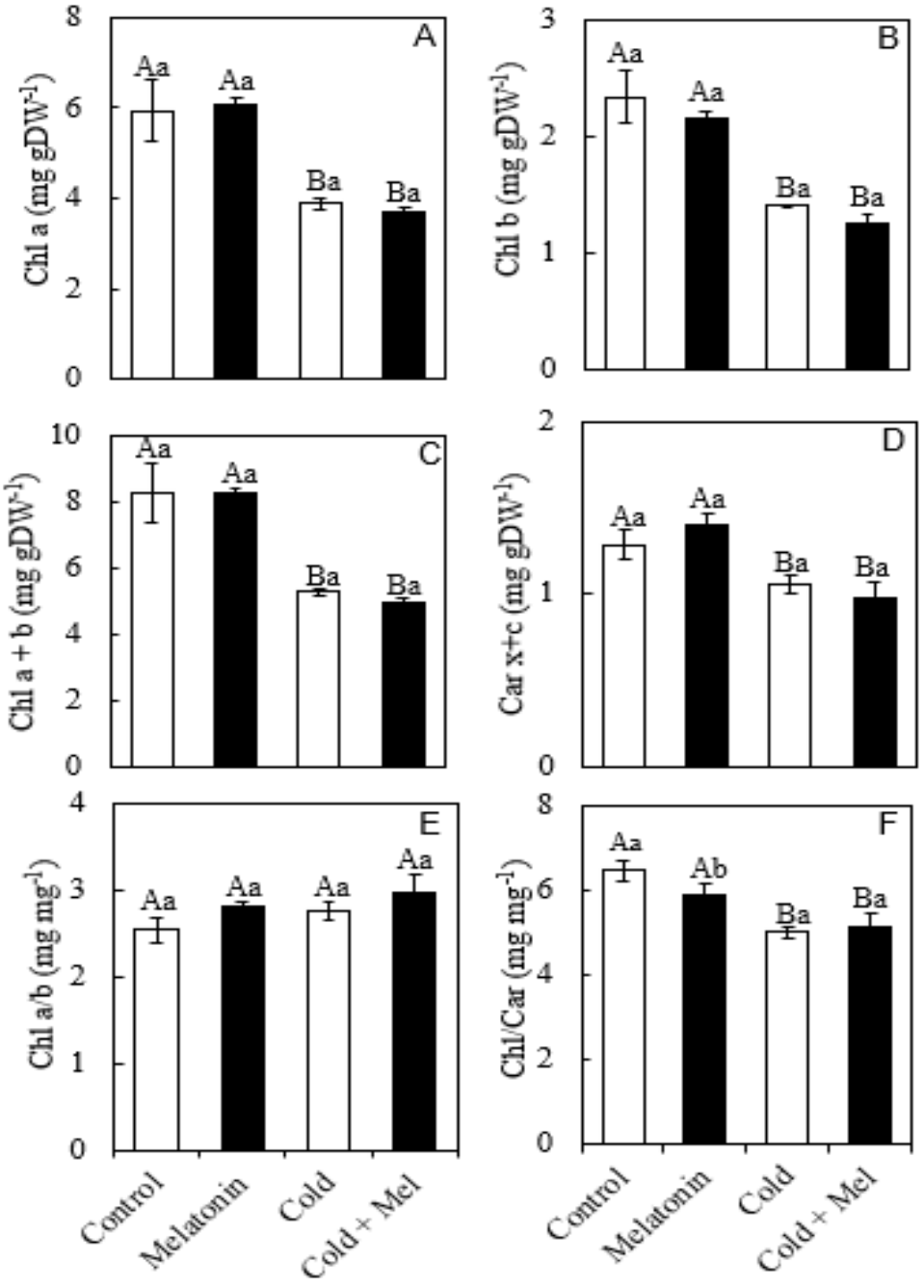


Fig. 26. Effect of exogenous melatonin and cold stress conditions on the contents of photosynthetic pigments; chlorophyll a (Figure 2A), chlorophyll b (Figure 2B), total chlorophylls (Fig. 2C), total carotenoids (Fig. 2D) as well as the ratios of chlorophyll a/b (Fig. 2E) and total chlorophyll /total carotenoid (Fig. 2F). Mel, melatonin. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within kinetin treatments ($p < 0.05$, Newman Keuls' test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

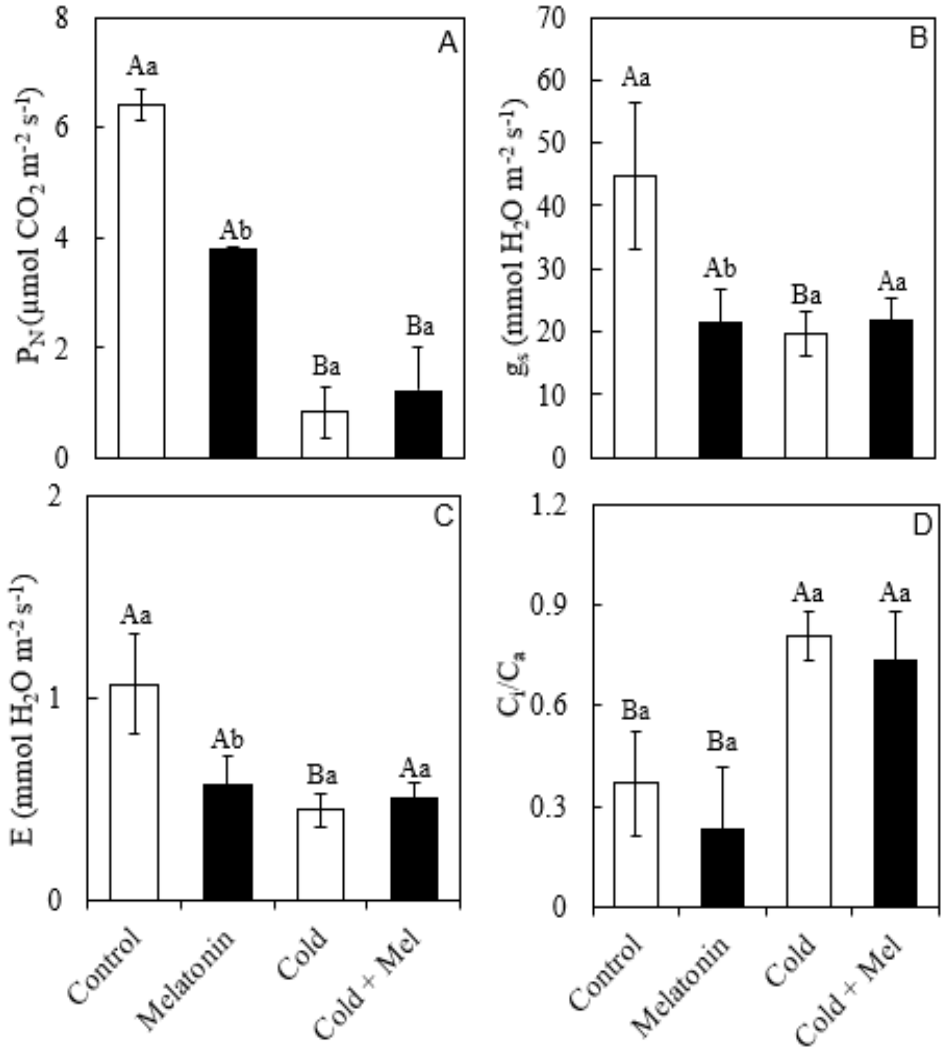


Fig. 27. Effect of exogenous melatonin and cold stress conditions on gas exchange parameters; net $CO_2$ assimilation (A), stomatal conductance (B), transpiration rate (C) and the ratio of internal to ambient $CO_2$ concentrations (D) in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25°C/20°C whereas cold stress conditions, 12°C/12°C. Mel, melatonin. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within melatonin treatments ($p < 0.05$, Newman Keuls' test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

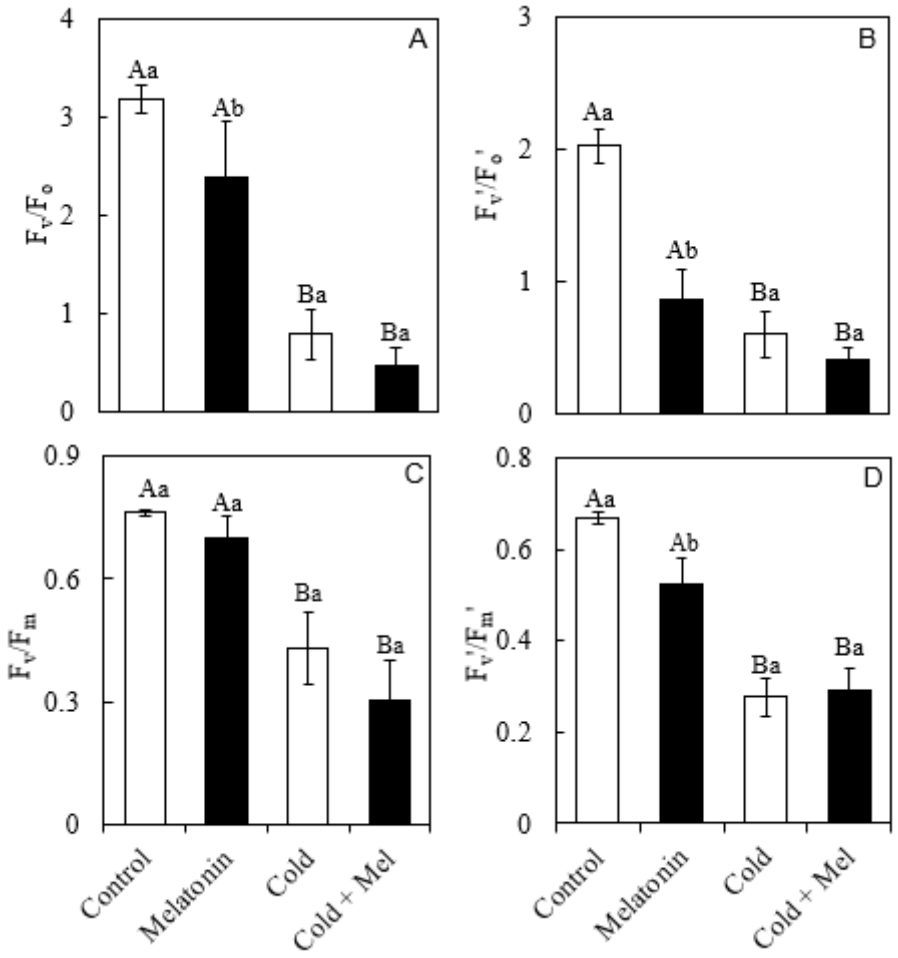


Fig. 28. Effect of exogenous melatonin and cold stress conditions on the maximum potential quantum efficiency of photosystem II during dark−adapted (A and C) and light−adapted states (B and D) in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25ºC/20ºC whereas cold stress conditions, 12ºC/12ºC. Mel, melatonin. Mel, melatonin. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within melatonin treatments ($p < 0.05$, Newman Keuls' test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean.

*5.4.3.5 HPLC content selected metabolite compounds*

Both cold induction and exogenous melatonin application caused various effects on the selected compounds determined by HPLC (Fig. 29). The concentration of caffeine was significantly lowest in the control treatment plants at 1.3 mg $gDW^{-1}$ whereas other treatments showed no significant differences amongst each other despite indicating a slight increase resulting from both cold and melatonin treatments (Fig. 29A). Similarly, the concentration of trigonelline was lowest at 0.5 mg $gDW^{-1}$ in the control treatment plants with both cold stress and melatonin treatment causing a slight increase in the concentration of trigonelline (Fig. 29B). The concentration of 5−CQA was lowest in the control treatment plants at 3.7 mg $gDW^{-1}$. Under optimum conditions, melatonin application increased the concentration of 5−CQA to 5.6 mg $gDW^{-1}$ while no eliciting no significant effects under cold stress conditions (Fig. 29C). A similar variation was observed for mangiferin and hence this compound also being lowest in the control treatment plants at 0.08 mg $gDW^{-1}$ (Fig. 29D). In absence of melatonin, instigation of cold stress conditions caused a significant increase in the concentration of mangiferin to 0.14 mg $gDW^{-1}$ while no significant effect was observed as a result of exogenous melatonin treatment under both optimum and cold stress conditions. Although the ratio of 5−CQA to caffeine was no significantly affected by temperature treatment, it was significantly increased by exogenous application of melatonin (Fig. 29E). Contrastingly, both temperature and exogenous melatonin application significantly increased the total content of HPLC metabolites with melatonin treatment having more effect under optimum conditions (Fig. 29F).

Table 19. Effect of exogenous melatonin and cold stress conditions photosystem II photochemistry; electron transfer rate (ETR), photosystem II operating efficiency ($\Phi_{PSII}$), fluorescence quenching ($q_N$), photochemical quenching ($q_P$), non–photochemical quenching (NPQ) and proportion of open photosystem II reaction centres in the leaves of coffee plants under optimum and cold stress conditions. Optimum temperature, 25ºC/20ºC whereas cold stress conditions, 12ºC/12ºC. Mel, Melatonin.

| Parameter | Optimum conditions | | Cold stress conditions | |
|---|---|---|---|---|
| | Control | Melatonin | Cold | Cold + Mel |
| ETR (µmol electrons $m^{-2}$ $s^{-1}$) | 26.0 ± 4.6 Aa | 28.1 ± 4.0 Aa | 8.62 ± 1.80 Ba | 12.9 ± 4.0 Ba |
| $\Phi_{PSII}$ | 0.12 ± 0.02 Aa | 0.13 ± 0.02 Aa | 0.04 ± 0.01 Ba | 0.06 ± 0.02 Ba |
| $q_P$ | 0.18 ± 0.03 Aa | 0.25 ± 0.04 Aa | 0.14 ± 0.02 Aa | 0.20 ± 0.05 Aa |
| $q_N$ | 3.03 ± 0.12 Aa | 2.12 ± 0.27 Ab | 1.39 ± 0.08 Ba | 1.41 ± 0.09 Ba |
| NPQ | 0.44 ± 0.09 Ab | 1.50 ± 0.35 Aa | 0.25 ± 0.05 Aa | 0.12 ± 0.11 Ba |
| $q_L$ | 0.47 ± 0.07 Aa | 0.35 ± 0.13 Aa | 0.29 ± 0.06 Aa | 0.27 ± 0.07 Aa |

Within a row, different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas treatment means followed by the same small letter, ($^{a}$) are not statistically significantly different between the melatonin treatments ($p < 0.05$, Newman Keuls' test). Data are expressed as means ± S.D ($n = 3$).

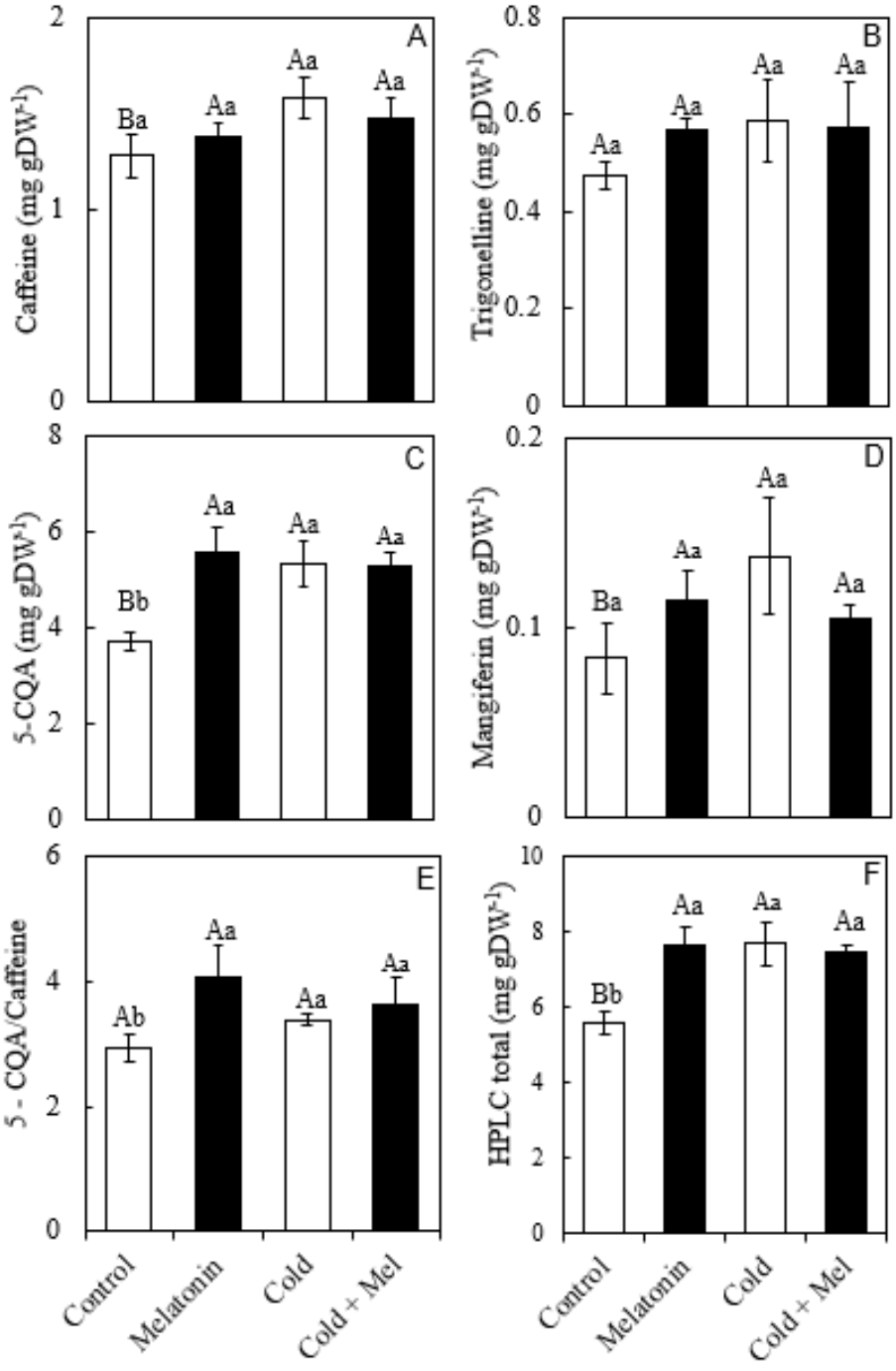


Fig. 29. Effect of exogenous melatonin and cold stress conditions on the concentrations of selected metabolites; caffeine (A), trigonelline (B), 5−caffeoylquinnic acid (C), mangiferin (D), ratio of caffeoylquinic acid to caffeine (E), and the HPLC total content (F) in the leaves of coffee plants . Mel, melatonin. Different capital letters, ($^{A,B}$) denote statistically significant differences between the parameter means within temperature treatments, whereas different small letters, ($^{a,b}$) denote statistically significant differences for the parameter means within melatonin treatments ($p < 0.05$, Newman Keuls' test). Means are a replicate of three independent samples ($n = 3$). Error bars represent the standard deviation of the mean

### 5.4.4 Discussion

Low temperature conditions cause severe stress to growth and productivity of coffee plants. Exogenous application of melatonin has been suggested to improve the tolerance of plants against several abiotic stress conditions including cold stress conditions (Gao *et al.,* 2018; Chen *et al.,* 2018; Campos *et al.,* 2019). Reactive oxygen species (ROS) which are by−products of photosynthesis and respiration are produced under both normal and abiotic stress conditions. However, under the latter conditions, the elevated production of ROS can stimulate membrane lipid peroxidation which results into damage to cell membrane (Mittler, 2002). In the current study, instigation of cold stress conditions resulted into an increase in the extent of membrane damage as indicated by higher ELI compared to than in plants under optimum conditions (Fig. 25). The current results agree with a number of studies where cold temperature instigation resulted into higher levels of membrane damage compared to plants growing under optimum temperature conditions (Gao *et al.*, 2018; Li *et al.*, 2018; Zhang *et al.*, 2018). In the current study under cold stress conditions, melatonin application increased the extent of membrane damage. This could be as a result of increased ROS resulting from exogenous melatonin application. It has been suggested that exogenous melatonin application generally reinforces the antioxidant system of plants through signal transduction by initiating the NADPH oxidase/ respiratory burst oxidative homolog (RBOH) a phenomenon known as ROS priming (Gong *et al.,* 2017). This mechanism involves an increase in the concentration of $H_2O_2$ which on one hand can result into formation of hydroxyl radicals ($OH^{\bullet-}$) via the Fenton reaction which causes severe damage to the cell membrane and increasing the leakage of cellular electrolytes (Mittler, 2017). Nevertheless, the perceived negative effect could be short lived since the increase in the ROS scavenging capacity resulting from elevated $H_2O_2$ levels via the cellular signalling pathway (Mittler, 2017) on another hand might counter the effects of cold stress in melatonin treated plants compared to untreated counter parts.

Both chlorophylls and carotenoids are susceptible to abiotic stress conditions (Ashraf and Harris, 2013). Under abiotic stress conditions, the reduction in the concentration of chlorophylls and carotenoid pigments results from both impaired pigment biosynthesis and accelerated rates of disintegration (Perveen *et al.,* 2010). In the current study, instigation of cold stress temperatures resulted into lower concentrations of chlorophylls and carotenoid pigments (Fig. 26). The increased concentration of ROS in the melatonin treatment also resulted into a decline in the concentration of especially chlorophyll b under optimum conditions and hence resulting into a lower ratio of total chlorophylls to carotenoids. The

current results are in contrast to those where melatonin improved the concentration of chlorophyll pigments under abiotic stress conditions (Han *et al.,* 2017; Li *et al.*, 2017, Li *et al.,* 2018). This discrepancy could be as a result of the duration of evaluation of melatonin effects for in the above unlike in the previous studies, the current study evaluated the effects of melatonin application after 5 days of cold stress instigation. Therefore, it is plausible that melatonin effects are not only concentration dependent but are also affected by both the duration of treatment as well as time of evaluation.

The reduction in the concentrations of the photosynthetic pigments was associated with similar variation in the photosynthetic activity as shown by low gas exchange parameters such as $P_N$, $g_s$ and E (Fig. 27A−C, respectively). Low temperatures induce both stomatal and non−stomatal limitations to photosynthesis with the latter including stomata closure which concomitantly results diminished $P_N$, $g_s$ and E (Tissue *et al.,* 2005). An increase in the $C_i/C_a$ was also observed under cold stress conditions (Fig. 27D). This could be owed to the increase in the $C_i$ as a result of a reduction in the ability to reduce absorbed $CO_2$ due to cold induced reduction in the activities of both the light and the light independent photosynthetic cycles (Cen and Sage, 2005; Ensminger *et al.,* 2006). Photosynthesis is a highly regulated physiological process and immensely sensitive to variations in the environmental conditions. In the current study, exogenous melatonin treatment reduced $P_N$ under optimum conditions possibly due to initiation of RBOH and or melatonin induced stomatal closure (Wei *et al.,* 2018). Nevertheless, consistent but slightly higher gas exchange parameters and lower $C_i/C_a$ were observed in the melatonin treated plants under cold stress conditions signifying the possible protective role of melatonin under cold stress conditions.

The operating efficiency of PSII photochemistry which chiefly modulates the photosynthetic activity of plants was significantly affected by both cold stress conditions and exogenous melatonin treatment (Fig. 28 and Table 19). Both cold stress treatment and melatonin application induced PSII photoinhibition as shown by reductions in the PSII maximum quantum efficiency ($F_v/F_m$ and $F_v/F_o$) during both dark and light adapted states. Such declines are associated with damages to the D1 protein subunit in the PSII photosystem which is highly sensitive to ROS accumulation induced by abiotic stress conditions including low temperatures (Murata *et al.,* 2007). The ability of the leaves to utilize the incident photosynthetically active radiation (PAR) is lowered abiotic stress conditions (Muller *et al.,* 2001). The resultant excess excitation energy from PAR translates into accumulation of ROS which directly inactivates the photochemical reaction centres of PSII (Murata, 2007). In the current study, this was evidenced

by decline in the ETR, $\Phi_{PSII}$, $q_P$ as well as the closure of the PSII reaction centres under cold stress conditions (Table 19). Under optimum conditions, the initiation of RBOH by exogenous melatonin application also reduced the operating efficiency PSII photochemistry. However, after 5 days a slight improvement in the PSII operating efficiency was observed in the melatonin treated plants. Exogenous melatonin application has been shown to reduce the decline in the operating efficiency of PSII reaction centres (Han *et al.,* 2017). This could be attribute to the scavenging ability of both the melatonin molecule as well as its modulation effect in the cells' secondary metabolism (Debnah *et al.,* 2019).

Plants have evolved several mechanisms to deal with excess accumulation of ROS during abiotic stress conditions in order to maintain a redox balance (Mittler, 2002; 2016). These ROS defence mechanisms comprise of both enzymatic and nonenzymatic antioxidant components (Asada, 2006). Due to the underdeveloped enzymatic antioxidant systems in the young leaves (Campa *et al.,* 2017) and also the suppression of enzymatic activity during low temperatures, coffee plants rely accumulation of nonenzymatic antioxidant compounds as the main mechanism of ROS detoxification (Ramalho *et al.,* 2018). These compounds especially the phenolics such as hydroxycinnamic acid esters and mangiferin possess electrophilic hydroxyl components and glycosylic linkages with potent ROS scavenging ability (Das and Roychoudhury, 2014). Moreover, other compounds such as trigonelline have shown to have antioxidant properties (Garg, 2006). In addition, this trigonelline (1,3,7−N−trimethylxanthine) is a NAD reservoir, a key coenzyme in energy metabolism hence important in cell adaptation to abiotic stress conditions (Ashihara, 2006; Zamani *et al.,* 2019). In the current study, the assessed compounds were generally increased by both melatonin and cold stress treatments (Fig. 29). This increment can be attributed to the ROS mediated signalling pathway which involves $H_2O_2$ mediated oxidation of cysteine residues on the protein resulting into changes in their structure and function (Mittler, 2017; Sies, 2017). In addition to this pathway melatonin has been shown to directly affect the cellular metabolism by affecting gene expression or suppression hence affecting metabolite accumulation in plants (Fan *et al.,* 2015).

The increased accumulation of ROS under cold stress conditions resulted into damage to the cellular membrane and caused disintegration of photosynthetic pigments. This was associated with a decline in both the rates of gas exchange and the activity of PSII photochemistry. However, these conditions resulted into accumulation of the quantified metabolites. On the other hand, under optimum conditions, exogenous melatonin application negatively affected the photosynthetic activity of the coffee plants by initiation of RBOH. Nevertheless, the

melatonin mediated initiation of RBOH increased in the contents of the assayed metabolites whose potency of ROS scavenging is well known. This resulted into an improvement in the both the gas exchange and the photochemistry of PSII after 5 days of cold stress conditions.

### 5.5 Titanium oxide nanoparticles

The advent of nanotechnology as a novel discovery of the 21$^{st}$ century is associated with tremendous possibilities of application in various scientific disciplines including agriculture (Siddiqui *et al.,* 2015). Nanotechnology refers to the manipulation of matter on nanoscale resulting into substances generally known as nanomaterials with at least a dimension of 5 to 100 nm in diameter. In addition to their inadvertent effects in the environment, nanomaterials are recently being applied in form of nano−fertilizers, pesticides as well as abiotic stress protectants also known as nanoagrochemicals. Use of these nanomaterials is normally intended for improved fertilizer use efficiency, molecular management of diseases or pests and enhanced tolerance against abiotic stress conditions (Farooqui *et al.,* 2016; Hayles *et al.,* 2017; Jampílek and Kralova, 2018; Kah *et al.,* 2018). Nanomaterials facilitate crop growth and development by improving germination, root growth and improvement in several physiological and metabolism aspects of plants under optimum and during abiotic stress conditions (Yasmeen *et al.,* 2015; Wang *et al.,* 2016; Liu *et al.,* 2016; Marslin *et al.,* 2017).

Nanoparticles, particularly those of titanium oxide ($TiO_2$ NPs) have received tremendous attention in improving growth under optimal and more so during abiotic pressures such as cold stress due to their unique physicochemical properties (Mohammadi *et al.,* 2013; Mohammadi *et al.*, 2014; Hasanpour *et al.,* 2015; Movafeghi *et al.,* 2018). Cold like other abiotic stresses is associated with production of toxic molecules especially reactive oxygen species (ROS) mainly in the chloroplasts as a result of limitations in gaseous exchanges, diminished photosynthetic efficiency as well as repressed enzymes involved in carbon fixation (Praxedes *et al.,* 2006; Hasanpour *et al.,* 2015; Demidchik, 2015). The accumulation of these toxic molecules poses a threat to cells and in case of failure in their homeostatic control, they lead to lipid peroxidation, oxidation of proteins, enzyme inhibition as well DNA and RNA damage in the living tissues (Mittler, 2002). $TiO_2$ NPs have been reported to rectify oxidative stresses by stimulating the activities of antioxidant enzymes such as superoxide dismutase which catalyse the conversion of the superoxide ($O_2^{\bullet-}$) to $H_2O_2$ and hydroxyl radicle ($OH^{\bullet-}$) which are further detoxified by ascorbate peroxidase and catalase (CAT) resulting into more tolerant membranes (Mohammadi *et al.,* 2014; Movafeghi *et al.,* 2018). In addition, $TiO_2$ NPs tended to halt the metabolic

limitations during cold stress conditions through enhancing in the activities of ribulose 1, 5 – bisphosphate and phosphoenolpyruvate carboxylase enzymes by triggering the expression of both small and large Rubisco subunits of the messenger RNA (Gao *et al.,* 2006; Xuming *et al.,* 2008; Hasanpour *et al.,* 2015). Titanium oxide NPs have also been reported to increase the content of the photosynthetic pigments and prolonging the age of chloroplasts even under oxidative stress conditions hence resulting into better photosynthetic rates (Hong *et al.*, 2005).

Coffee is one of the world's most traded primary product whose production is increasingly getting threatened by cold stress conditions associated with the recent trend in the global climatic changes (Ramalho *et al.,* 2014). Arabica coffee which accounts for over 70% of the global coffee production is mainly cultivated in highland areas in the subtropical regions such as in Brazil where temperatures as low as 5ºC are becoming not uncommon during winter conditions (Pompelli *et al.*, 2010). Cold stress affects all aspects of coffee physiology resulting into poor growth and diminished yield. Nevertheless, coffee plants are endowed with a defence mechanism against cold induced oxidative stresses. However, it has recently been demonstrated that coffee plants rely on the accumulation of powerful nonenzymatic compounds to scavenge the ROS due to repressed antioxidant defence enzymes during cold stress (Ramalho *et al.,* 2018). These compounds contain hydroxyl components as well as glycosylic linkages that scavenge ROS (Das and Roychoudhury, 2014). Amongst the compounds, anthocyanins, caffeine, mangiferin, chlorogenic acids and trigonelline are particularly important in adapting to excess excitation energy, a major source of the ROS during oxidative stress conditions (Kumar *et al.,* 2015; Campa *et al.,* 2017). Such studies hence suggest that reinforcement of the nonenzymatic antioxidant defence system is of importance in enhancing tolerance of coffee plants against abiotic stresses such as suboptimal temperatures.

The purpose of this study was therefore to assess the potential of $TiO_2$ NPs foliar sprays in protection against cellular leakage and the integrity of the photosynthetic pigments as well as the reinforcement of the nonenzymatic antioxidant defence system with concomitant improvement the photosynthetic physiology of young coffee plants exposed to cold stress conditions. Results of this study may facilitate the adoption of nanotechnology particularly use of $TiO_2$ NPs in improving coffee productivity under abiotic stress conditions.

### 5.5.1 Materials and methods

*5.5.1.1 Plant material and growing conditions*

Plant materials and growing conditions have been described in the previous chapter (see section 2.2).

*5.5.1.2 Titanium oxide nanoparticles*

The Aeroxide $TiO_2$ P25 NPs were provided by the department of Engineering of Tottori University (Tottori, Japan). Grain and crystal properties of the fine white powder were examined using a scanning electron microscope (SEM) (S–4500, Hitachi, Japan) at 3.0 kV and X−ray diffraction (XRD) (RINT TTR – III, RIGAKU, Japan) in the 2θ range of $5^o$ – $80^o$ at a voltage of 50 kv and a current of 100 mA by mounting a small quantity of the nanoparticles powder directly on to a sample holder in each case (Fig. 30 and Fig. 31, respectively). The size of the primary crystals was calculated according to the Sherrer equation (Eqn. 6). The results indicated that the $TiO_2$ NPs consisted of a mixture of anatase and rutile forms at a proportion of 89.2% and 10.8% respectively with an average size of 20.92 nm. The presence of the rutile form rendered the nanoparticles less toxic than those used elsewhere (Mohammadi *et al.,* 2013; Mohammadi *et al.,* 2014).

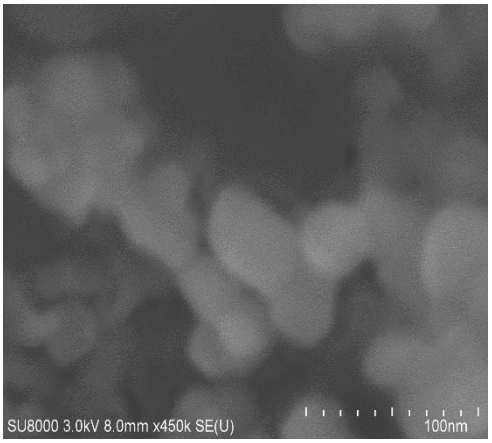


Fig. 30. SEM image of $TiO_2$ NPs. The particle size in diameter of the $TiO_2$ NPs was about 20 nm (bar, 100 nm).

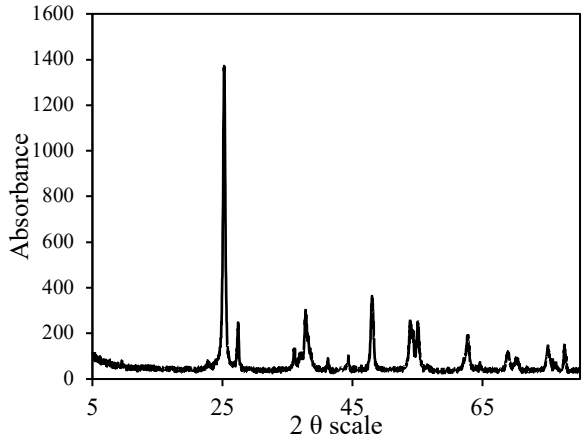


Fig. 31. XRD examination indicated presence of both anatase and rutile phases of $TiO_2$ NPs

$$Dp = \frac{K\lambda}{\beta cos\theta}$$

Equation 6. Sherrer equation for determination of $TiO_2$ nanoparticles size

Where Dp is the average crystallite size (nm), K is the Sherrer constant (0.94 for spherical crystallite with cubic symmetry), λ is the X−ray wavelength (Cu Kα average 1.54178 Å for mini XRD), β is the full width at half maximum (FWHM which was calculated according to Williamson−Hall method [$B_{obs} = B_{size} + B_{inst} + B_{strain}$] where B is broadening) and θ is the XRD peak position which is one half of 2θ (Hall *et al.,* 2000).

*5.5.1.3 Experimental design*

When they had attained at least 5 pairs of leaves, the plants were transferred and acclimated in a growth chamber at 25/20$^{o}$C, day/night respectively with an irradiance of 250 µmol $m^{-2}$ $s^{-1}$ provided by white luminescent lamps at a 12 hours photoperiod and a 70% relative humidity for 30 days. Cold treatments were initiated by suddenly decreasing the temperature of the growth chamber to 4/4$^{o}$C for a period of 10 days.

The experiment was a completely randomised design consisting of 6 treatments and 4 replications. The experiment included 5 cold treatments established by varying the concentrations of $TiO_2$ NPs and a control retained treatment in which neither cold treatment nor $TiO_2$ NPs foliar sprays were administered. The concentrations of the $TiO_2$ NPs in the treatments were 0, 5, 10, 15, and 20 ppm. In all the treatments 0.1% isopropyl alcohol (Kao Global Chemicals, Tokyo, Japan) was used as the surfactant which was added to the solution to reduce the surface tension between the leaves and the$TiO_2$ solution thereby increasing absorption of the nanoparticles (Castro *et al.,* 2013). For each treatment, all the 4 plants were sprayed with 100 mL of the solution on the leaf surfaces at 6:00 pm just before the fluorescent bulbs turned off. For 0 ppm treatment, only distilled water and the surfactant was sprayed on the plants. The concentration treatments were administered twice; 24 h before and 24 h after cold treatments so as to increase bioaccumulation of the nanoparticles in the coffee plants (Mohammadi *et al.,* 2013). Measurements and sampling were done on each of the 4 plants.

*5.5.1.4 Photosynthetic measurements*

Gaseous and chlorophyll *a* fluorescence measurements were done as previously described in the previous chapter (Subsection 4.2.3) and the subsequent calculation made as previously described in subsection 5.2.1.3.

*5.5.1.5 Electrolyte leakage Index*

Electrolyte leakage index was assessed as previously described in the previous chapter (subsection 5.2.1.4).

*5.5.1.6 Sample preparation for biochemical assays*

For biochemical analysis, the leaves were sampled and processed as previously described (subsection 3.2.3).

*5.5.1.7 $TiO_2$ Bioaccumulation in coffee leaves*

Leaf powder samples were digested on a hot block with $HNO_3$ for 1 hr at 120$^oC$ and analysed using an inductively coupled plasma atomic spectrometer (ICP−AES SPECTRO CIROS CCD, Japan) for Titanium (Ti) as described by Karanassios (Gibson *et al.,* 2006) from which $TiO_2$ NPs bioaccumulation were calculated.

*5.5.1.8 Photosynthetic pigments*

Chlorophylls and carotenoids were extracted from 50 mg of freeze−dried power using ice−cold 2.5 mM sodium phosphate 80% (v/v) buffered acetone (pH 7.8) and quantified spectrophotometrically (Hitachi ratio beam spectrophotometer, U−5100, Japan). The concentrations of chlorophylls *a*, *b* and their totals were calculated according to Porra et al. (1989) while carotenoids were quantified as a total of xanthophyll and carotene (x + c). according to Lichtenthaler and Buschmann (2001).

*5.5.1.9 Content of HPLC selected metabolite*

Caffeine and trigonelline were simultaneously extracted as previously described (Ky et al. 2001) with slight modifications. 50 mg powder sample were put in glass capped tubes to which 500 mg of MgO and 20 mL of ultrapure water were added. The tubes and their contents were autoclaved at 105$^oC$ for 1 hr, cooled to room temperature and transferred to 50 ml Falcon conical centrifuge tubes (Thermo Fisher Scientific, Massachusetts, USA). Centrifugation was done at 29300 ×g and 10$^oC$ for 15 minutes (Hitachi CR21N, Tokyo, Japan). The extracts were filtered using 0.22 µm pore for further analysis.

Quantification was done on a 10 µL extract using high−performance liquid chromatography (HPLC) system (Hitachi L−2490, Japan) equipped with a UV detector and an ODS3 column (0.6 x 150 mm and 5µm particle size) at 50$^oC$ at a flow rate of 0.4 mL $min^{-1}$ and a wavelength of 270 nm. The elution programme for analysing the two alkaloids consisted of sonicated and degassed solvents; A (Water: acetic acid, 98:2% respectively) and B (100% methanol). The two solutions A and B were mixed at a ratio of 65:35% respectively. The resultant retention time for trigonelline and caffeine was 3.87 and 8.82 minutes respectively.

Mangiferin and 5 – caffeoylquinic acid (5–CQA) which represents 80% of the total chlorogenic acids in coffee plants were extracted as described by Campa et al. (Campa et al. 2012) with a few modifications. The two phenolic compounds were extracted by sonicating 50 mg freeze

dried powder sample in 25 ml 80% methanol solution in Falcon tubes. Centrifugation was done at 29300 ×g and 10°C. Extraction was repeated 3 times per sample and all the extracts pooled together then filtered using 0.22 µm pore filters.

HPLC analysis was done using the same system and flow liquid used for caffeine and trigonelline but with different elution programs. Chlorogenic acid was eluted with *A* to *B* mixed at 65: 35% respectively while mangiferin was eluted with *A* to *B* mixed at 74:26% respectively. The retention time for 5 – caffeoylquinic acid was 7.47 minutes while mangiferin yielded two inseparable peaks due to co−existence of two isomers that is to say mangiferin and iso−mangiferin at 16.63 minutes. Quantification of the HPLC metabolites was done by peak area measurement of the samples in comparison with standard compounds (Sigma−Aldrich, St. Louis, Mo., USA). Calibration curves for each compound was established using 4 replicate points.

*5.5.1.10 Anthocyanin content*

Anthocyanin content was assayed as previously described in the previous chapter (subsection 5.2.4.7).

### 5.5.2 Statistical analysis

Experimental data was computed and analysed using one−way analysis of variance (ANOVA) in Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). The differences between the treatment means were compared for statistical significance amongst the $TiO_2$ NPs and cold treatment duration by the Newman−Keuls test ($P \leq 0.05$). Unless otherwise indicated, data are expressed as means ± S.D, n = 4, error bars indicate standard deviation of the mean.

### 5.5.3 Results

*5.5.3.1 Bioaccumulation of $TiO_2$ NPs in the leaf tissues*

The content of $TiO_2$ NPs increased with increasing concentration of the nanoparticles sprayed on the leaves of the coffee seedlings (Table 20) although this accumulation was not significant among seedlings treated with 5, 10 and 15 ppm $TiO_2$ NPs. However, 20 ppm caused a 120% increase in the accumulation of $TiO_2$ NPs in the leaf tissues compared with 15 ppm. On the other hand, $TiO_2$ NPs were absent in the seedlings which were not sprayed.

*5.5.3.2 Membrane damage alleviation*

The extent of cell membrane injury assessed as electrolyte leakage index indicated that imposition of cold temperatures significantly increased the leakage of the intracellular electrolytes compared to optimum conditions (Fig. 32). Titanium oxide NPs however showed a protective effect against membrane damage. Moreover, higher concentrations of the NPs restored the membrane integrity to values not significantly different from those at optimum temperature conditions.

*5.5.3.3 Photosynthesis and chlorophyll fluorescence improvement*

Assessment of the photosynthetic activity indicated that, regardless of the $TiO_2$ NP treatments, the net photosynthesis ($P_N$) of the coffee plants decreased significantly on imposition of cold temperatures with least values recorded on the day 4 after initiation of cold treatment (Fig. 33). Nevertheless, a recovery in the $P_N$ was observed on day 6 in all the plants with no significant differences amongst $TiO_2$ NPs treatments. However, on a given day of the measurements, the net photosynthesis increased with increasing concentration of the NPs with the increment becoming more evident on day 2 and 4 of cold treatment.

Table 20. Bioaccumulation of $TiO_2$ NPs in the leaves of *Coffea arabica* L. plants under both optimum temperature (control retained) and 10 days after cold treatment. Optimum temperature and cold treatment temperatures were 25°C and 4°C respectively. Leaf samples were collected at the 6th hour of illumination.

| **Treatment** | **$TiO_2$ NPs bioaccumulation (µg/100 g)** |
|---|---|
| Control retained | 0 c |
| 0 ppm | 0 c |
| 5 ppm | 0.27 ± 0.05 b |
| 10 ppm | 0.28 ± 0.03 b |
| 15 ppm | 0.33 ± 0.06 b |
| 20 ppm | 0.74 ± 0.25 a |

Different letters denote significant differences between the means ($P \leq 0.05$, Newman−Keuls' test). Data are expressed as means ± S.D, n = 4.

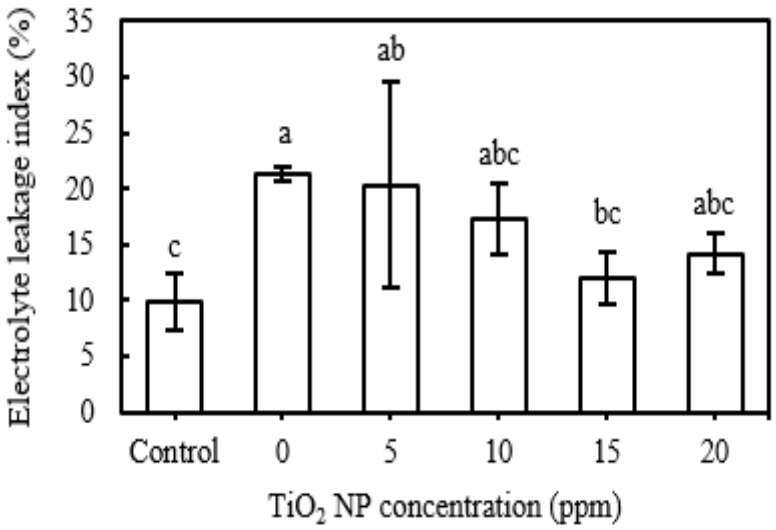


Fig. 32. Effect of $TiO_2$ NPs concentrations on electrolyte leakage index (ELI) in coffee leaves under both optimum temperature (control) and 10 days after thermal treatment. Optimum and thermal treatment temperatures were 25/20°C and 4/4°C respectively. Samples were collected at the 6$^{th}$ hour of illumination before and on the 10$^{th}$ day after thermal treatment. Absence of letters denote non−significant differences between the means ($P \leq 0.05$, Newman−Keuls' test). Error bars indicate standard deviation of the mean (n = 4).

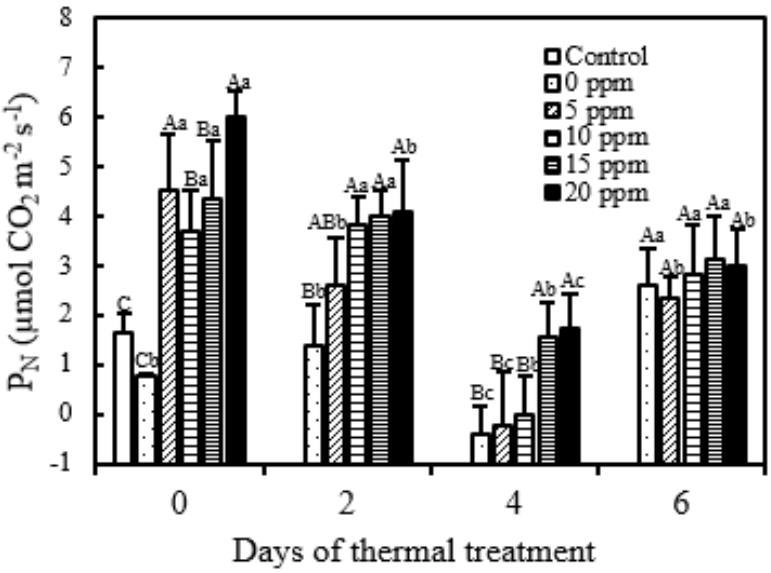


Fig. 33. Changes in $P_N$ of coffee seedlings treated with different $TiO_2$ NPs concentrations during optimum and cold stress conditions. Measurements were done at the 6th hour of illuminance on the first fully expanded leaf for each plant before thermal treatment (0 day) and after 2, 4 and 6 days of thermal treatment. Optimum and thermal treatment temperatures were 25ºC and 4ºC respectively. Different capital letters denote significant differences between the means of net photosynthesis for $TiO_2$ NPs treatments while different small letters denote significant differences in the net photosynthesis at different days of measurement ($P \leq 0.05$, Newman−Keuls' test). Error bars indicate standard deviation of the mean, n = 4.

Electron transfer rate (ETR) gradually decreased with time after instigation of the cold temperatures (Fig. 34A) despite slight increases in the values within the $TiO_2$ NP treated plants. A similar trend was observed for the operating efficiency of photosystem II ($\Phi_{PSII}$) and the proportion of absorbed PAR allocated to photochemistry ($q_P$) with the highest concentration of $TiO_2$ NPs causing the highest values in the parameters on all measurement days (Fig. 34B and Fig. 34C respectively). These changes were followed by similar surges in the fraction of open photosystem II centres ($q_L$) which was highest in the highest $TiO_2$ NPs concentration treated plants on all the measurement days despite the values decreasing with the duration of cold treatment (Fig. 34D).

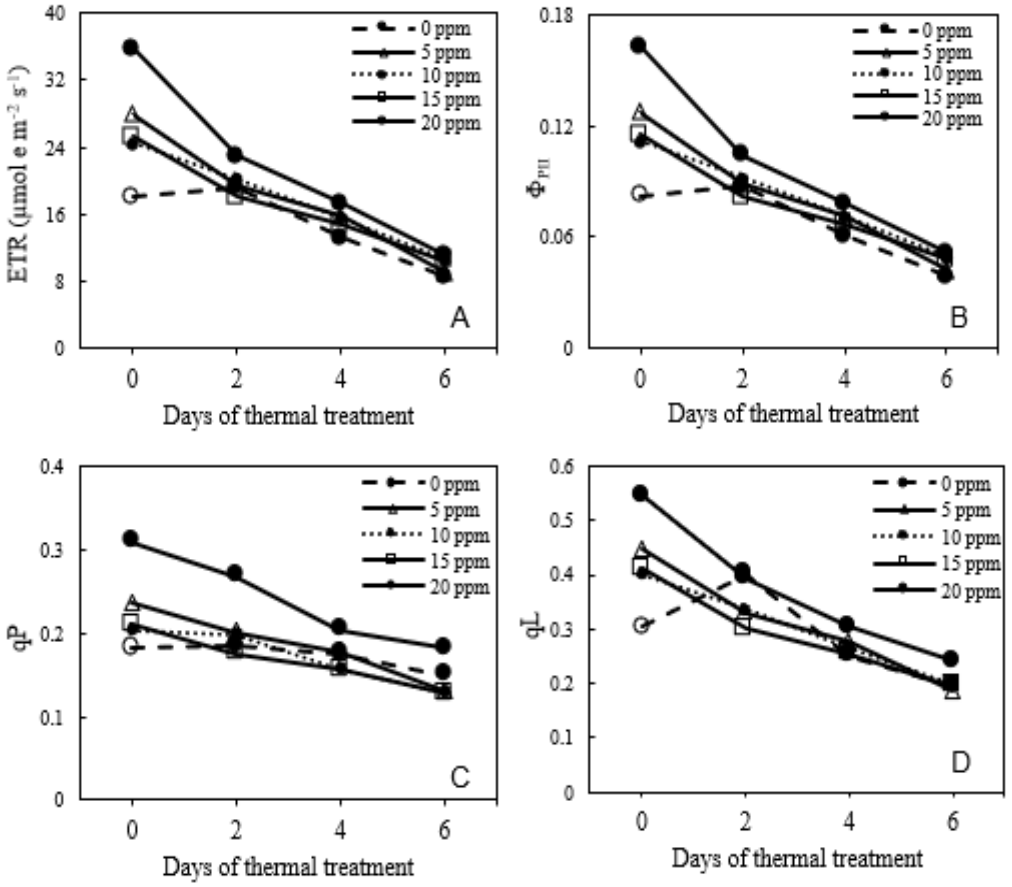


Fig. 34. Effect of $TiO_2$ and cold stress conditions on the photosystem II photochemistry; electron transfer rate (Fig. 5A), photosystem II operating efficiency (Fig. 5B), photochemical quenching (Fig. 5C) and the proportion of PSII open centres (Fig. 5D) in the leaves of coffee plants treated with different $TiO_2$ NPs concentrations. Measurements were done at the $6^{th}$ hour of illuminance on the first fully expanded leaf for each seedling before (0 day) and after 2, 4 and 6 days of thermal treatment. Optimum and thermal treatment temperatures were 25/20°C and 4/4°C, respectively.

Table 21. Effect of $TiO_2$ NPs concentrations on the light−adapted quantum efficiency of PSII in coffee leaves. Measurements were done at the 6$^{th}$ hour of illuminance on the first fully expanded leaf for each plant before cold treatment (0 day) and after 2, 4 and 6 days of cold treatment. Optimum and cold treatment temperatures were 25/20ºC and 4/4ºC, respectively.

| Parameter | $TiO_2$ NPs concentration in ppm | | | | | |
|---|---|---|---|---|---|---|
| | Control retained | 0 | 5 | 10 | 15 | 20 |
| | | | **Day 0** | | | |
| Fo' | 641.9 ± 35.3 A | 670.9 ± 48.5 Ab | 680.0 ± 41.4 Aab | 695.1 ± 20.6 Ab | 693.2 ± 3.8 Ab | 683.4 ± 51.1 Aa |
| Fm' | 1065.9 ± 110.4 B | 1237.5 ± 152.6 ABa | 1468.4 ± 293.1 Aa | 1555.8 ± 132.9 Aa | 1548.8 ± 99.4 Aa | 1465.5 ± 205.4 Aa |
| Fv' | 424.0 ± 77.4 C | 566.7 ± 109.4 BCa | 679.4 ± 132.0 ABa | 860.7 ± 126.6 Aa | 855.6 ± 96.0 Aa | 782.1 ± 154.7 Aa |
| Fs | 966.9 ± 103.9 B | 1137.8 ± 163.8 ABa | 1271.1 ± 188.7 Aa | 1381.9 ± 126.3 Aa | 1371.5 ± 108.2 Aa | 1229.2 ± 200.3 ABa |
| Fv'/Fm' | 0.40 ± 0.03 C | 0.46 ± 0.03 Ba | 0.53 ± 0.07 Aa | 0.55 ± 0.03 Aa | 0.55 ± 0.03 Aa | 0.53 ± 0.03 Aa |
| Fo'/Fs | 0.67 ± 0.04 A | 0.59 ± 0.05 Bc | 0.54 ± 0.05 Bb | 0.51 ± 0.04 Bb | 0.51 ± 0.04 Bc | 0.56 ± 0.05 Bc |
| | | | **Day 2** | | | |
| Fo' | | 625.6 ± 27.4 Aab | 647.5 ± 11.9 Ab | 649.5 ± 20.0 Ac | 653.7 ± 10.8 Ac | 642.5 ± 33.3 Aa |
| Fm' | | 1106.8 ± 74.2 Aab | 1154.8 ± 127.6 Ab | 1222.0 ± 64.0 Ab | 1239.3 ± 84.4 Ab | 1055.8 ± 91.1 Ab |
| Fv' | | 463.7 ± 54.4 Aab | 507.3 ± 117.5 Ab | 572.4 ± 60.0 Ab | 585.5 ± 76.2 Ab | 413.4 ± 70.8 Ab |
| Fs | | 1010.6 ± 79.6 ABa | 1050.8 ± 95.0 ABa | 1109.2 ± 66.1 ABb | 1137.8 ± 84.4 Ab | 945.0 ± 78.8 Bb |
| Fv'/Fm' | | 0.42 ± 0.03 Aa | 0.43 ± 0.06 Ab | 0.47 ± 0.03 Ab | 0.47 ± 0.03 Ab | 0.39 ± 0.04 Ab |
| Fo'/Fs | | 0.64 ± 0.02 ABc | 0.62 ± 0.05 ABa | 0.59 ± 0.03 Bab | 0.58 ± 0.04 Bb | 0.68 ± 0.04 Ab |
| | | | **Day 4** | | | |
| Fo' | | 682.2 ± 45.0 Aab | 674.1 ± 6.0 Aab | 681.2 ± 20.1 Abc | 686.6 ± 11.4 Ab | 669.5 ± 45.2 Aa |
| Fm' | | 1047.6 ± 75.9 Cb | 1123.8 ± 65.0 BCb | 1255.8 ± 66.8 Ab | 1200.6 ± 61.8 ABb | 1093.6 ± 75.1 BCb |
| Fv' | | 365.5 ± 52.4 Cb | 449.7 ± 61.7 BCb | 574.6 ± 52.2 Ab | 514.0 ± 56.9 ABbc | 424.1 ± 36.9 BCb |
| Fs | | 985.3 ± 89.2 Aa | 1043.1 ± 52.5 Aa | 1166.6 ± 70.3 Ab | 1120.0 ± 54.1 Ab | 1007.0 ± 66.6 Ab |
| Fv'/Fm' | | 0.35 ± 0.03 Cb | 0.40 ± 0.03 Bb | 0.46 ± 0.02 Ab | 0.43 ± 0.03 ABb | 0.39 ± 0.02 Bb |
| Fo'/Fs | | 0.69 ± 0.04 Ab | 0.65 ± 0.03 ABa | 0.58 ± 0.02 Cab | 0.61 ± 0.02 BCab | 0.66 ± 0.01 Ab |
| | | | **Day 6** | | | |
| Fo' | | 723.8 ± 51.8 Aa | 707.6 ± 10.7 Aa | 732.9 ± 24.0 Aa | 730.4 ± 33.2 Aa | 701.5 ± 33.2 Aa |
| Fm' | | 976.2 ± 56.0 Bb | 1063.1 ± 123.6 ABb | 1199.5 ± 97.2 Ab | 1169.0 ± 73.3 Ab | 981.5 ± 58.7 Bb |
| Fv' | | 252.4 ± 16.1 Bc | 355.6 ± 118.0 ABb | 466.6 ± 112.4 Ab | 438.6 ± 59.7 Ac | 280.0 ± 48.9 Bb |
| Fs | | 938.6 ± 66.5 Aa | 1018.6 ± 119.3 Aa | 1140.9 ± 92.8 Ab | 1112.9 ± 72.8 Ab | 931.1 ± 52.5 Ab |
| Fv'/Fm' | | 0.26 ± 0.02 Bc | 0.33 ± 0.07 ABb | 0.39 ± 0.07 Ac | 0.37 ± 0.03 Ac | 0.28 ± 0.04 ABc |
| Fo'/Fs | | 0.77 ± 0.01 Aa | 0.70 ± 0.07 ABa | 0.65 ± 0.07 Ba | 0.66 ± 0.03 Ba | 0.75 ± 0.04 Aa |

Different capital letters denote significant differences between the means of the parameters for $TiO_2$ NP treatments on each day while different small letters denote significant differences in the parameters on different days of measurement ($P \leq 0.05$, Newman−Keuls' test). Data are expressed as mean ± S.D (n = 4).

The light adapted state of chlorophyll *a* fluorescence indicated lower values of the fluorescence parameters resulting into low fractions of variable to maximum and initial to steady state fluorescence (Table 21). In fact, both $F_v'/F_m'$ and $F_o'/F_s$ ratios in all the plants throughout the experiment with typical values around 0.46 and 0.60, respectively (Table 21). Although the two ratios increased with increasing concentration of $TiO_2$ NPs, the variation in the two ratios during cold temperatures was antagonistic to each other with $F_v'/F_m'$ reducing for example from 0.46 to 0.26 while $F_o'/F_s$ increased from 0.59 to 0.77 on day 0 and day 6 of cold treatment respectively. On the other hand, only minor changes were noticeable in other fluorescence parameters with minimal fluorescence ($F_o'$) increasing with the duration of the cold stress while others such as maximal fluorescence ($F_m'$), variable fluorescence ($F_v'$) and the steady state level of fluorescence ($F_s$) decreased with time. Nevertheless, the parameters showed slight increases with increasing concentration of the $TiO_2$ NPs.

The predawn $F_v/F_m$ ratio was relatively high at around 0.74 and further increasing with $TiO_2$ NPs concentration (Table 22) and although the ratio dropped sharply to values as low as 0.48 for the non−treated plants, the drop was less sharp in the $TiO_2$ NPs treated plants. In addition, other predawn fluorescence parameters such as the dark−adapted minimal fluorescence ($F_o$), maximal fluorescence ($F_m$), and variable fluorescence ($F_v$) showed a similar trend by increasing gradually with $TiO_2$ NPs concentration and falling with the duration of cold temperature treatment.

#### *5.5.3.4 Mitigation of photosynthetic pigments degradation*

The concentration of chlorophyll *a* was neither significantly affected by instigation of cold temperatures nor by $TiO_2$ NPs sprays (Table 23). On the other hand, instigation of cold temperatures reduced the concentration of chlorophyll *b* only in the non $TiO_2$ NPs treated plants. Similarly, the total concentrations of both chlorophylls and carotenoids were only degraded in the non $TiO_2$ NPs treated plants when cold temperatures where instigated. On the contrary, the ratio of chlorophyll *a*/*b* was highest in plants in the non−treated plants while both control plants and $TiO_2$ NPs treated plants had similar values. The ratio of chlorophylls to carotenoids was barely affected under all circumstances.

Table 22. Effect of $TiO_2$ NPs concentrations on the dark−adapted quantum efficiency of PSII in coffee leaves. Measurements were during the dark just before illuminance (pre−dawn) on the first fully expanded leaf for each plant before cold treatment (0 day) and after 2, 4 and 6 days of cold treatment. Optimum and cold treatment temperatures were 25ºC and 4ºC, respectively.

| | $TiO_2$ NPs concentration in ppm | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | Control retained | 0 | 5 | 10 | 15 | 20 |
| | | | **Day 0** | | | |
| Fo | 802.6 ±75.6 A | 757.6 ± 92.2 Aa | 762.4 ± 26.4 Aa | 720.5 ± 66.4 Aa | 711.3 ± 99.1 Aa | 761.2 ± 54.5 Aa |
| Fm | 3074.2 ±144.6 A | 3171.0 ± 182.3 Aa | 2961.7 ± 633.4 Aa | 3179.7 ± 210.4 Aa | 3213.8 ± 321.2 Aa | 3152.7 ± 70.0 Aa |
| Fv | 271.5 ± 166.6 A | 2413.4 ± 92.3 Aa | 2275.4 ± 483.6 Aa | 2459.2 ±158.6 Aa | 2502.5 ± 226.5 Aa | 2391.4 ±73.5 Aa |
| Fv/Fm | 0.74 ± 0.03 B | 0.76 ± 002 ABa | 0.77 ± 0.01 ABa | 0.77 ± 0.01 ABa | 0.78 ± 0.01 Aa | 0.76 ± 0.02 ABa |
| | | | **Day 2** | | | |
| Fo | | 774.1 ± 65.1 Aa | 765.8 ±38.7 Aa | 739.2 ± 33.9 Aa | 752.9 ±13.0 Aa | 758.3 ± 49.8 Aa |
| Fm | | 2651.9 ± 303.1 Ab | 3026.7 ± 296.5 Aa | 3072.6 ± 135.3 Aa | 3030.9 ± 222.1 Aa | 2993.7 ± 64.9 Aa |
| Fv | | 1877.8 ± 305.2 Ab | 2261.0 ± 301.2 Aa | 2333.5 ± 137.6 Aa | 2293.1 ± 207.6 Aa | 2235.4 ± 58.3 Aa |
| Fv/Fm | | 0.71 ± 0.04 Aa | 0.74 ± 0.03 Aa | 0.76 ± 0.02 Aa | 0.75 ± 0.01 Aa | 0.75 ± 0.01 Aa |
| | | | **Day 4** | | | |
| Fo | | 763.5 ± 55.0 Aa | 791.2 ± 26.7 Aa | 776.6 ± 26.3 Aa | 777.3 ± 22.8 Aa | 787.3 ± 58.7 Aa |
| Fm | | 1484.0 ± 242.1 Bc | 1973.4 ± 465.6 Ab | 2511.6 ± 218.6 Ab | 2139.7 ± 221.5 Ab | 2032.7 ± 203.1 Ab |
| Fv | | 720.4 ± 207.3 Cc | 1182.2 ± 450.0 Bb | 1735 ± 203.0 Ab | 1362.4±200.7 ABb | 1245.4 ± 179.4 ABb |
| Fv/Fm | | 0.48 ± 0.06 Bb | 0.58 ± 0.09 Ab | 0.69 ± 0.02 Ab | 0.63 ± 0.03 Ab | 0.61 ± 0.03 Ab |

Different capital letters denote significant differences between the means of the parameters for $TiO_2$ NPs treatments on each day while different small letters denote significant differences in the parameters on different days of measurement ($P \leq 0.05$, Newman−Keuls' test). Data are expressed as mean ± S.D, n = 4.

Table 23. Effect of $TiO_2$ NPs and cold stress conditions on the concentrations photosynthetic pigments; chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophylls (Chl a + b) and total carotenoids (Car x + c) as well as the ratios of chlorophyll A to chlorophyll B (Chl a/b) and chlorophyll to carotenoids (Chl/Car) in the leaves of Coffea arabica seedlings under optimum temperature (control retained) and 10 days after cold treatment. Optimum temperature and cold treatment temperatures were 25ºC and 4ºC respectively. Samples were collected during the dark period just before illumination (predawn)

| Parameter | $TiO_2$ NPs concentration in ppm | | | | | |
|---|---|---|---|---|---|---|
| | Control retained | 0 | 5 | 10 | 15 | 20 |
| Chl a (mg g $DW^{-1}$) | 3.14 ± 0.75 a | 1.92 ± 0.35 a | 3.27 ± 0.78 a | 3.14 ± 0.68 a | 2.68 ± 0.49 a | 2.94 ± 0.58 a |
| Chl b (mg $gDW^{-1}$) | 1.36 ± 0.28 a | 0.68 ± 0.11 b | 1.39 ± 0.41 a | 1.33 ± 0.26 a | 1.14 ± 0.22 a | 1.26 ± 0.20 a |
| Chl a + b (mg g $DW^{-1}$) | 4.50 ± 1.03 a | 2.61 ± 0.46 b | 4.66 ± 1.19 a | 4.47 ± 0.93 a | 3.82 ± 0.71 ab | 4.20 ± 0.78 ab |
| Car (mg $gDW^{-1}$) | 0.59 ± 0.03 a | 0.34 ± 0.08 b | 0.58 ± 0.14 a | 0.56 ± 0.12 a | 0.50 ± 0.05 a | 0.56 ± 0.10 a |
| Chl a/b (mg $mg^{-1}$) | 2.29 ± 0.11 b | 2.81 ± 0.13 a | 2.38 ± 0.16 b | 2.36 ± 0.09 b | 2.36 ± 0.08 b | 2.32 ± 0.14 b |
| Chl/car (mg $mg^{-1}$) | 7.63 ± 1.72 a | 7.76 ± 0.61 a | 8.04 ± 0.44 a | 7.97 ± 0.57 a | 7.56 ± 0.58 a | 7.49 ± 0.28 a |

Different letters denote significant between the means for each parameter ($P \leq 0.05$, Newman−Keuls' test). Data are expressed as means ± S.D (n= 4).

*5.5.3.5 Nonenzymatic antioxidants reinforcement*

Caffeine was the highest in the plants at 3.0% on dry matter basis (dmb) and although the content increased after instigation of cold temperatures by around 17%, a gradual decrease was observed with increasing concentration of $TiO_2$ NPs to values below those at optimum temperature (Table 24). Trigonelline on the hand peaked at 15 ppm with the content decreasing from 2.3% to 1.5% dmb with further increase in $TiO_2$ NPs concentration (Table 24). The content of 5 – caffeoylquinic acid increased with increase in the concentration of the nanoparticles and was significantly highest in plants treated with 20 ppm $TiO_2$ NPs. Mangiferin – the other phenolic compound was the least in coffee leaves at about 0.32% dmb and sharply dropping by 34% on initiation of the cold temperatures for the 0 ppm (Table 24). However, application of $TiO_2$ NPs restored mangiferin content with values remaining fairly constant regardless of the increasing concentration of the nanoparticles.

High accumulation of anthocyanins was observed when the coffee seedlings were subjected to cold stress conditions especially in the non $TiO_2$ NPs treated plants (Table 24). Despite being non−significantly different amongst the treatment when expressed as YGM – 5b equivalent, cold treatment elicited significantly the highest contents of anthocyanins when expressed as Cy3G equivalent in absence of $TiO_2$ NPs. On the other hand, $TiO_2$ NPs sprays maintained the concentration of the anthocyanin at levels similar to those in plants under control conditions.

Table 24. Effect of $TiO_2$ NPs and cold stress conditions on the concentration of selected metabolite compounds; caffeine, trigonelline, 5 – caffeoylquinic acid, mangiferin and total anthocyanins expressed as both YGM – 5b and Cy3G equivalents as well as the ratio of 5 – caffeoylquinic acid to caffeine (5 – CQA/Caffeine) in the leaves of *C. arabica* seedlings under both optimum temperature (control retained) and 10 days after cold treatment. Optimum temperature and cold treatment temperatures were 25°C and 4°C respectively. Samples were collected at the 6th hour of illumination

| Parameter | $TiO_2$ NPs concentrations in ppm | | | | | |
|---|---|---|---|---|---|---|
| | Control | 0 | 5 | 10 | 15 | 20 |
| Caffeine (mg g $DW^{-1}$) | 3.38 ± 0.57 ab | 3.95 ± 0.47 a | 3.49 ± 0.33 ab | 3.09 ± 0.28 b | 2.83 ± 0.12 b | 3.03 ± 0.14 b |
| Trigonelline (mg $gDW^{-1}$) | 1.53 ± 0.34 b | 1.39 ± 0.18 b | 1.41 ± 0.24 b | 1.61 ± 0.28 b | 2.29 ± 0.54 a | 1.45 ± 0.13 b |
| 5 – CQA (mg $gDW^{-1}$) | 1.66 ± 0.14 b | 1.59 ± 0.27 b | 1.59 ± 0.27 b | 1.66 ± 0.08 b | 1.71 ± 0.16 b | 2.29 ± 0.03 a |
| Mangiferin (mg $gDW^{-1}$) | 0.32 ± 0.06 ab | 0.21 ± 0.10 b | 0.42 ± 0.08 a | 0.35 ± 0.08 ab | 0.31 ± 0.04 ab | 0.39 ± 0.08 a |
| 5 − CQA/Caffeine | 0.53 ± 0.12 ab | 0.41 ± 0.09 b | 0.46 ± 0.11 ab | 0.54 ± 0.08 ab | 0.60 ± 0.06 ab | 0.68 ± 0.16 a |
| YGM – 5b eq. (mg $gDW^{-1}$) | 1.10 ± 0.19 a | 1.98 ± 0.50 a | 1.14 ± 0.37 a | 1.42 ± 0.86 a | 1.09 ± 0.41 a | 1.09 ± 0.09 a |
| Cy3G eq. (mg $gDW^{-1}$) | 0.39 ± 0.24 b | 0.92 ± 0.23 a | 0.54 ± 0.17 ab | 0.67 ± 0.39 ab | 0.49 ± 0.19 ab | 0.51 ± 0.04 ab |

Different letters denote significant differences between the means for each parameter ($P \leq 0.05$, Newman−Keuls' test). Data are expressed as means ± S.D (n = 4).

### 5.5.4 Discussion

The results of this current study have demonstrated the potential of foliar application of $TiO_2$ NPs in ameliorating the negative effects of cold temperatures on the photosynthetic physiology of coffee plants. Exogenous application of $TiO_2$ NPs on plant tissues causes their penetration into cellular components particularly into cell vacuoles and chloroplasts (Mohammadi *et al.,* 2013). This intake into plant tissues and subsequent localization is dependent on the size of the nanoparticles. The nanoparticles used in the current study were spherical−shaped and consisted of both anatase and rutile phases with an average diameter of about 21 nm (Fig. 30 and 31). In solution, Tada−Oikawa *et al.* (2016) reported that P25 has a hydrodynamic size of about 185 nm and a zeta potential of 12.68 mV. Application of $TiO_2$ NPs solutions on to plant tissues therefore likely modified the plant cell wall facilitating their incorporation into the leaf tissues. Moreover, despite acting as a barrier against entry of foreign materials, cell walls normally have pores that can allow penetration of small sized materials (Singh *et al.,* 2012; Mohammadi *et al.,* 2013). $TiO_2$ NPs have also been reported to create pores in the cell wall that act as new entry points for their intake (Jampílek and Kráľová, 2018).

Titanium oxide NPs are recognized amongst nanomaterials for special ability to speed−up photoreactions, a phenomenon referred to as photocatalysis (Jain and Vaya, 2017). In cold stressed plants, suppression of the photosynthetic efficiency associated with limited gas exchanges and/or repressed carboxylic enzymes implies that any incident light on leaf tissues is potentially in excess and therefore is likely to result into excess excitation energy which lead to formation of toxic molecules such as ROS as well as charged states of chlorophyll molecules (Asada, 2006). Accumulation of these molecules results into oxidation of cellular components which is characteristically associated with breakdown of the cell membranes causing leakage of the intracellular components. By speeding up photochemical reactions in the chloroplasts, $TiO_2$ NPs reduced the formation of these toxic substances hence protecting the cells against oxidation resulting into low electrolyte leakages under cold conditions. $TiO_2$ NPs improved the photochemical efficiency as indicated by the fluorescence parameters in the treated plants. Moreover, continued exposure to cold conditions indicated that $TiO_2$ NPs treated plants were more tolerant to the negative effects of cold stress than their untreated counterparts.

The current study also revealed that $TiO_2$ NPs treated plants tended to maintain higher concentrations of chlorophylls. This result agrees with Singh *et al.* (2012) and Mohammadi *et al.* (2014) who reported significantly higher concentrations of the photosynthetic pigments in

$TiO_2$ NPs treated plants under optimum and cold stress conditions. Such results could be attributed to the suppression of oxidative molecules as well as reinforcement of antioxidant defence mechanism (Mohammadi *et al.,* 2014). Moreover, $TiO_2$ NPs enhanced the metabolism of nitrogen which is an important component of amino acids and chlorophylls (Yang *et al.,* 2006). In additions, $TiO_2$ NPs treated plants tended to have higher concentrations of carotenoids which serve as a protective molecule against excess excitation energy which would otherwise degrade the photosynthetic pigments (Hong *et al.*, 2005).

The enhancement in the content of the photosynthetic pigments and the photocatalytic ability of the $TiO_2$ NPs led to improvement in the net photosynthesis on all the measurement days before and after instigation of cold temperatures. $TiO_2$ NPs have been reported to improve absorbance, acceleration and transformation of light energy through modifying the micro−environment of PSII leading to facilitation of energy transfer among amino acids within PSII protein complex and acceleration of energy transport from tyrosine to chlorophyll *a* (Mingyu *et al.,* 2007). Similar to what was observed in the current study, such effects lead to enhancement in the fluorescence quantum yield as well improved net $CO_2$ assimilation. $TiO_2$ NPs protect chloroplasts against aging hence prolonging their photosynthetic activity(Hong *et al.*, 2005) all which lead to sustained carbon assimilation during cold stress. In addition to getting involved in the electron transfer reactions in PSI and PSII, $TiO_2$ NPs cancels the effects of linolenic acids whose accumulation under abiotic stress conditions is associated with inhibition of photoreduction of the whole chain electron transport of the two photosystems and also the evolution of oxygen (Mingyu *et al.,* 2000).

Similar studies reported that $TiO_2$ NPs significantly improved the activity of ribulose 1, 5−bisphophate and phosphoenolpyruvate carboxylase through triggering the expression of both the small and large Rubisco subunits of the messenger RNA in the nano−anatase treated plants (Xuming *et al.,* 2008; Hasanpour *et al.,* 2015). These enzymes catalyse the oxidation and oxygenation of ribulose 1, 5−bisphosphate which is the first committed step in the competitive metabolic pathways of photorespiration and photosynthetic $CO_2$ fixation in higher plants (Mauser *et al.,* 2001).

It is further suggested that, $TiO_2$ NPs improved tolerance of the treated plants to cold stress by stimulating the nonenzymatic antioxidant compounds evaluated in this study. Recently, it has been observed that coffee plants rely on nonenzymatic antioxidant system which is comprised of powerful antioxidant molecules such as anthocyanins, 5–caffeoylquinic acid, mangiferin,

trigonelline and caffeine for protection due to repression of the antioxidant enzymes during cold stress (Ramalho *et al.,* 2018). These compounds contain a number of hydroxyl components as well as glycosylic linkages which neutralize the reactive oxygen species to water (Das and Roychoudhury, 2014). Instigation of cold stress stimulated the content of anthocyanins in coffee leaves. However, $TiO_2$ NPs downregulated the contents of these flavonoid compounds possibly due to enhancement of other protection mechanisms during cold stress. On the other hand, $TiO_2$ NPs tended to enhance the metabolism of 5–caffeoylquinic acid while supressing that of caffeine resulting into an increase in the ratio of 5–CQA/caffeine with increasing $TiO_2$ NPs concentration. 5 – caffeoylquinic acid is the main chlorogenic acid in coffee plants representing over 80% of the phenolic compound with a very high potency in scavenging the ROS (Campa *et al.,* 2017). Although the mechanism under which these compounds are stimulated or supressed is yet to be understood, the localisation of the titanium nanoparticles in the cell vacuoles suggests their possible selective effect on the metabolites' biosynthetic genes and/or enzymes. Ghosh *et al.* (2019) have recently reported that $TiO_2$ NPs degrade caffeine in solution in presence of light. Therefore, its plausible that $TiO_2$ NPs have a similar effect on caffeine in the leaves of coffee plants. On the other hand, $TiO_2$ NPs stimulated the production of total polyphenols in micro−algal species which is in agreement with our findings of increased 5–caffeoylquinic acid and mangiferin with increasing $TiO_2$ NPs concentration in the leaf tissues (Comotto *et al.,* 2014). The current results therefore suggest, that in addition to generation of less ROS treated due to improvements in the photosynthetic efficiency, the metabolic adjustments induced by $TiO_2$ NPs also contribute to increased tolerance of coffee plants to cold stress conditions.

In the current study, instigation of cold stress temperatures caused photosynthetic limitations with its impacts increasing with increasing time of exposure resulting into production ROS that caused damage to the cell membrane. Coffee plants treated with $TiO_2$ NPs maintained higher photosynthetic rates and associated chlorophyll *a* fluorescence parameters in a concentration dependent manner. It is plausible therefore that the improved photosynthetic efficiency induced by the $TiO_2$ NPs resulted into less generation of the ROS hence causing less membrane damage. In addition, $TIO_2$ NPs protected the stability of the chlorophylls and carotenoids thereby prolonging the lifespan of the chloroplasts resulting into improved photosynthetic rates. Moreover, $TiO_2$ NPs elicited the production of nonenzymatic antioxidants such as mangiferin and 5–caffeoylquinic acid which scavenge the ROS that are produced during cold stress conditions. The results of this study suggest that $TiO_2$ NPs application exhibit potential in

ameliorating cold−induced stress. Nevertheless, detailed studies under field conditions are still necessary to establish their efficacy over a long time without resulting into phytotoxicity and other unintended consequences.

### 5.6 Conclusion

Results of the current study indicated that exposure to cold stress conditions negatively impacted the physiology and metabolic processes of coffee plants. The ROS generated as a result of oxidative stress associated with cold stress conditions caused disintegration of the cell membranes and photosynthetic pigments. This was accompanied by diminished photosynthetic efficiency of the PSII with concomitant reductions in the gas exchange and photosynthetic activity in the leaves of coffee plants. On the other hand, the exogenous foliar application of kinetin, salicylic acid, melatonin and $TiO_2$ $NP_S$ improved the antioxidative capacity of the coffee plants by upregulating the metabolism of the nonenzymatic antioxidant compounds. This was associated with increased reactive species scavenging capacity in the treated plants. Exogenous application of the substances slightly increased the photochemical and mesophyll efficiency for $CO_2$ fixation in addition to maintaining somewhat higher gas exchanges parameters under cold stress conditions compared to the nontreated plants. Therefore, on the basis of the results presented in this chapter current study, it is suggested that exogenous application of elicitor compounds has a potential to modulate the growth of the coffee plants under cold stress conditions, and therefore more studies should be conducted to explore its efficacy under field conditions. Exploring cold mitigation or tolerance enhancement is not only useful in the face of unpredictable climatic changes, but also is likely to expand coffee cultivation to new production areas, whose environmental conditions are currently unsuitable for coffee production.

## Chapter six

## Combined nitrogen and melatonin improve the photosynthetic physiology of coffee plants via modulation of the nonenzymatic antioxidant system during cold stress conditions

### 6.1 Introduction

By combining the results of chapter 4 where foliar nitrogen recovered the photosynthetic apparatus and chapter 5 in which the foliar application of the elicitor compounds (such as melatonin) reinforced the nonenzymatic antioxidant system, chapter 6 evaluated the effects of the two substances on improvement of cold tolerance of coffee plants.

Coffee plants evolved as an understorey species under moderate temperature and light conditions (Charrier and Berthaud, 1985). Traditionally, coffee cultivation has been done under shade although in some regions, unshaded coffee plantations exist (DaMatta, 2004). Coffee cultivation has also expanded to a wide range of regions which experience more several environmental conditions and fluctuations (DaMatta and Ramalho, 2006). These conditions such as low non−freezing temperatures (4–18°C) and high light intensity trigger overproduction of ROS which induce oxidative damage in the leaves of coffee plants (Mittler, 2002). Abiotic stress conditions induce several limitations to photosynthetic productivity of coffee plants (DaMatta *et al.,* 2006). Such effects are associated with low net $CO_2$ assimilation induced by both diffusive and biochemical constraints emanating from cold stress conditions (Pompelli *et al,* 2010).

Low temperatures thermodynamically affect the activity of photosynthetic enzymes such as restraining the recovery of the activation state of RuBisCO which is consistent with a regulatory feedback reflecting limitations in RuBP regeneration (Yamori and Caemmerer, 2009). These reductions are consistent with parallel decreases in $CO_2$ assimilation (Mishra *et al.,* 2019). Moreover, cold stress conditions also lower the electron transport rate which affects the products of the photochemical reactions (NADPH and ATP) regeneration which further curtail RuBP regeneration (Sage and Kubien, 2007). In addition, under temperatures below 15°C, $P_N$ is also limited by $P_i$ regeneration capacity (Cen and Sage, 2005). These decreases in the biochemical consumption of the photochemical reaction products creates a situation as if there is high radiance even under lower PPFD which cause elevated excitation state (Mishra *et al.,* 2019). This creates several stress responses such as increase in the cyclic electron transport

around the photosystem, the water – water cycle and production of ROS (Asada, 1999; Endo *et al.,* 1999; Mishra *et al.,* 2019).

The heightened production of ROS during photooxidative is associated with charged states of chlorophylls, namely $^{3}$Chl* and $^{1}$Chl in the chloroplasts resulting into chlorophyll degradation (Asada, 2006). The generation of ROS is not only restricted to chloroplasts, but rather occurs in other organelles such as mitochondria and the peroxisomes due to presence of several oxidative and electron transport reactions in these organelles. Although ROS are produced under normal cellular metabolism as signalling molecules, their exacerbated production under stress conditions causes the oxidation and disintegration of lipids, proteins, pigments and DNA, as well as the inactivation of the enzymes of the photosystems and might culminate in cell death. However, plants are endowed with an elaborated antioxidant system which is comprised of both enzymatic and nonenzymatic components (Asada, 2006). It has recently become clear that these components are modulated by ROS whose production is exacerbated by oxidative stress conditions and or by the RBOHs (Kreslavski *et al.,* 2012; Baxter *et al.,* 2014; Mittler, 2017; Sies, 2017). Due to underdeveloped enzymatic antioxidant system, coffee plants rely on accumulation of powerful nonenzymatic antioxidant compounds such as anthocyanins, flavonoids, flavonols, chlorogenic acids and xanthonoids for ROS scavenging and maintenance of homeostatic conditions (Campa *et al.,* 2017; Ramalho *et al.,* 2018). In addition, leaves contain carotenoids (molecules), which dissipate excess excitation energy away from the PSII through de−epoxidation of the xanthophyll cycle in which violaxanthin is converted to xanthophyll, hence avoiding the generation of ROS (Niyogi *et al.,* 1997).

Nitrogen plays an important role in enhancing tolerance of coffee plants to oxidative stress conditions (Pompelli *et al.,* 2010). Therefore, the significance of nitrogen metabolism and its role in *C. arabica* L. physiology has been a subject of tremendous research (Carelli *et al.,* 2006). Several reports indicating the plasticity of coffee plants to different light regimes have indicated that this high adaptability of coffee plants is dependent on the change in the content and/or composition of the photosynthetic apparatus a process directly related to nitrogen partitioning (Long *et al.,* 1994; Ramalho *et al.,* 1997; 1999; 2000). A close association between light−saturated photosynthetic rate and nitrogen contents of leaves due to allocation of high amounts nitrogen to photosynthetic elements has been reported (Evans, 1989). On the other hand, nitrogen deficiency would cause a reduction in the cellular concentration of pigments, photosynthetic units and enzymes hence causing a decline in carbon assimilation hence increasing susceptibility to photoinhibition (Sukenik *et al.,* 1987). Moreover, nitrogen

deficiency amplifies oxidative stresses resulting from even mild deflections from the normal environmental conditions (Verhoeven *et al.,* 1997; Carelli *et al.,* 2006).

In addition, melatonin (N–acetyl–5–methoxy tryptamine) a pleiotropic ubiquitous hormone with multifunctional effects in both animals and plants has also been applied as a natural bio−stimulating treatment for field crops under abiotic stress conditions (Arnao and Hernández−Ruiz, 2014). Being an amphipathic molecule, exogenously applied melatonin readily diffuses across the cell membrane into the cytoplasm and into the subcellular compartments where it triggers several processes that confer tolerance to abiotic stress (Zhang *et al.,* 2015). This melatonin conferred tolerance has also been shown to be as a result of enhanced nitrogen metabolism during abiotic stress conditions (Gao *et al.,* 2016; Zhang *et al.,* 2017).

The current study, therefore investigated the combined effects of exogenous nitrogen and melatonin on the physiology of coffee plants. The study examined the underlying mechanisms of cold acclimation of the photosynthetic physiology of coffee plants via modulation of the nonenzymatic antioxidant compounds as affected by exogenous application of foliar nitrogen and melatonin.

## 6.2 Materials and Methods

### 6.2.1 Plant Material and Growing Conditions

Plant materials and growing conditions have been described in the previous chapter (see section 2.2).

### 6.2.2 Experimental design

After acclimation, 16 plants were selected and divided in two groups of eight plants each. One group was sprayed with urea solution containing 10 mM nitrogen adopted from chapter 4. whereas another group was sprayed with distilled water. All the foliar sprays together with 0.1% isopropyl alcohol surfactant (Kao Global Chemicals, Tokyo, Japan) were made once a weak just before the dark phase of the growth chamber for 90 days. The plants were again divided into two groups of eight plants, and one group sprayed with 100 μM melatonin solution whereas another group was sprayed with distilled water. The sprays with a 0.1% surfactant were made three times every after 5 days (chapter 5). The resultant exogenous foliar treatments therefore were; (1) control, for which the plants were sprayed with distilled water; (2) nitrogen, plants receiving nitrogen; (3) melatonin, plants receiving melatonin, (4) Nit + Mel, plants

receiving both nitrogen and melatonin. Twenty−four hours after the last melatonin application, photosynthetic measurements were taken after which samples were collected for biochemical analyses. The plants were thereafter challenged with cold stress conditions (12/12°C, day and night, respectively) while maintaining other ambient conditions constant for 15 days after which photosynthetic measurements and sampling for biochemical analysis were made.

### 6.2.3 Photosynthetic Measurements

Measurements were performed with a portable Li–6800 photosynthesis system (LiCOR Inc. NE, USA) fitted with infra−red gas analyser (IRGAs) chamber with a 2 $cm^2$ cuvette. The two response curves $A/C_i$ and light response were measured under ambient environmental conditions consecutively on the same portion of the leaf. The Li–6800 $CO_2$ response program was used for the A/Ci curve by setting the $CO_2$ concentration to 0, 50, 100, 200, 400, 500, 800, 1000, 1500, 2000, 400 ppm. After determining the $A/C_i$, the light response curves were evaluated using the Li–COR light response program with the photosynthetic photon flux density PPFD set at 0, 20, 50, 100, 200, 500, 1000, 1500, 2000, 2500, 3000, 0 μmol $m^{-2}$ $s^{-1}$. The minimum and the maximum wait times were set to 60 and 300 s, respectively during each program based on the stability of $CO_2$ net assimilation rate and the difference between sample and $CO_2$ concentrations (Coursolle *et al.,* 2019). The IRGAs were matched before the measurements at each concentration. From the $A/C_i$ and the light response data, the following parameters were estimated using the EFT model developed by Bellasio *et al.* (2016); light respiration, initial PSII (photosystem II) photochemical yield, initial quantum yield for $CO_2$ fixation, fraction of incident light harvested by PSII, initial quantum yield for electron transport, electron transport rate, rate of photorespiration, stomatal limitation, Rubisco (ribulose 1·5−bisphosphate carboxylase/oxygenase) rate of carboxylation and oxygenation, Rubisco specificity factor, mesophyll conductance to $CO_2$ diffusion, light and $CO_2$ compensation point, Rubisco apparent Michaelis–Menten constant, and Rubisco $CO_2$−saturated carboxylation rate.

### 6.2.4 Electrolyte Leakage Index determination

Electrolyte leakage index was assessed as previously described in the previous chapter (subsection 5.2.1.4).

### 6.2.5 Sample preparation for biochemical assays

For biochemical analysis, the leaves were sampled and processed as previously described (subsection 3.2.3).

### 6.2.6 Photosynthetic pigments

Chlorophylls and carotenoids pigments were assayed and determined as previously described in the previous chapter (subsection 3.2.4).

### 6.2.7 Determination of nonenzymatic antioxidant compounds

*6.2.7.1 Total anthocyanin content (TAC)*

Anthocyanin content was assayed as previously described in the previous chapter (subsection 5.2.1.8).

*6.2.7.2 Total flavonoids and total flavonols*

Total flavonoids and flavonols were extracted and assayed according to Miliauskas *et al.* (2004) with a few modifications. Extraction was done from 50 mg of the freeze−dried sample using pure methanol solution (99.5%). The mixture was sonicated in 50 mL Falcon tubes for 30 minutes in an ice−cold water bath and thereafter centrifuged at 29, 300 ×g at 4°C. Extraction was repeated three times and all the supernatants pooled together in 25 mL mess−up flasks.

The content of total flavonoids was determined using rutin compound as a reference. For the assays, 1 mL of the plant extract was mixed with 1 mL of aluminium chloride solution (20% w/v) and then diluted with pure ethanol (99.5%) to the mark in 25 mL flasks. Blank sample were prepared from the plant extract with 1 drop of acetic acid and diluted with ethanol to 25 mL. Rutin standards of serial concentrations were similarly prepared using 50 mg of rutin hydrate ($C_{22}H_{30}O_{16}$. $xH_2O$) stock solution in pure methanol. Spectrophotometric measurements were made at 415 nm after incubation at room temperatures for 40 minutes. The concentration of flavonoids in rutin equivalent (RE) was then calculated using the formula using the calibration curve developed from the rutin standards (Eqn. 7).

$$(X = (AxMo)) / ((AoxM))$$

Equation 7. Determination of total flavonoids

Where X is flavonoid content in mg $gDW^{-1}$ in RE; A is absorption of standard rutin solution; $A_o$ is the absorption of standard rutin solution; M is the weight of plant extract (mg); Mo is the weight rutin in the solution (mg).

Total flavonols content was determined using rutin reference standard. 1 mL of methanol sample extract and/or serial concentrations of rutin standards were reacted with 1 mL of 20%

(w/v) $AlCl_3$ ethanol solution and 5 mL of 50% (w/v) sodium acetate ethanol solution. The mixture was then incubated at room temperature for 2.5 hrs and thereafter absorbance read at 440 nm. The content of flavonols in RE was then calculated using the following formula (Eqn. 8).

$$(X = C.V) \ / \ m$$

Equation 8. Determination of flavonols

Where X is flavonols content in mg $gDW^{-1}$ in RE; C is concentration of rutin solution established from the calibration curve in mg $mL^{-1}$; V and m is volume and mass of plant extracts in ml and mg, respectively.

*6.2.7.3 HPLC analysis of selected metabolites*

Selected alkaloids and phenolic compounds were assayed and determined as previously described in the previous chapter (subsection 2.2.4.4).

*6.2.7.4 Total phenolic content (TPC)*

TPC was assayed as previously described in the previous chapter (subsection 2.2.4.6).

### 6.2.8 Total radical scavenging capacity

Total radical scavenging capacity of the coffee leaves was assayed using 2,2−diphenyl−1−Picryl−Hydrazyl (DPPH) radical assay as previously described in the previous chapter (subsection 2.2.4.7).

### 6.2.9 Statistical analysis

The experimental data was arranged in a completely randomized design in a 2x2x2 factorial (2 nitrogen, 2 melatonin and 2 temperature treatments). Each treatment consisted of 4 experimental plots with each plot containing a single coffee plant in one container. The parameter differences were compared for statistical significance amongst the treatments using the Newman–Keuls test at $p \leq 0.05$. Assumptions of normality were tested using the Kolmogorov–Smirnov test, and the data transformed accordingly to attain a normal distribution whenever necessary. All the statistical analyses were done using the Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). Data are expressed as means ± SE ($n \geq 3$).

## 6.3 Results

### 6.3.1 Net assimilation

The photosynthetic rates ($P_N$) were higher under optimum temperature conditions as shown by both the A/$C_i$ and the light response curves (Fig. 35). Under optimum temperature conditions, plants maintained showed higher $P_N$ and low intercellular $CO_2$ ($C_i$) concentration compared to those under cold stress conditions while lower $P_N$ and higher $C_i$ (Fig. 35A and B, respectively). Under the former temperature conditions, plants under the nitrogen treatment maintained the highest photosynthetic rates and showed the highest saturation point followed by those under Nit + Mel treatment, reaching saturation at over 6 µmol $CO_2$ $m^{-2}$ $s^{-1}$. Plants under control and melatonin showed low photosynthetic rates with maximum $P_N$ at around 2 µmol $CO_2$ $m^{-2}$ $s^{-1}$. Contrastingly, under cold stress conditions, highest $P_N$ rates were observed in the Nit + Mel plants followed by plants under melatonin and nitrogen and being lowest in the control plants. Nevertheless, all the treatments showed a saturation in $P_N$ rates of around 1 µmol $CO_2$ $m^{-2}$ $s^{-1}$ in response to external $CO_2$ supply.

The light−response curves for all the treatments under both optimum and cold stress conditions indicated hyperbolic response of $P_N$ with increasing PPFD, and generally indicating saturation before 500 µmol $m^{-2}$ $s^{-1}$ (Fig. 35C and D). For all the treatments, $P_N$ in response to increasing PPFD was higher in coffee plants under optimum compared to cold stress conditions. Under optimum conditions (Fig 35C) $P_N$ was highest in the plants under Nitrogen treatment peaking at 2.0 µmol $m^{-2}$ $s^{-1}$ followed by plants under Nit + Mel which peaked at 1.0 µmol $m^{-2}$ $s^{-1}$. $P_N$ was low in plants under Control and least in the Melatonin treatment with the two showing peak at below 0.8 µmol $m^{-2}$ $s^{-1}$. On the contrary, under cold stress conditions, plants under the Nit + Mel treatment maintained the highest $P_N$ followed by those in the Melatonin and Control treatments while those in Nitrogen treatment was least (Fig. 35D). Nevertheless, the $P_N$ in the treatments was all well below 0.4 µmol $m^{-2}$ $s^{-1}$.

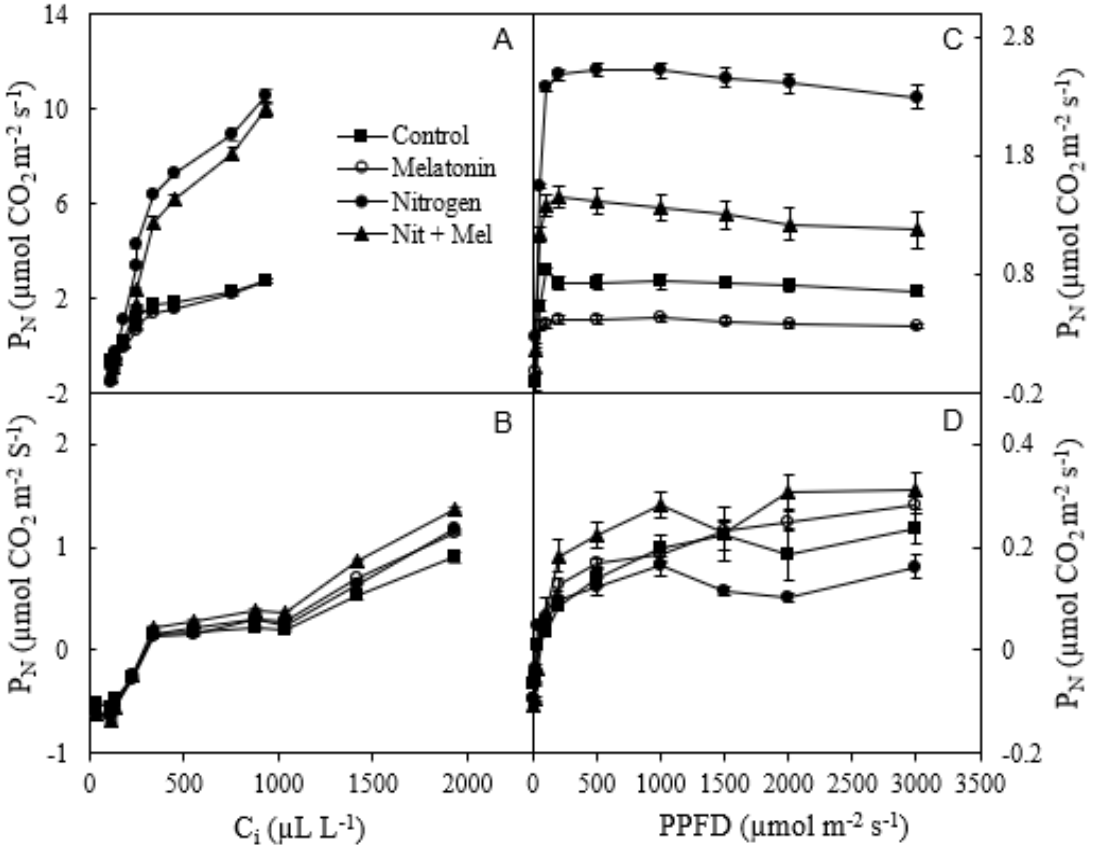


Fig. 35. Effect of exogenous nitrogen and melatonin on A/Ci and light-response curves during optimum and cold stress conditions; $CO_2$ and light response net photosynthetic curves (A,B and C,D, respectively) for the treatments: (i) Control – filled squares, (ii) Melatonin – open circles, (iii) Nitrogen – filled circles, (iv) Nit + Mel – filled rectangles; under optimum (A and B) and 15 days of cold stress conditions (C and D). Optimum conditions were 25/20$^{o}$C whereas cold stress conditions were 12/12$^{o}$C, day and night respectively. Data points represent measurements from 3 individual plants. Error bars represent standard error of the mean, (n = 3).

### 6.3.2 Gross assimilation

The gross assimilation rate (GA) estimated as the sum of photosynthesis and photorespiration in shown in Fig. 36. Generally, GA increased sharply with minor increments in the PPFD under the two temperature conditions, showing saturation at around 500 µmol $m^{-2}$ $s^{-1}$. In the respective treatments however, GA showed higher saturation under optimum temperature conditions (Fig. 36A) than under cold stress conditions (Fig. 36B). Under optimum temperature conditions, nitrogen treated plants showed the highest GA saturation point at 2.5 µmol $CO_2$ $m^{-2}$ $s^{-1}$. This was followed by plants under Nit + Mel with 1.0 µmol $CO_2$ $m^{-2}$ $s^{-1}$ whereas plants under the control and melatonin treatments showed the least GA at below 0.5 µmol $CO_2$ $m^{-2}$ $s^{-1}$ despite the former showing slightly higher values. On the other hand, despite GA showing saturation at lower levels, plants under melatonin treatments showed the highest saturation at over 0.4 µmol $CO_2$ $m^{-2}$ $s^{-1}$ followed by plants under Nit + Mel and control treatments at around 0.3 µmol $CO_2$ $m^{-2}$ $s^{-1}$ whereas GA saturation was least for nitrogen treated plants at around 0.2 µmol $CO_2$ $m^{-2}$ $s^{-1}$.

### 6.3.3 Photosystem II photochemical yield

Variation in the quantum yield of PSII ($Y_{(II)}$) in response to PPFD and intracellular $CO_2$ concentration amongst the treatments in shown in Fig. 37. Generally, $Y_{(II)}$ decreased with increasing PPFD in all the treatments (Fig. 37A and C). This decline was more apparent in the simulated $Y_{(II)}$ variation in under cold stress conditions compared to variation in the actual variation in $Y_{(II)}$ under optimum conditions. On the other hand, $Y_{(II)}$, increased with increasing $C_i$ (37B and D). Nevertheless, variation in $Y_{(II)}$ in response to both PPFD and $C_i$ showed no clear disparity amongst the treatments both under optimum and cold stress conditions.

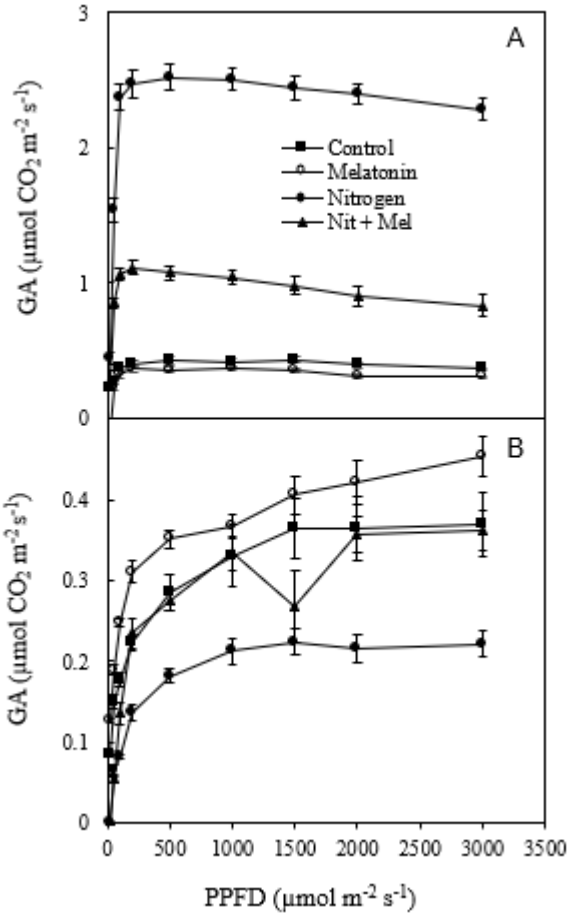


Fig. 36. Effect of exogenous nitrogen and melatonin on gross assimilation during optimum and cold stress conditions; (i) Control – filled squares, (ii) Melatonin – open circles, (iii) Nitrogen – filled circles, (iv) Nit + Mel – filled rectangles; under optimum (A) and 15 days of cold stress conditions (B). Optimum conditions were 25/20ºC whereas cold stress conditions were 12/12ºC, day and night respectively. Data points represent measurements from 3 individual plants. Error bars represent standard error of the mean.

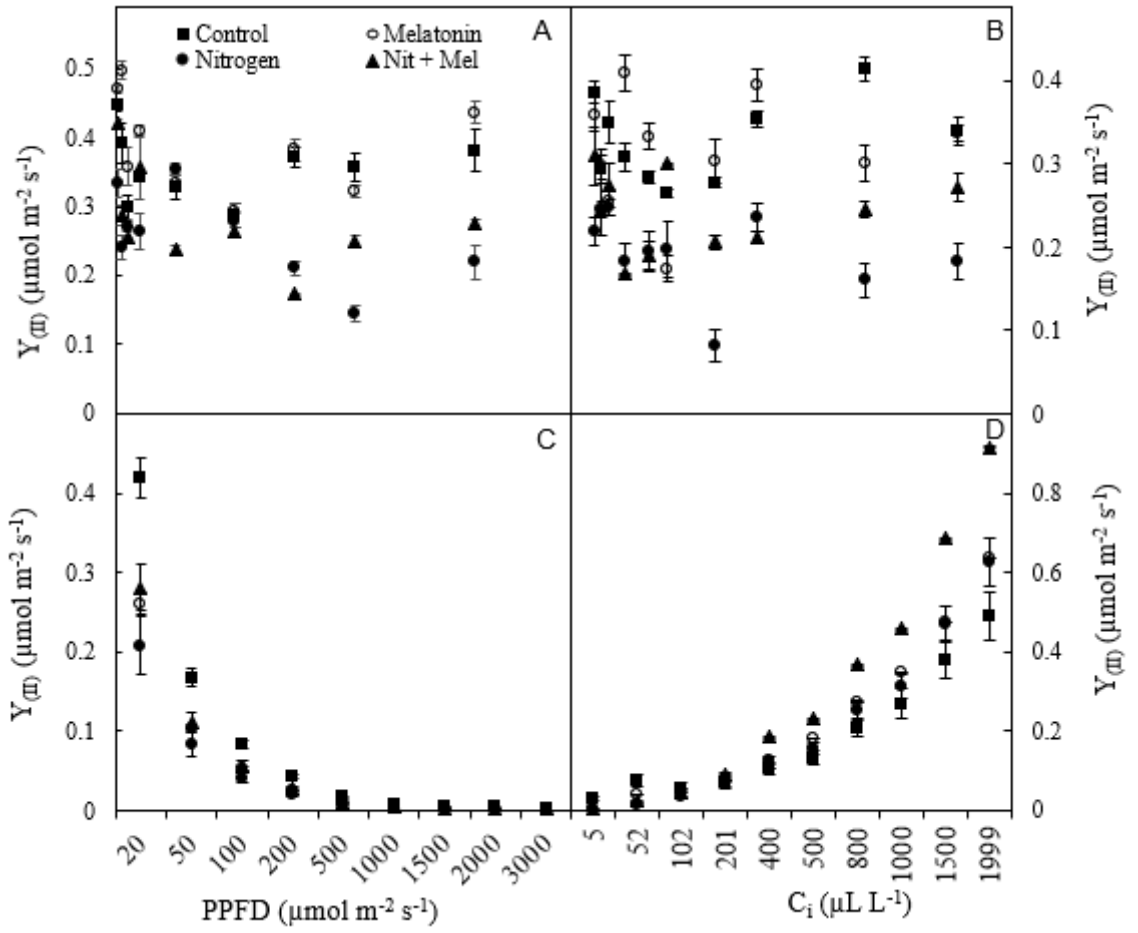


Fig. 37. Effect of exogenous nitrogen and melatonin on the photochemical yield of photosystem II during optimum and cold stress conditions; in response to photosynthetic photon flux density (A and C) and to intracellular $CO_2$ concentration (B and D) amongst the treatments: (i) Control– filled squares, (ii) Melatonin – open circles, (iii) Nitrogen – filled circles, (iv) Nit + Mel – filled rectangles; under cold stress conditions (25/20ºC, day and night respectively). Data points represent measurements from 3 individual plants. Error bars represent standard error of the mean, (n = 3).

### 6.3.4 RuBisCO kinetics

*6.3.4.1 Fo and $V_o/V_c$*

The photorespiratory $CO_2$ (Fo) release and the ratio of oxygenation to carboxylation rate ($V_o/V_c$) in the coffee plants in response to PPFD and $C_i$ during optimum and cold stress conditions is shown in Fig. 38. Photorespiratory $CO_2$ release showed a gradual increase in the with increasing PPFD especially under optimum conditions (Fig. 38A). Under optimum conditions, Fo was highest in the plants under the Melatonin treatment showing a maximum value of 50 µmol $m^{-2}$ $s^{-1}$ at 3000 µmol $m^{-2}$ $s^{-1}$ PPFD. This was followed by plants under the control treatment with Fo at 40 µmol $m^{-2}$ $s^{-1}$ and then those under Nit + Mel at 30 µmol $m^{-2}$ $s^{-1}$. Fo was least in plants under Nitrogen treatment with 20 µmol $m^{-2}$ $s^{-1}$ at 3000 µmol $m^{-2}$ $s^{-1}$ under optimum conditions. The Fo under cold stress conditions was generally low and barely increasing above 0 µmol $m^{-2}$ $s^{-1}$ despite increasing PPFD. With respect to $C_i$, Fo also generally showed a gradual increase with increasing $C_i$ being more apparent under cold stress conditions whose Ci was generally higher than plants under optimum conditions. At the highest $C_i$ of 2000 µL $L^{-1}$, Fo was highest at 17 µmol $m^{-2}$ $s^{-1}$ in plants under Nit + Mel followed by those under Melatonin at 12 µmol $m^{-2}$ $s^{-1}$. Plants under nitrogen treatment had the least Fo at 10 µmol $m^{-2}$ $s^{-1}$ while those under control had a maximum Fo value of 11 µmol $m^{-2}$ $s^{-1}$ at 2000 µL $L^{-1}$ $C_i$. The ratio of oxygenation to carboxylation rate ($V_o/V_c$) also known by Φ was estimated by the equation (Eqn. 9).

$$\Phi = \frac{Vo}{Vc} = \left(\frac{1}{Sc/o}\right)\frac{O}{C} = \left(\frac{Vc,max}{Ko}\frac{Kc}{Vc,max}\right)\frac{O}{C}$$

Equation 9. Determination of RuBisCO rate of carboxylation and oxygenation

Where: $S_{c/o}$ is the relative specificity of RuBisCO; C and O are the chloroplastic $pCO_2$ and $pO_2$; $V_{c,max}$, $V_{o,max}$, $K_c$ and $K_o$ are the maximal rates and Michaelis−Menten constants of carboxylation and oxygenation, respectively.

The apparent RuBisCO constant estimated as from the equations for the coffee plants was 550. Variation in $V_o/V_c$ amongst the treatments during optimum and cold stress conditions in response to PPFD and $C_i$ (Fig. 38C and D) generally showed a parallel trend especially after 500 µmol $m^{-2}$ $s^{-1}$ and 400 µL $L^{-1}$ in response to increasing PPFD and $C_i$, respectively. Generally, plants under optimum conditions had higher $V_o/V_c$ values despite showing no clear differences amongst the individual treatments. This distinction between optimum and cold stressed plants was more observable in response to PPFD where the $V_o/V_c$ values were above

1 whereas those for plants under cold stress condition where about 1 or below. Under the latter conditions, $V_o/V_c$ was least in the plants under Nitrogen and Nit + Mel conditions whereas those under Control and Melatonin treatment had slightly higher $V_o/V_c$ values (Fig. 38C). In response to Ci, $V_o/V_c$ showed no clear distinctions amongst both temperature and foliar spray treatments (Fig. 38D).

*6.3.4.2 $J_{max}$, $V_{c,max}$ and TPU*

The relationship between $J_{max}$, $V_{c,max}$ and TPU is indicated in Fig. 5. $J_{max}$, $V_{c,max}$ and TPU were highest in plants under optimum conditions and were greatly depressed in cold stressed plants. Moreover, these parameters showed a significantly positive correlation amongst each other with the Plants under Nitrogen treatment indicating the highest $J_{max}$, $V_{c,max}$ and TPU followed by those under Nit + Mel, Control and Melatonin, respectively under optimum conditions. In addition to being low under optimum conditions, these parameters showed no distinctive differences amongst the treatments despite being slightly higher in plants under Nit + Mel treatment (Fig 39A and B).

### 6.3.5 Electrolyte leakage Index

Under optimum conditions, ELI was highest in the plants under the Control treatment and least in those under Nitrogen treatment whereas the two Melatonin treatments showed intermediate values (Fig. 40). Exposure of the coffee plants to cold stress conditions increased the leakage of cellular electrolytes in all the treatments. This percentage increment was highest in the plants under Control and Nitrogen treatments at 41% and least in Melatonin and Nit + Mel treatments at 30 and 15%, respectively.

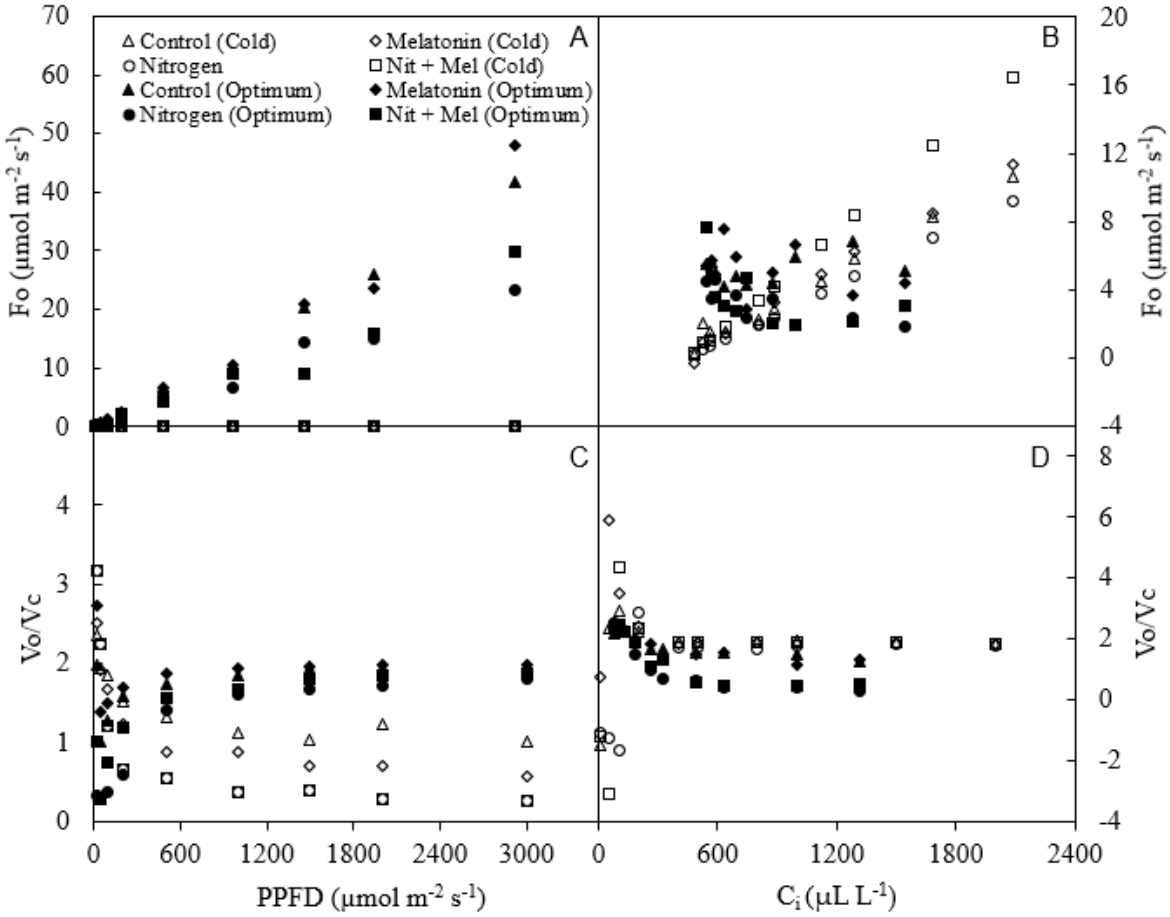


Fig. 38. Effect of exogenous nitrogen and melatonin on RuBisCO kinetics during optimum and cold stress conditions; photorespiratory $CO_2$ release (A and B) and ratio of RuBisCO oxygenase to carboxylase (C and D) in response to PPFD (A and C) and to $C_i$ (B and D) amongst the treatments under optimum (25/20ºC) and cold stress (12/12°C) conditions. Data points represent measurements from 3 individual plants. Error bars represent standard error of the mean.

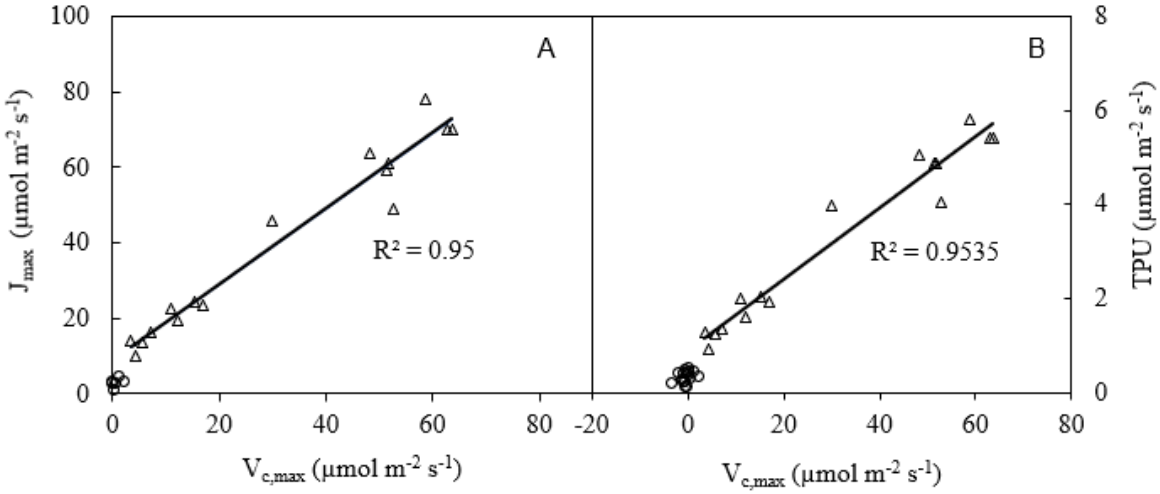


Fig. 39. Effect of exogenous nitrogen and melatonin the relationship between Jmax and Vc,max (A) and between TPU and Vc,max (B) in coffee plants during optimum and cold stress conditions; Open triangles represent optimum conditions (25/20°C, day and night respectively) and open circles represent cold stress conditions (12/12°C).

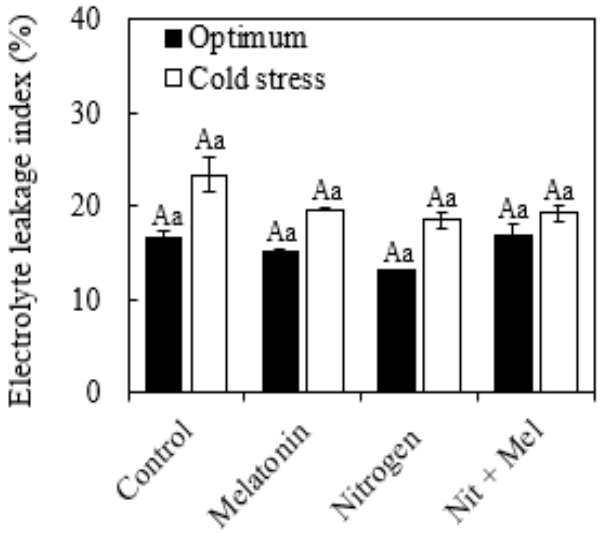


Fig. 40. Effect of exogenous nitrogen and melatonin on the electrolyte leakage index during optimum and cold stress conditions; Optimum conditions were 25/20°C whereas cold stress conditions were 12/12°C, day and night respectively. Different capital letters denote significant differences among means for nitrogen treatment (Nitrogen effect) whereas different small letters represent significant differences amongst means of melatonin treatment (Melatonin effect). For each treatment, means of cold stressed plants followed by an asterisk (*) are significantly differ from those under optimum conditions (Temperature effect). Statistical comparisons were made using Newman−Keuls' test at $P \leq 0.05$. Error bars represent standard error of the mean, (n = 3).

### 6.3.6 Photosynthetic pigments

The concentrations of chlorophylls *a* and *b* together with their respective total (a + b) were generally highest in the Nitrogen and Nit + Mel treatments under optimum conditions although these did not show significant differences amongst the treatments (Fig. 41). These pigments were increased by 8, 4 and 7%, respectively as a result of nitrogen application (Fig. 41A–C, respectively). This increment was compounded by melatonin application which increased the concentration of chl a, b and a + b by 43, 42 and 44%, respectively. Amongst all the treatments, cold stress conditions induced a decline in the concentrations chl a, b resulting into a percentage decline of 11, 3, 6 and 10% in the Nit + Mel, Nitrogen, Melatonin and Control treatments, respectively. Although the concentration of carotenoids did not vary significantly amongst the treatments (Fig 41D), exposure of the coffee plants to cold stress conditions consistently increased the concentration of carotenoids in all the treatments by 30, 42, 37 and 30% in the Control, Melatonin, Nitrogen and Nit + Mel treatments, respectively. The ratio of chl a to b showed no significant differences with both exogenous application of nitrogen and melatonin and with instigation of cold stress conditions (Fig. 41E). Nevertheless, this ratio slightly declined in all the treatments as a result of cold stress treatment except int the Nit + Mel treatment. On the other hand, despite showing no significant differences in the ratio of chlorophylls to carotenoids amongst the treatments under the respective temperature, a dramatic decline of 17, 30, 33 and 17% in Control, Melatonin, Nitrogen and Nit + Mel treatment, respectively was observed on exposure of the coffee plants to cold stress conditions (Fig. 41F).

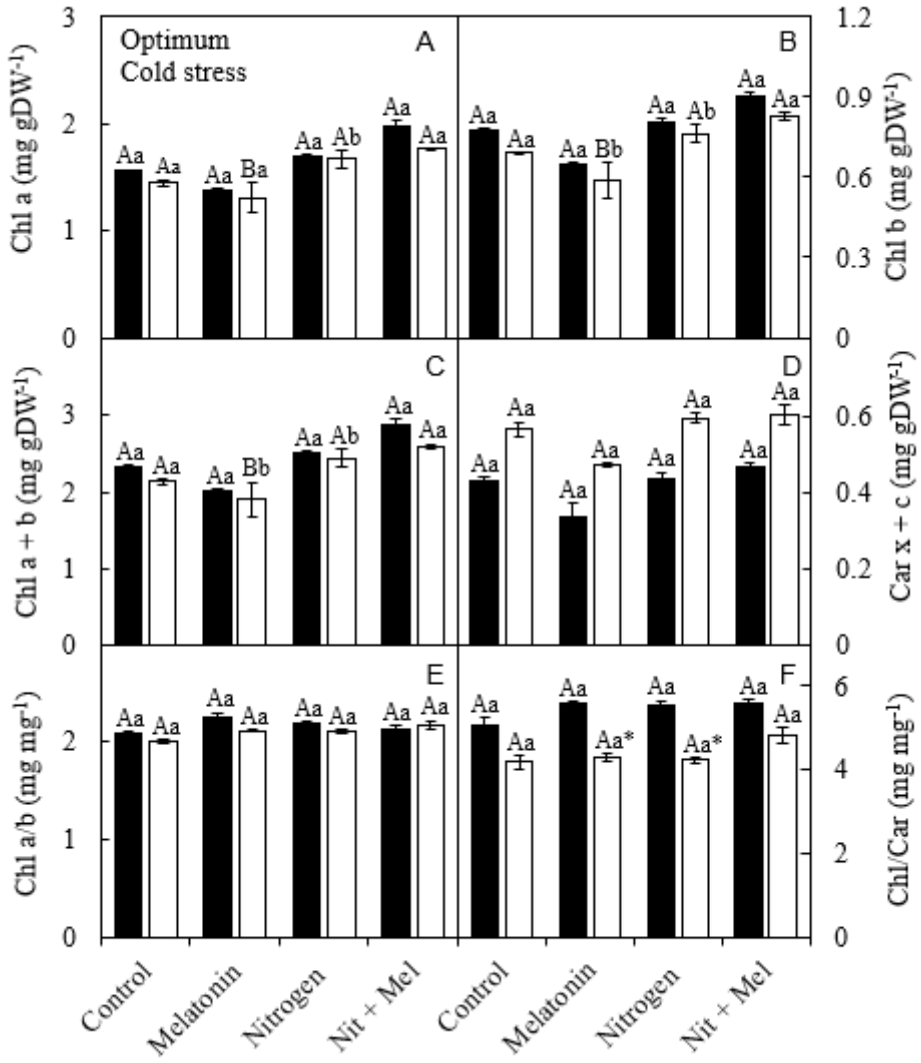


Fig. 41. Effect of exogenous nitrogen and melatonin on the concentration of the photosynthetic pigments during optimum and cold stress conditions; chlorophyll a (A), chlorophyll b (B), chlorophyll a + b (C), carotenoids (D), chlorophyll a/b (E) and chlorophyll/carotenoids (F) amongst the treatments under optimum (black bars) and after 15 days of cold stress conditions (white bars). Optimum conditions were 25/20°C whereas cold stress conditions were 12/12°C, day and night respectively. Different capital letters denote significant differences among means for nitrogen treatment (Nitrogen effect) whereas different small letters represent significant differences amongst means of melatonin treatment (Melatonin effect). For each treatment, means of cold stressed plants followed by an asterisk (*) are significantly differ from those under optimum conditions (Temperature effect). Statistical comparisons were made using Newman−Keuls' test at $P \leq 0.05$. Error bars represent standard error of the mean, (n = 3).

### 6.3.7 Nonenzymatic antioxidant compounds

*6.3.7.1 Total anthocyanin content (TAC)*

Accumulation of total anthocyanins expressed on the basis of peonidin – 3 − (6 – caffeoyl − sophoride) − glucoside (YGM–5b) and cyanidin−3−gluocoside (Cy3G) equivalents is indicated in Fig 42A and B, respectively. The concentration of total anthocyanins was not significantly affected by exogenous application of nitrogen or melatonin under optimum conditions. Nevertheless, total anthocyanin concentration tended to decline in the plants under Nit + Mel treatment compared to those under Nitrogen as indicated by a 17% decline in both Cy3G and YGM−5b equivalents. On the other hand, cold stress conditions caused a non−significant but consistent decline in the concentration of the total anthocyanins except in plants under Nit + Mel treatment. Moreover, under these conditions, the concentration of total anthocyanins was significantly least in plants under Melatonin treatment as indicated by both Cy3G and YGM−5b equivalents (Fig. 42A and B, respectively).

*6.3.7.2 Total flavonoids and flavonols content*

The concentration of total flavonoids and flavonols expressed as rutin equivalents are indicated in Fig 42C and D. Despite varying non−significantly amongst the treatments under both optimum and cold stress conditions, the concentration of both flavonoids and flavonols were highest under cold stress conditions in the plants under Nitrogen treatment at 44 and 18 mg $gDW^{-1}$, respectively. These were least in plants under the Melatonin treatment during optimum conditions at 30 and 15 mg $gDW^{-1}$ for flavonoids and flavonols, respectively. Exposure of the coffee plants to cold stress conditions generally resulted into an increment in the concentration of flavonoids by 14, 0.9 and 9% in the plants under Control, Melatonin and Nitrogen treatments respectively while a decline of 4% was observed in plants under Nit + Mel treatment (Fig. 42C). On the other hand, a minor but consistent surge was observed in the concentration of total flavonols in all the treatments which indicated an increment of 17, 4, 10 and 3% in plants under Control, Melatonin, Nitrogen and Nit + Mel treatments, respectively as a result of cold stress cold stress conditions instigation (Fig. 42D). This indicated that the percentage change in the concentration of both flavonoids and flavonols was more observable in the non−melatonin treated plants. Moreover, exogenous melatonin treatment tended to instigate a decline in the concentration of flavonoids by 20 and 25% and flavonols by 11 and 8% in non−nitrogen and nitrogen treated plants, respectively under cold stress conditions.

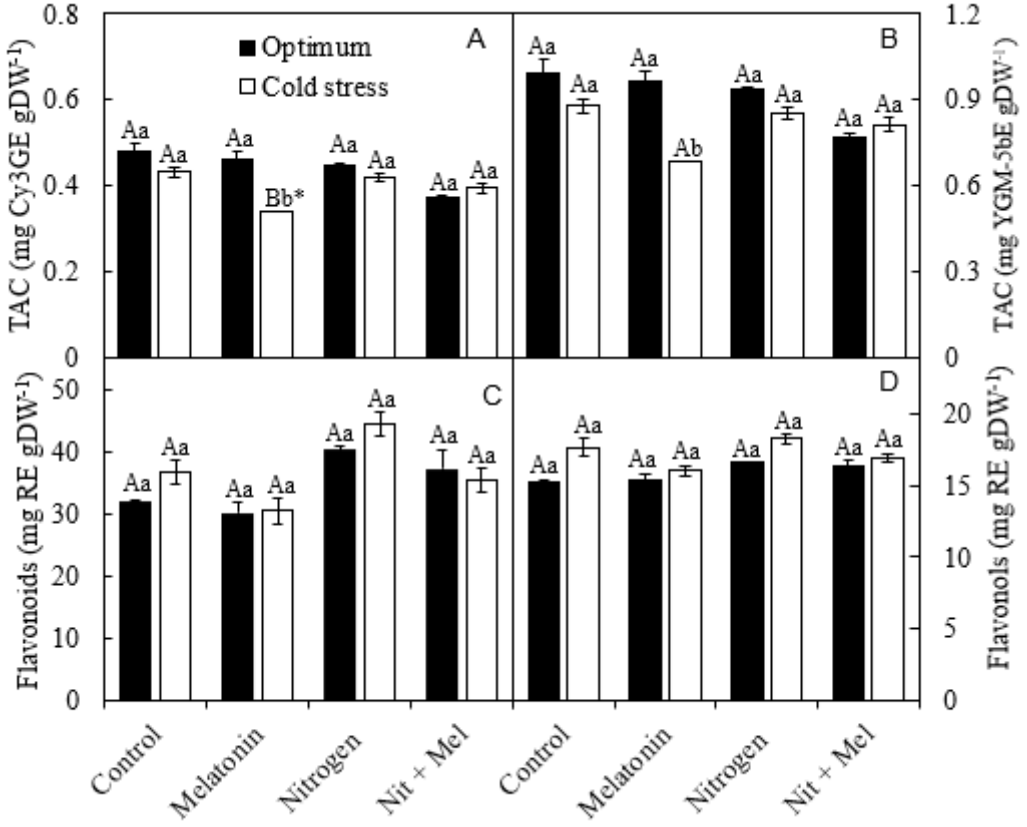


Fig. 42. Effect of exogenous nitrogen and melatonin on the concentration of total anthocyanin in coffee leaves during optimum and cold stress conditions; expressed on the basis of peonidin−3−(6−caffeoyl−sophoride)−glucoside and cyanidin 3 glucoside (A and B, respectively) and total flavonoid content (C) and total flavonols content amongst the treatments under optimum (black bars) and after 15 days of cold stress conditions (white bars). Optimum conditions were 25/20˚C whereas cold stress conditions were 12/12˚C, day and night respectively. Different capital letters denote significant differences among means for nitrogen treatment (Nitrogen effect) whereas different small letters represent significant differences amongst means of melatonin treatment (Melatonin effect). For each treatment, means of cold stressed plants followed by an asterisk (*) are significantly differ from those under optimum conditions (Temperature effect). Statistical comparisons were made using Newman−Keuls' test at $P \leq 0.05$. Error bars represent standard error of the mean, (n = 3).

*6.3.7.3 HPLC selected metabolites*

The concentration of some of the most abundant metabolites that is to say trigonelline, mangiferin and 5−caffeoylquinnic acid as determined by the HPLC is indicated in Fig. 43. The concentration of trigonelline in coffee plants was highest under optimum conditions in all the treatments and was consistently reduced by 43, 27, 19 and 12% in plants under Control, Melatonin, Nitrogen and Nit + Mel treatments on instigation of cold stress despite varying non−significantly amongst all treatments (Fig. 43A). Both under optimum and cold stress conditions, the concentration of trigonelline was highest in plants under Nitrogen treatment at 0.37 mg $gDW^{-1}$ followed by those under Melatonin and Nit + Mel at 0.33 and 0.28 mg $gDW^{-1}$, respectively and was least in plants under Control treatment at 0.26 mg $gDW^{-1}$. Similarly, the concentration of mangiferin was highest in plants under nitrogen treatment at 0.39 mg $gDW^{-1}$ under cold stress conditions and least in those under Control treatment at 0.23 mg $gDW^{-1}$ under the same conditions (Fig. 43B). Unlike in Control and Melatonin treatments where the concentration of mangiferin more or less did not vary between temperature treatments, instigation of cold stress conditions caused an increment of 18 and 23% in Nitrogen and Nit + Mel treatments, respectively. The concentration of 5−caffeoylquinic acid (5–CQA) was significantly highest in plants under Nitrogen and Nit + Mel treatments at 5 and 5.4 mg $DW^{-1}$ during optimum conditions and 4.8 and 5.1 mg $gDW^{-1}$ during cold stress conditions, respectively. 5–CQA concentration was lowest in plants under Control and Melatonin treatments at 4.0 and 3.6 mg $gDW^{-1}$ during optimum and 4.2 and 4.11 mg $gDW^{-1}$ during cold stress conditions, respectively (Fig. 43C). This implied that in all the treatments, instigation of cold stress conditions tended to stimulate increase in the concentration of 5–CQA. As a result, the concentration of total metabolites was also significantly highest in the Nitrogen and Nit + Mel treatments at 6.1 and 5.5 mg $gDW^{-1}$ during cold stress conditions and 5.8 and 5.5 mg $gDW^{-1}$ during optimum conditions (Fig. 43D). Total metabolite content was lowest at 4.6 mg $gDW^{-1}$ in both Control and Melatonin treatments during cold stress conditions and 4.1 and 4.5 mg $gDW^{-1}$, respectively during optimum conditions.

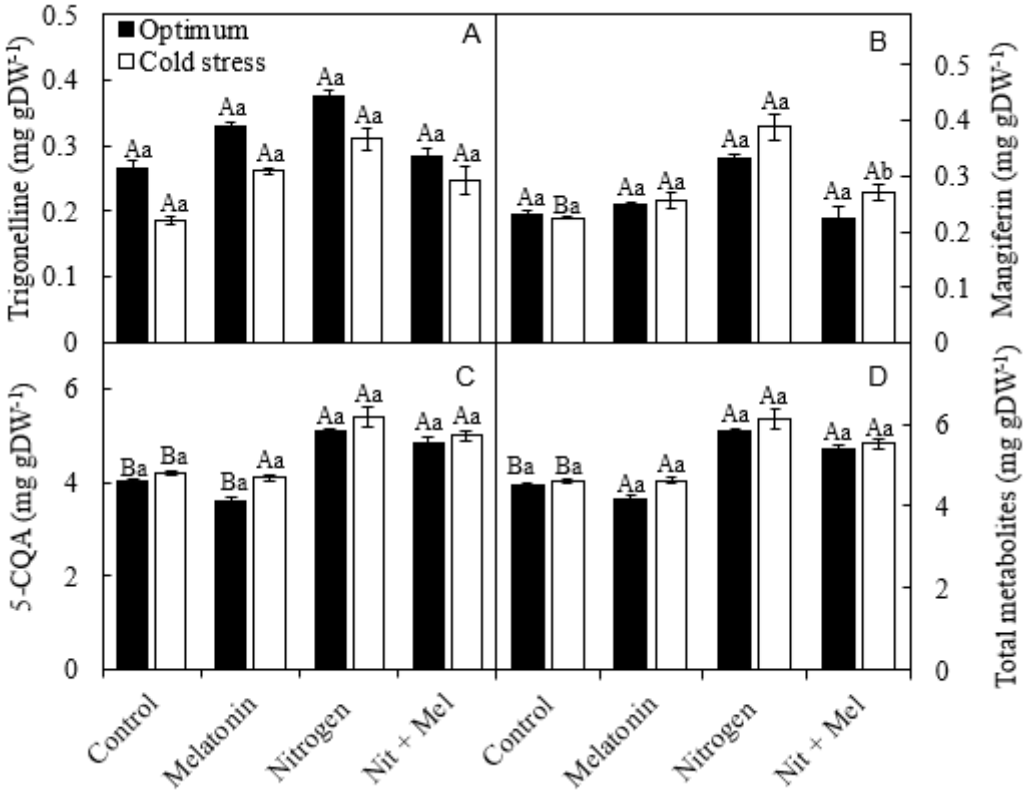


Fig. 43. Effect of exogenous nitrogen and melatonin on the concentration of selected HPLC metabolites in coffee leaves during optimum and cold stress conditions; trigonelline (A), mangiferin (B), 5–caffeoylquinic acid (C) and total metabolites amongst the treatments under optimum (black bars) and after 15 days of cold stress conditions (white bars). Optimum conditions were 25/20˚C whereas cold stress conditions were 12/12˚C, day and night respectively. Different capital letters denote significant differences among means for nitrogen treatment (Nitrogen effect) whereas different small letters represent significant differences amongst means of melatonin treatment (Melatonin effect). For each treatment, means of cold stressed plants followed by an asterisk (*) are significantly differ from those under optimum conditions (Temperature effect). Statistical comparisons were made using Newman−Keuls' test at $P \leq 0.05$. Error bars represent standard error of the mean, (n = 3).

*6.3.7.4 Total phenolic content (TPC)*

Accumulation of phenolic compounds in the coffee plants expressed on the basis of gallic acid equivalent (GAE) is indicated in Fig. 44. Total phenolic content was significantly least in the coffee plants treated with Melatonin under optimum conditions at 148 mg GAE $gDW^{-1}$ which also indicated significantly lower TPC concentration compared to cold stress conditions at 167 mg GAE $gDW^{-1}$ in the same foliar treatment. On the other hand, in addition to not varying significantly amongst the temperature treatments, TPC was high in plants under Control, Nitrogen and Nit + Mel treatments at 180, 179 and 180 mg GAE $gDW^{-1}$, respectively under optimum conditions and 182, 186 and 175 mg GAE $gDW^{-1}$, respectively under cold stress conditions.

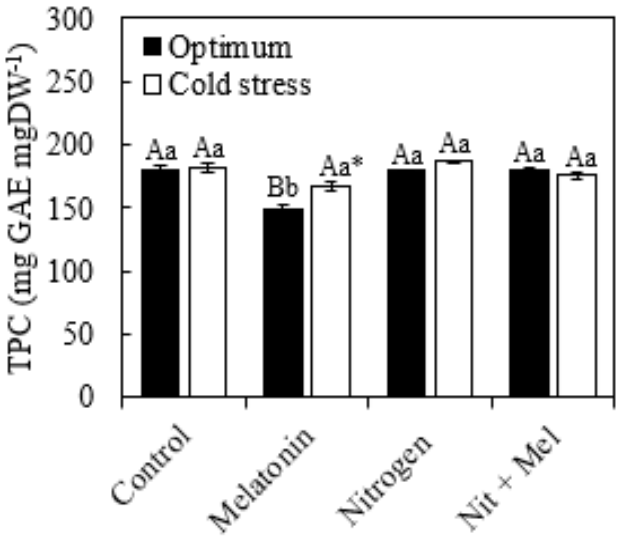


Fig. 44. Effect of exogenous nitrogen and melatonin on the concentration of total phenolic compounds in coffee leaves during optimum and cold stress conditions; Optimum conditions were 25/20°C whereas cold stress conditions were 12/12°C, day and night respectively. Different capital letters denote significant differences among means for nitrogen treatment (Nitrogen effect) whereas different small letters represent significant differences amongst means of melatonin treatment (Melatonin effect). For each treatment, means of cold stressed plants followed by an asterisk (*) are significantly differ from those under optimum conditions (Temperature effect). Statistical comparisons were made using Newman−Keuls' test at $P \leq 0.05$. Error bars represent standard error of the mean, (n = 3).

### 6.3.8 Total ROS scavenging capacity

The radical scavenging capacity of the leaf sample extracts of the plants in the different treatments is indicated in Fig. 45. The half maximal concentration that scavenge 50% of the DPPH free radical (DPPH $IC_{50}$) which is an inverse of the free radical scavenging activity was significantly higher under optimum conditions in plants under Nit + Mel and Nitrogen treatments at 80 and 72 µg $mL^{-1}$ and lower at 58 and 54 µg $mL^{-1}$ in plants under Control and Melatonin treatments (Fig. 45A). Under cold stress conditions, DPPH $IC_{50}$ was lower in the melatonin treated in presence or absence of nitrogen at 43 and 47 µg $mL^{-1}$ in Nit + Mel and Melatonin treatments, respectively compared to non−melatonin treated plants at 52 and 58 µg $mL^{-1}$ in Nitrogen and Control treatments, respectively despite this variation being non−significant. This indicated therefore, the exposure of the coffee caused a substantial decline in the DPPH $IC_{50}$ mostly in plants under Nit + Mel treatments with a percentage reduction of 45% compared to 29 and 13% in plants under Nitrogen and Melatonin treatments while no observable effect was seen in those under Control treatment. DPPH TEAC which is an indicator the actual radical scavenging capacity of the sample extracts was significantly higher under cold stress conditions compared to optimum conditions (Fig. 45B). This variation was observed more so in melatonin treated plants in presence or absence of nitrogen at 664 and 617 µmol Trolox $gDW^{-1}$ in Nit + Mel and Melatonin treatments compared to 561 and 517 µmol Trolox $gDW^{-1}$ in the non−melatonin treated plants for Nitrogen and Control treatments, respectively under cold stress conditions. This is compared to 376, 409, 541 and 502 in plants under Nit + Mel, Nitrogen, Melatonin and Control treatments, respectively.

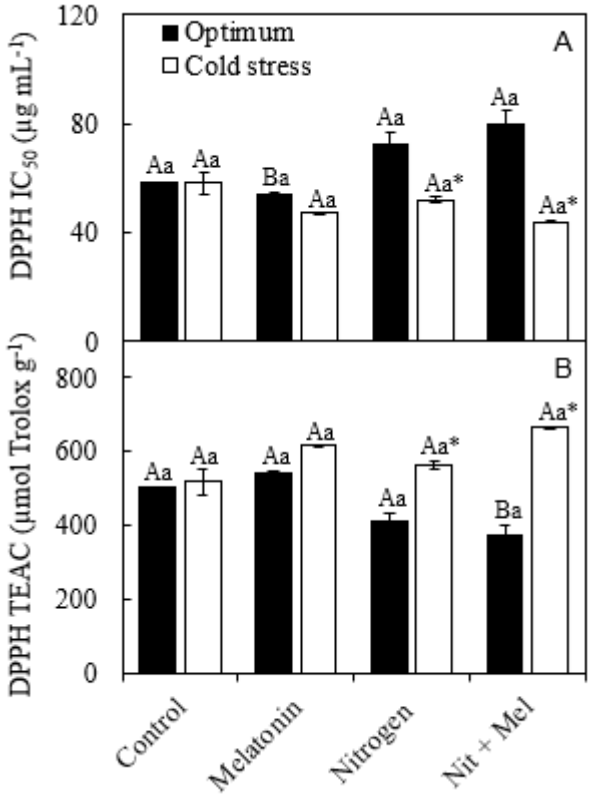


Fig. 45. Effect of exogenous nitrogen and melatonin on ROS scavenging capacity in coffee leaves during optimum and cold stress conditions; expressed as DPPH $IC_{50}$ (A) and DPPH TEAC (B) amongst the treatments under optimum (black bars) and after 15 days of cold stress conditions (white bars). Optimum conditions were 25/20˚C whereas cold stress conditions were 12/12˚C, day and night respectively. Different capital letters denote significant differences among means for nitrogen treatment (Nitrogen effect) whereas different small letters represent significant differences amongst means of melatonin treatment (Melatonin effect). For each treatment, means of cold stressed plants followed by an asterisk (*) are significantly differ from those under optimum conditions (Temperature effect). Statistical comparisons were made using Newman−Keuls' test at $P \leq 0.05$. Error bars represent standard error of the mean, (n = 3).

## 6.4 Discussion

Coffee plants evolved as an understorey species under moderate temperature (25 – 20°C) and light conditions (300–700 µmol $m^{-2}$ $s^{-1}$) in the tropical rainforest in Africa (Davis *et al.,* 2006). Traditionally, coffee cultivation is done under shaded conditions although in some regions of the globe, unshaded coffee plantations exist (DaMatta, 2004). During cultivation under extreme environmental conditions, nitrogen supply has been shown to confer photoprotection due to its direct role in preserving a balance between enzyme (such as rubisco) and light harvesting system (such as chlorophylls and carotenoids) metabolism (Farquhar *et al.,* 1980; Wullscleger *et al.,* 1993). In the current study, nitrogen treated plants maintained the highest maximum rates of both net and gross $CO_2$ assimilation in repose to both $C_i$ and PPFD compared to those with no foliar nitrogen treatment (Fig. 35A–D and Fig. 36, respectively). The current results concur with those reported in a previous study where foliar urea sprays containing 10 mM of nitrogen enhanced the photosynthetic performance of coffee plants recovering from cold stress conditions (Chapter 4). Furthermore, this enhancement was associated with accumulation of nitrogen and photosynthetic pigments, chlorophylls and carotenoids (see chapter 4). Melatonin application under optimum conditions caused a decline in the maximum net and gross $CO_2$ assimilation in response to both $C_i$ and PPFD (Fig. 35A–D and Fig. 36). Although exogenous melatonin is generally known for its improvement in the photosynthetic activity of many plants (Li *et al.,* 2018; Zhang *et al.,* 2017; Yu *et al.,* 2018), its mechanism of action involves initiation of RBOH which increase the concentration of ROS hence reducing the photochemical efficiency of PSII (Gong *et al.,* 2017; Chen *et al.,* 2018).

Furthermore, the $A/C_i$ curve analysis indicated a decline in the maximum rate of $CO_2$ assimilation under cold stress conditions in response to controlled increase in $C_i$ (Fig. 35A and B). A similar decline was observed in the light−response curve analysis which also indicated saturation below 1000 µmol $m^{-2}$ $s^{-1}$ under both cold and optimum conditions (Fig. 35C and D, respectively). This decline in the maximum rate of $CO_2$ assimilation is normally associated with both diffusivity and biochemical constraints emanating from low temperature stress (Pompelli *et al.,* 2010). Moreover, while accounting for both photorespiration and dark respiration, GA which also represents true photosynthesis or carboxylation followed a typical trend, being higher under optimum conditions in all the foliar treatments (Fig. 36A and B, respectively). Similar impacts of low temperature conditions on gross $CO_2$ assimilation and $O_2$ evolution have been reported in several plants (Flexas *et al.,* 1999; Mishra *et al.,* 2019). In

addition to lowering the stomatal conductivity, cold stress conditions cause substantial decline in the PSII photochemical efficiency, thylakoid electron transport, enzyme activity and the metabolism of both carbon and nitrogen (Partelli *et al.,* 2009). In the current study, nonetheless, the photochemical yield showed less variation amongst the temperature treatments in contrast to the previous studies (Fig. 37A–D). This could however be attributed to the recovery in the PSII photochemistry as has been reported to occur in some varieties of arabica with better cold tolerance (Partelli *et al.,* 2009).

In the current study, cold−stressed plants had a lower photorespiratory $CO_2$ release and diminished values of RuBisCO oxygenase to carboxylase which is normally associated with low photosynthetic rate (Fig. 38). This decline in the maximum $CO_2$ assimilation could be attributed more to the cold−induced thermodynamic reduction in the Calvin−Benson cycle enzymes thus limiting the supply of $NADP^+$ and ADP for phosphorylation (Rasool *et al.*, 2014). Moreover, the capacities of RuBP carboxylation and regeneration ($V_{c,max}$ and $J_{max}$, respectively) together with the triose phosphate utilization rate (TPU) which represent the main 3 limitations to photosynthesis (Fabre *et al.*, 2019) were all dampened by cold stress conditions (Fig. 39). The sensitivity of these parameters to cold stress has been indicated in several plants (McClain and Sharkey, 2018). TPU is situated at the interface between photosynthetic production and consumption, therefore photosynthesis can only be as fast the plants can remove the resources away from the chloroplasts (Sharkey *et al.,* 2019).

The decline in the photosynthetic activity of the coffee plants under cold stress conditions was more prominent in the plants under nitrogen treatment. Under optimum conditions, the role of nitrogen in photosynthetic regulation is an undisputed phenomenon. However, in a previous study, we reported a decline in the content of nonenzymatic antioxidant compounds in the nitrogen treated plants compared to those under control conditions (see chapter 4). This was attributed to the improvement in the efficiency of the photosynthetic apparatus in the nitrogen treated plants thereby reducing the need for an elaborated antioxidant system due to low levels of ROS in the photosynthetic apparatus. Subjection of such plants to cold stress conditions as in this study showed a substantial decline in the maximum $CO_2$ assimilation. On the other hand, melatonin improved the photosynthetic activity of coffee plants under cold stress conditions. The current results concur with a number of reports that have indicated positive effects on the photosynthetic physiology of exogenous melatonin in several plants (Szafranska *et al.,* 2017; Zhang *et al.,* 2017; Yu *et al.,* 2018). Similar to the above studies, exogenous melatonin improved the maximum $CO_2$ assimilation via enhancement of the quantum yield of PSII

photochemistry. Melatonin priming has also been shown to upregulate the gene expressions of enzymes responsible for ATP, NADPH, RuBisCO activase as well directly improving the activity of RuBisCO and hence facilitating photosynthetic activity during abiotic stress conditions (Erdal, 2019).

Membrane damage analysis expressed as electrolyte leakage index indicated a surge in ROS on exposure of coffee plants to cold stress conditions especially in plants under control treatment (Fig. 40). A corresponding decline in the content of the photosynthetic pigment was in the cold stressed plants compared to their optimum counterparts (Fig. 41A−F). The photosynthetic apparatus of coffee plants is highly dependent on nitrogen metabolism and therefore the risk of photoinhibition is highest in nitrogen deficient plants (see chapter 4). This reduction in the photosynthetic capacity is further worsened by exposure to cold stress conditions which result into heightened production of ROS which cause cell damage and disintegration of chlorophyll pigments while increasing the photoprotective molecules such as carotenoids (Sharma *et al.,* 2012; Tripathy and Oelmuller, 2012). In addition to improving photosynthetic activity during cold stress conditions, melatonin is an antioxidant with ROS scavenging abilities (Arnao and Hernandez−Ruiz, 2006). These protective role of melatonin on the damage to photosynthetic apparatus were more apparent in the nitrogen treated plants which tended to maintain a higher chlorophyll/carotenoid ratio (Fig. 41F).

Coffee plants rely on accumulation of powerful nonenzymatic antioxidant compounds for ROS scavenging and maintenance of homeostatic conditions (Campa *et al.,* 2017; Ramalho *et al.,* 2018). In the current study, the content of anthocyanins only declined in plants under Melatonin treatment while no significant variation was observed amongst other treatments under the two temperature conditions (Fig. 42A and B). Despite showing no significant variation amongst the treatments, flavonoids and flavonols tended to be highest in the plants under Nitrogen which also had the highest photosynthetic rate under optimum conditions (Fig. 42C and D). Its plausible therefore, that during high rates of photosynthetic activity, coffee plants upregulate the synthesis of flavonoids for ROS scavenging. Flavonoids and flavonols have been shown to surge under oxidative stress conditions (Sarmadi *et al.,* 2018).

HPLC analysis of the selected metabolites also indicated highest accumulation of trigonelline in plants treated with nitrogen (Fig. 43A). Trigonelline (1−methylpyridinium−3−carboxylate) a pyridine alkaloid and therefore contains nitrogen and directly benefits from nitrogen metabolism. This compound is NAD reservoir and therefore its accumulation might have a

direct effect on the content of NADP, a key intermediate in the $CO_2$ fixation pathway (Perchat *et al.,* 2018). Trigonelline accumulation in the coffee plants was however dampened by low temperature due to a general decline in general plant metabolism under cold stress conditions (Hussain *et al.,* 2018). Mangiferin ((1S)−1,5−Anhydro −1−(1,3,6,7 – tetrahydroxy – 9 – oxo − 9H – xanthen – 2 − yl) – D −glucitol) and 5−caffeoylquinic acid as well as the total of the HPLC metabolites were also generally highest in the nitrogen treated plants although unlike trigonelline, the two phenolic compounds were slightly increased by cold stress conditions (Fig. 43B–D). The current results agree those observed in chapter 4 where a dramatic increase of these compounds in coffee plants during oxidative stress conditions.

Total phenolic content (TPC) analysis generally showed no significant differences in the content of TPC amongst the treatments except in plants under Melatonin treatment during optimum conditions (Fig. 44). Nevertheless, on the basis of the anthocyanins, flavonoids, flavonols, mangiferin and 5−cafeeoylqunic acid, subjection of cold of plants to cold stress conditions elevated accumulation of these phenolic compounds. It has recently become clear that these components are modulated by ROS whose production is exacerbated by oxidative stress conditions and or by the RBOHs initiated by melatonin burst (Kreslavski *et al.,* 2012; Baxter *et al.,* 2014; Mittler, 2017; Sies, 2017). Due to underdeveloped enzymatic antioxidant system, coffee plants rely on accumulation of powerful nonenzymatic antioxidant compounds for ROS scavenging and maintenance of homeostatic conditions (Campa *et al.,* 2017; Ramalho *et al.,* 2018). Antioxidant analysis indicated an increase in the ROS scavenging capacity of the coffee plants after subjection to cold stress conditions (Fig. 45). The current results agree with those in chapter 4 who reported a positive relationship between accumulation of phenolic compounds during cols stress conditions and the ability to neutralise ROS. Phenolic compounds possess electrophilic glycosylic linkages and hydroxyl components that scavenge ROS (Das and Roychoudhury, 2014). Moreover, this ROS scavenging ability was highest in plants under Nit + Mel during cold stress conditions. This suggests an elevated ability to quench the ROS compared to their Nitrogen only treated counterparts.

### 6.5 Conclusion

Nitrogen improved the photosynthetic physiology of the coffee plants by enhancing both the net and gross maximum $CO_2$ assimilation rates in response to increasing $C_i$ and PPFD compared to non−nitrogen treated plants. This was associated with a higher nitrogen partitioning for both the metabolism of photosynthetic pigments and the RuBisCO enzymes

and other nitrogenous compounds such as trigonelline. Moreover, plants treated the foliar nitrogen exhibited a high capacity for RuBP carboxylation and regeneration under optimum conditions. Nevertheless, cold stress conditions caused strong reductions in the photosynthetic physiology of coffee plants which caused a tremendous damage to the cell membranes. This damage was however less prominent in melatonin treated plants which also had high contents of phenolic nonenzymatic antioxidant compounds such as anthocyanins, flavonoids, chlorogenic acids, mangiferin. Accumulation of these compounds caused a higher ROS scavenging capacity in coffee plants especially those treated with melatonin. Therefore, the combined effects of nitrogen and melatonin indicate a possible remedy for mitigating cold−induced decline in the photosynthetic physiology of coffee plants.

## Chapter seven

## ROS/RBOH−induced response of the nonenzymatic antioxidant system of coffee plants during oxidative stress conditions

### 7.1 Introduction

Finally, in this chapter the mechanism through which the nonenzymatic antioxidant system during cold stress conditions by either oxidative stress or RBOH induced ROS in coffee plants was evaluated.

Reactive oxygen species (ROS) are partially reduced or excited forms of atmospheric oxygen that are produced as by–products of aerobic metabolism (Mittler, 2002, Luis *et al.,* 2006; Pandhair and Sekhon, 2006). ROS are produced mainly in the chloroplasts by photosynthesis, mitochondria by aerobic respiration and in other cellular compartments with a high enough redox potential to excite or donate an electron to atmospheric oxygen (Mittler, 2017). ROS formation is initiated when atmospheric oxygen (dioxygen $O_2$) undergoes excitation or reduction to form singlet oxygen ($^1O_2$) or superoxide radical ($O_2^{\bullet-}$), respectively. The superoxide radical then dismutases to form hydrogen peroxide ($H_2O_2$) a reaction catalysed by an enzyme superoxide dismutase (SOD). The $H_2O_2$ reacts with $Fe^{2+}$ via the Fenton reaction (Eqn. 10) to produce hydroxide ($OH^-$) and the hydroxyl ($OH^{\bullet-}$) radical (Gupta *et al.,* 2016).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet-}$$

Equation 10. Production of $OH^{\bullet-}$ during Fenton reaction

This reaction and subsequent formation of the highly toxic $OH^{\bullet-}$ radical triggers a series of reactions that culminate into oxidation of DNA, RNA and protein molecules as well as several membranes (Cordeiro *et al.,* 2014).

Nevertheless, recent studies have revealed that ROS play a paradoxical role in cell metabolism being toxic in some instances while on one hand being involved in regulation of several basic biological processes (Czarnocka and Karpinski, 2018). Toxicity of ROS is driven by their heightened production during changes in the normal environment hence causing oxidative stress which culminates into cellular injury (Singh and Tuteja, 2010; Sies, 2015; Choudhury *et al.,* 2017). On the other hand, by acting as important signalling messenger molecules, ROS play active roles in regulation of a broad range of physiological processes such as cellular growth and development as well as acclimating to changes in the ambient environment

fluctuations (Karkonen and Kutchitsu, 2015). In the former role, ROS modulates both the enzymatic and the nonenzymatic antioxidants systems to protect the plants against oxidative stress (Singh and Tuteja, 2010).

In coffee leaves whose enzymatic antioxidant system is still immature and or suppressed by low temperature during cold stress conditions, coffee plants rely particularly on the nonenzymatic antioxidant system which is composed of ascorbic acid, phenolics, alkaloids, tocopherols among others for restoration of cellular ROS homeostasis (Campa *et al.,* 2017; Ramalho *et al.,* 2018). These antioxidant compounds contain redox buffers and redox signalling components that interact with biomembranes−related compartments. In addition, they provide essential information on cellular redox state and regulate gene expression associated with biotic and abiotic stress response to optimize defence and survival (Shao *et al.,* 2008). As a result, ROS priming by exogenous treatment with elicitor compounds such as by foliar application of phytochemicals e.g. melatonin, kinetin and salicylic acid is increasingly becoming popular under abiotic stress conditions (Kerchev *et al.,* 2020). These management practices result into reinforcement of the nonenzymatic antioxidant system via activation of NADPH oxidative/respiratory burst oxidative homologs (RBOH). The ROS produced by aerobic metabolism or RBOH result into accumulation of $H_2O_2$ that mediates the oxidation of cysteine residues on proteins, affecting their structure and function and hence triggering cellular signalling pathway (Mittler, 2017; Gong *et al.,* 2017).

Although a number of reviews on ROS metabolism are present, particular role of ROS in stimulation of the nonenzymatic antioxidant system in plants such as coffee during cold stress conditions remain unknown. This study therefore investigated the relationship between ROS toxicity assessed as membrane damage and the signalling function of ROS induced by RBOH on the contents of selected antioxidant compounds namely; alkaloids, phenolic compounds and ascorbic acid in the leaves of coffee plants during cold stress conditions.

## 7.2 Materials and methods

### 7.2.1 Plant material and growing conditions

Plant materials and growing conditions have been described in the previous chapter (see section 2.2).

### 7.2.2 Experimental design

After acclimation, 96 plants were selected and sprayed with 10 a urea solution containing 10 mM nitrogen for a period of 90 days as according to the previous study (chapter 4). In the previous study, foliar nitrogen supply improved the efficiency of the photosynthetic apparatus hence resulting into production of lower levels of ROS. The plants were then divided into four groups of 24 plants each and labelled Kinetin, Melatonin, Nitrogen and Salicylic acid treatments. The plants in each treatment were then sprayed every after 3 times every after 5 days with 0.35, 0.1, 10 and, 0.1 mM solution containing kinetin, melatonin, nitrogen and salicylic acid, respectively. The selected concentrations were determined from previous studies (chapter 4 and 5). All the foliar sprays together 0.1% isopropyl alcohol (Kao Global Chemicals, Tokyo, Japan) as a surfactant were made once a weak just before the dark phase of the growth chamber. Twenty−four hours after the last treatments, leaf samples were collected for biochemical analyses and thereafter challenged with cold stress conditions (12/12°C, day and night, respectively) while maintaining other ambient conditions constant for 10 days. For analysis, sample were collected once during optimum conditions and every after the other for a period of 10 days during cold stress conditions.

### 7.2.3 Electrolyte Leakage Index determination

Electrolyte leakage index was determined as previously described in the previous chapter (subsection 5.2.1.4).

### 7.2.4 Sample preparation for antioxidant compound assays

The leaf samples were grinded in a mortar with a pestle together with liquid nitrogen into a fine powder and thereafter stored under –80°C immediately until further analysis. For all the antioxidant assays, the powdered samples were freeze−dried at –20°C (Eyela DRC 1000−FDU 1110, Tokyo, Japan).

#### *7.2.4.1 Ascorbic acid extraction and determination*

For ascorbic acid assay, 100 mg of the freeze−dried powder were homogenized in 6 mL of an ice−cold solution of 2% metaphosphoric acid (w/v) and 100% acetonitrile mixed at a ratio of 1:1. The contents were agitated vigorously and thereafter sonicated in an ice−cold bath for 30 minutes. The samples were then centrifuged at 29,300 ×g for 10 minutes at 4°C and the contents filtered at 0.22µm Millipore before HPLC analysis. Ascorbic acid concentration was determined from a 10 µL sample using a high−performance liquid chromatography (HPLC) system equipped with a UV detector at a wavelength of 254 nm (Hitachi L–2490, Hitachi,

Tokyo, Japan). Ascorbic acid was detected by an Inertsil HILIC column (5µm particle size, 4.6 x 150 mm) in a thermostatic oven at a temperature of 20°C (Sigma–Aldrich, Tokyo, Japan) with a binary phase mobile gradient at a flow rate of 0.4 mL $min^{-1}$. The mobile phase consisted of two filtered (0.22µm Millipore), sonicated and degassed solvent A (0.68M ammonium acetate buffer of pH 6) and B (100% acetonitrile) with linear evolution of the gradient profile to a set program (Table 25). The retention time for ascorbic acid under these HPLC conditions was 14.31 min. The calibration curve for ascorbic acid was determined from 3 replicate points for the standard compound and its concentration in both the standards and the samples quantified by peak area measurement. Each standard or sample was eluted for 40 minutes followed by idling for 20 minutes. All analytical standards and organic solvents used were of analytical grade (Sigma−Aldrich, Tokyo, Japan).

*7.2.4.2 Alkaloid and phenolic compounds HPLC determination*

Selected alkaloids and phenolic compounds were assayed and determined as previously described in the previous chapter (subsection 2.2.4.4).

*7.2.4.3 Total flavonoids and total flavonols determination*

The leaf content of total flavonoids and total flavonols was assayed as described in the previous chapter (subsection 6.3.7.2).

*7.2.4.4 Total phenolic content assay*

The content of the total phenolic compounds was assayed and determined as previously described in the previous chapter (subsection 2.2.4.6).

Table 25. Linear evolution of the gradient profile program for HPLC determination of ascorbic acid

| Time (min) | 14.0 | 14.5 | 20.5 | 21.0 | 34 |
|---|---|---|---|---|---|
| Ammonium acetate buffer (%) | 80 | 80 | 50 | 80 | 80 |
| Acetonitrile (%) | 20 | 20 | 50 | 20 | 20 |

### 7.2.5 Statistical Analysis

The experimental data was arranged in a completely randomized design consisting of four exogenous treatments (Kinetin, Melatonin, Nitrogen and Salicylic acid) and 6 temperature treatments. The parameter differences for the temperature treatments were compared for statistical significance using the Newman–Keuls test at $p \leq 0.05$. Assumptions of normality were tested using the Kolmogorov–Smirnov test, and the data transformed accordingly to attain a normal distribution whenever necessary. All the statistical analyses were done using the Stata 12.0 statistics and data analysis program (Stata Corp, Texas USA). Data are expressed as means ± SE (n = 4).

## 7.3 Results

### 7.3.1 Increased membrane damage

Membrane damage assessed as electrolyte leakage index indicated a consistent increase with progression of cold stress conditions compared to optimum conditions (Fig. 46). Therefore, in the treatments, ELI was lowest under optimum conditions at an average of 13%. Amongst the foliar treatments, ELI was lowest in plants under nitrogen treatment at 11% and highest in those under melatonin and salicylic acid treatment at 14% while those under kinetin had moderate values of ELI at 12% under optimum conditions. Under cold stress conditions, ELI peaked on the 4$^{th}$ day after instigation of cold stress conditions at an average of 20%. Within the foliar treatments, on day 4 of cold stress conditions, ELI was still highest in plants under melatonin and salicylic acid at 23 and 21%, respectively and lowest in plants under kinetin and nitrogen at 17 and 19%, respectively. Further progression of cold stress caused no further increase in the ELI in all the treatments maintaining an average of 20% from day 6 to day 10 of cold stress conditions.

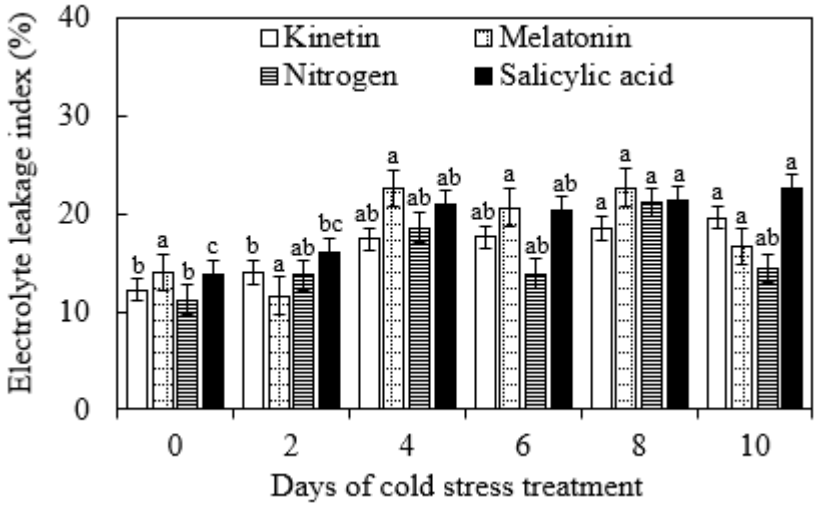


Fig. 46. Effect of ROS/RBOH on the integrity of cellular membrane assessed as electrolyte leakage index (%) in coffee leaves in the exogenous treatments. Different letters indicate significant differences amongst the temperature treatments for each exogenous treatment ($p \leq 0.05$). Error bars indicate standard error of the mean, n = 4. Cold stress conditions (12/12°C) were instigated 24 hours after the last foliar treatment which were all applied during optimum conditions (25/20°C, day and night, respectively).

### 7.3.2 Response of antioxidant compounds

*7.3.2.1 Ascorbic acid*

Variation in the concentration of ascorbic acid (ASC) in the treatments with progression of cold stress is indicated in Fig. 47. ASC was lowest under optimum conditions in all the treatments with an average of 0.75 mg $gDW^{-1}$. Under optimum conditions, within the foliar treatments, ASC was lowest in the kinetin and nitrogen treatment at 0.63 and 0.83 mg $gDW^{-1}$ and highest in the highest in plants under melatonin and salicylic acid treatments at 0.69 and 0.94 mg $gDW^{-1}$, respectively. Instigation and progression of cold stress conditions caused a consistent increase in the ASC after day 4 of cold stress which indicated an average ASC content in the treatments of 1.12 mg $gDW^{-1}$. ASC peaked on day 8 of cold stress conditions in the treatments with an average of 1.33 mg $gDW^{-1}$ and thereafter indicating a plateau on day 10 of cold stress conditions.

*7.3.2.2 HPLC content selected metabolites*

Variation in the contents of the 2 alkaloids (caffeine and trigonelline) and 2 phenolic compounds (5–caffeoylquinic acid and mangiferin) with progression of cold stress conditions is indicated in Fig. 48. Among the alkaloids, both caffeine and trigonelline varied non−significantly with progression of cold stress conditions showing an average concentration of 0.76, 1.08, 1.12, and 0.92 mg $gDW^{-1}$ for caffeine and 0.53, 0.67, 0.64 and 0.59 mg $gDW^{-1}$ for plants under nitrogen, salicylic acid, melatonin and kinetin treatments, respectively (Fig. 48A and B, respectively). Although caffeine variation showed no peculiar trend amongst the treatments, trigonelline tended to decline with progression of cold stress conditions in all the treatments (Fig. 48A). On the other hand, 5–caffeoylquinic acid tended to increase in all the treatments with progression of cold stress conditions despite showing no significant differences (Fig. 48C). Therefore, the concentration of 5–caffeoylquinic acid was lowest under optimum conditions at an average of 3.9 mg $gDW^{-1}$ thereafter increasing after instigation of cold stress conditions to 4.0 mg $gDW^{-1}$ on day 2. The concentration of 5–caffeoylquinic acid was highest with further progression of cold stress conditions with an average of 5.1, 4.7 and 4.2 on day 6, 8 and 10, respectively. Mangiferin, another phenolic compound showed neither peculiar trend nor significant differences in the treatments with an average concentration of 0.10 mg $gDW^{-1}$ (Fig. 48D).

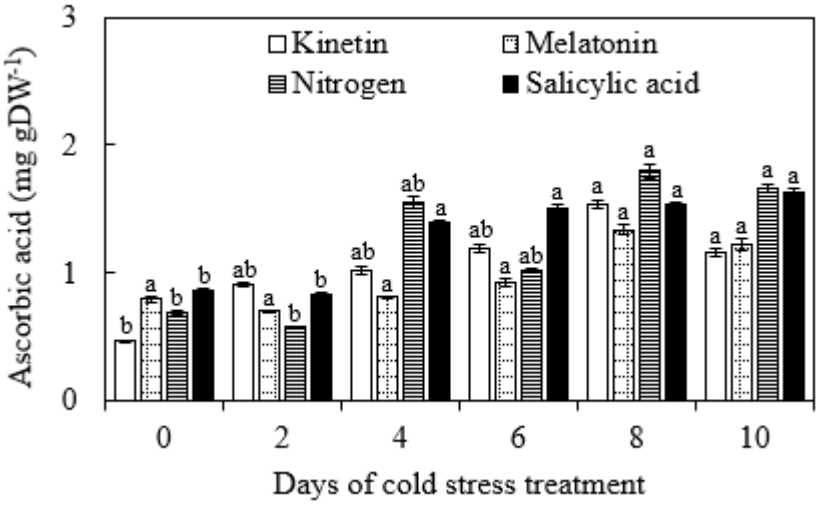


Fig. 47. Effect of ROS/RBOH on the concentration of ascorbic acid in the leaves of coffee plants. Different letters indicate significant differences amongst the temperature treatments for each exogenous treatment ($p \leq 0.05$). Error bars indicate standard error of the mean, n = 4. Cold stress conditions (12/12°C) were instigated 24 hours after the last foliar treatment which were all applied during optimum conditions (25/20°C, day and night, respectively).

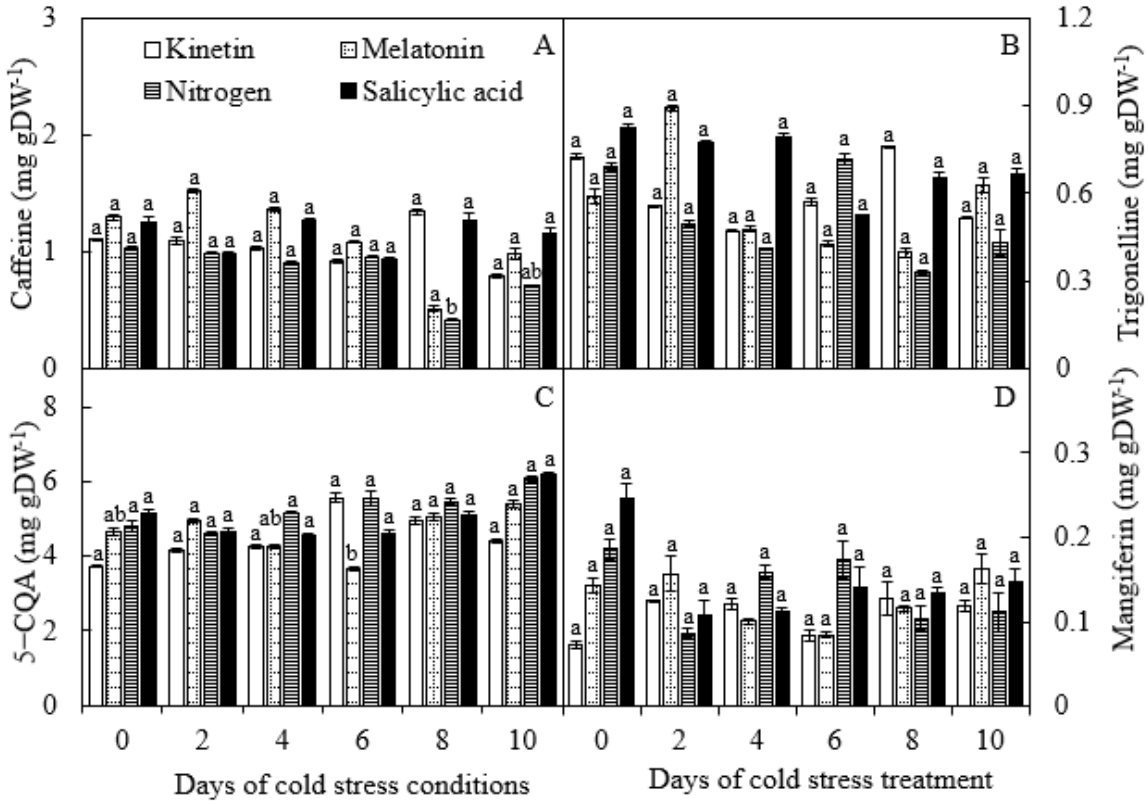


Fig. 48. Effect of ROS/RBOH on the concentration of selected HPLC metabolites. Different letters indicate significant differences amongst the temperature treatments for each exogenous treatment ($p \leq 0.05$). Error bars indicate standard error of the mean, n = 4. Cold stress conditions (12/12°C) were instigated 24 hours after the last foliar treatment which were all applied during optimum conditions (25/20°C, day and night, respectively).

*7.3.2.3 Flavonoids and Flavonols*

Variation in the concentration of flavonoids and flavonols in the leaves amongst the treatments with progression of cold stress conditions is indicated in Fig. 49. Both flavonoids and flavonols showed neither significant variation nor peculiar trend in variation amongst the foliar treatments and on instigation or progression of cold stress conditions. Nevertheless, on average, the concentrations of flavonoids were 14, 17, 16 and 18 mg RE $gDW^{-1}$ in plants under kinetin, melatonin, nitrogen and salicylic acid treatments whereas that of flavonols was about 14 mg RE $gDW^{-1}$ in all the treatments (Fig. 49A and B, respectively).

*7.3.2.4 Total phenolic content*

The concentration of total phenolic content in the leaves as affected by different exogenous treatments and progression of cold stress conditions is shown in Fig. 50. Generally, TPC showed no significant variation amongst treatments and or with progression of cold stress conditions. Within the treatments, TPC was highest at 137 mg GAE $gDW^{-1}$ in plants under salicylic acid followed by those under nitrogen at 133 mg GAE $gDW^{-1}$. TPC was lowest in plants under kinetin and melatonin treatments at 124 and 128 mg GAE $gDW^{-1}$, respectively. On the other hand, TPC was highest at day and 8 and 10 after instigating cold stress conditions at 137 and 133 mg GAE $gDW^{-1}$ while the previous days stimulated low concentration of TPC with an average of 129 mg GAE $gDW^{-1}$.

***ROS damage and signalling during cold stress conditions***

The relationship between the ROS induced damage of the cellular membrane assessed as ELI and the assayed antioxidant compounds is indicated in Table 26. Amongst all the antioxidant compounds, ASC showed the strongest positive correlation with ELP at 0. 74, 0.66, 0.55 and 0.4 in the salicylic acid, kinetin, nitrogen and melatonin treatments, respectively. Although the correlation for the other compounds was low, TPC indicated a positive correlation with ELP at 0.46 in the nitrogen treatment unlike other treatments which indicated a low correlation between the compounds and ELP.

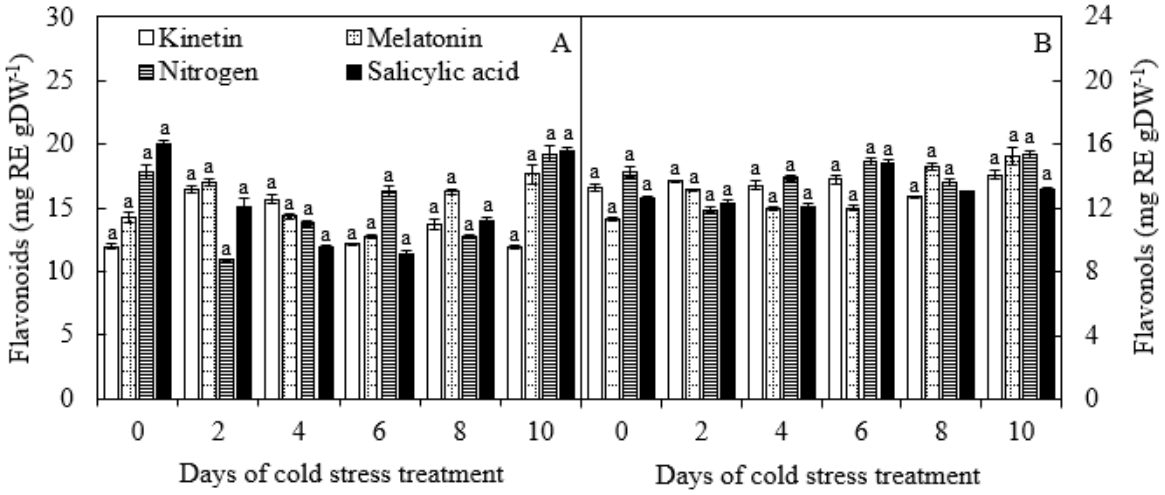


Fig. 49. Effect of ROS/RBOH on the concentration of flavonoids (A) and flavonols (B). Different letters indicate significant differences amongst the temperature treatments for each exogenous treatment ($p \leq 0.05$). Error bars indicate standard error of the mean, n = 4. Cold stress conditions (12/12°C) were instigated 24 hours after the last foliar treatment which were all applied during optimum conditions (25/20°C, day and night, respectively).

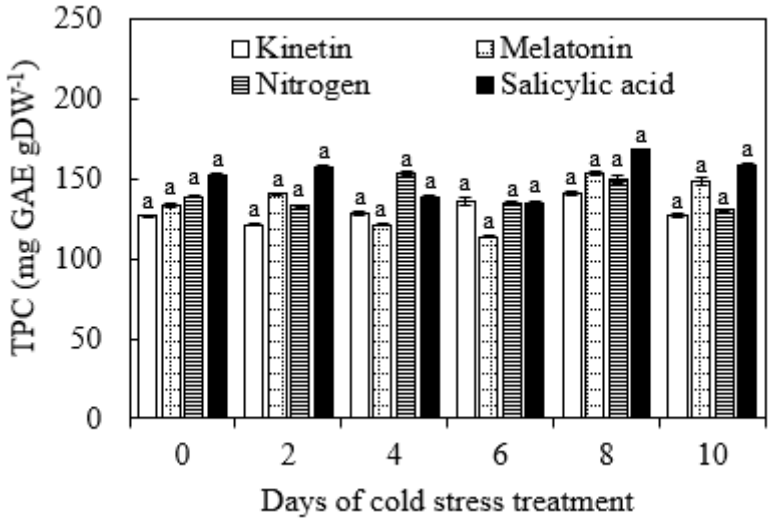


Fig. 50 Effect of ROS/RBOH on the concentration of total phenolic compounds. Different letters indicate significant differences amongst the temperature treatments for each exogenous treatment ($p \leq 0.05$). Error bars indicate standard error of the mean, n = 4. Cold stress conditions (12/12°C) were instigated 24 hours after the last foliar treatment which were all applied during optimum conditions (25/20°C, day and night, respectively).

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Table 26. Pearson correlation co−efficient ($r^2$) of the electrolyte leakage index and the contents of the compounds in the leaves of coffee plants during cold stress conditions as affected by ROS/RBOH initiation

| Analyte | Nitrogen | Salicylic acid | Melatonin | Kinetin |
|---|---|---|---|---|
| Trigonelline | −.21 | −.28 | .03 | −.42* |
| Caffeine | −.30 | .22 | .02 | −.43* |
| Mangiferin | −.18 | −.42* | .25 | −.05 |
| 5–caffeoylquinic acid | −.04 | .06 | −.11 | .25 |
| Ascorbic acid | .55** | .74** | .41* | .66** |
| Flavonoids | −.09 | −.42* | −.15 | −.07 |
| Flavonols | .13 | .02 | .14 | −.00 |
| TPC | .46* | .14 | −.12 | .37 |

**. Correlation is significant at the 0.01 level (2−tailed).
*. Correlation is significant at the 0.05 level (2−tailed).

## 7.4 Discussion

ROS have become an integral part of many living organisms due to the abundance of $O_2$ in the atmosphere. Although produced under normal cellular metabolism, excessive production of ROS occurs during abiotic stress conditions such as low temperature often resulting into a condition known as oxidative stress (Mittler, 2002). For coffee plants, cold stress conditions are prompted when the ambient temperature conditions fall below 18°C (Ramalho *et al.,* 2014). Subjection of the coffee plants to 12/12°C therefore, increased the cellular ROS by reducing the photosynthetic capacity causing a mismatch between energy capture and C–assimilation (Asada, 2006). This led to erroneous energy transfer to chlorophylls triggering their excitation into $^3$Chl* and $^1$Chl as well as formation of $^1O_2$ and $O_2^{\bullet-}$ in the PSI and PSII (Khorobrykh *et al.,* 2020). Moreover, ROS are also generated at other sites such as mitochondria and peroxisomes due to presence of several electron transport reactions (Lismont *et al.,* 2015). By SOD, the formed $O_2^{\bullet-}$ dismutases to $H_2O_2$ which is partly neutralised to $H_2O$ by APX and CAT. However, via the Fenton reaction, the remaining $H_2O_2$ forms the highly toxic $OH^{\bullet-}$ which despite its short life span, reacts readily with cellular membrane casing their rapid disintegration. The results of the current study indicated an increase in ELP within 4 days after instigation of cold stress conditions (Fig. 46). This agrees with previous results were subjection to cold stress conditions caused a spike in the ELP in the leaves of the coffee plants.

However, after reaching a maximum by day 6 of cold stress conditions, ELP indicated a plateau despite a further progression of cold stress conditions. This coincided with elevated amounts of antioxidant compounds especially ascorbic acid (Fig. 47) and to a less extent 5–caffeoylquinic acid while generally, the other assayed compounds showed nonsignificant response (Fig. 48). Ascorbic acid a powerful antioxidant compound with very high free radical scavenging activity and therefore forms the most immediate response during oxidative stress conditions (Wang *et al.,* 2019). On the other hand, phenolic compounds like 5–caffeoylquinic acids, mangiferin, flavonoids and flavonols constitute the secondary response given their slow accumulation during oxidative conditions (Fig. 48, 49 and 50 respectively).

Nevertheless, despite the reinforced nonenzymatic antioxidant system, cold stress conditions slowed down the metabolism of nitrogen which was evidenced by a consistent decline in the nitrogenous compound such as N−methyl nicotinate (trigonelline) (Perchat, 2018). The results of the current study therefore suggest that, the $H_2O_2$ formed by ROH and RBOH and its subsequent involvement in the signal transduction pathway causes various effects on coffee

plants secondary metabolism (Mittler, 2017). While the metabolism of antioxidant compounds such as ascorbic acid and phenolic compounds are promoted under such conditions, the $H_2O_2$ induced change in the protein structures might slow down the metabolism of some compounds such as trigonelline as indicated in Table 26.

### 7.5 Conclusion

Excessive production of ROS instigated by cold stress conditions caused membrane damaged and supressed the metabolism of nitrogen containing compounds especially trigonelline. However, accumulation of ROS initiated by both cold stress and RBOH also triggered nonenzymatic antioxidant protective mechanism which led to increase in the levels of especially ascorbic acid and 5–caffeoylquinic acid which restored ROS to homeostatic levels and thereby preventing further oxidation of the cellular membrane.

## Chapter eight

## General discussion

*Coffea* species contain several phytochemicals such as caffeine, trigonelline, chlorogenic acids, mangiferin, sucrose (Fig. 3) which render their wide exploitation for pharmacological and health promoting benefits (Patay *et al.,* 2016). The current study revealed that raw beans contained the highest amounts of the phytochemicals especially 5–CQA, sucrose, caffeine and trigonelline. Coffee seeds remain the most important organ in coffee trade because of their extensive use in the coffee beverage processing (Pereira *et al.,* 2019). Phytochemicals in seeds accumulate as a result of metabolism within the fleshy parts of the fruits during maturation but also due to deposition having been processed from leaves and young buds of the coffee plant (Farah and Donangelo, 2006; Koshiro *et al.,* 2006; Koshiro *et al.,* 2007; Farah *et al.,* 2008). Coffee seeds contain mainly carbohydrates, sucrose being the main constituent whose role is to provide nourishment for the embryo in case the seeds germinate (Xu *et al.,* 2010). Also, similar to the findings in the current study (Table 2 and 4), high amounts of phenolic compounds have been reported in the coffee fruits and seeds. Chlorogenic acids are the main phenolic compounds that accumulate in the beans during maturation of coffee fruits and seeds (Farah and Donangelo, 2006; Farah *et al.,* 2008). Of these, 5–CQA forms the main constituent of these hydroxycinnamic acid esters (Campa *et al.,* 2012).

Leaves are associated with high rates of metabolism due to their role in photosynthesis. For this, they contain chlorophylls to facilitate photosynthetic activity. The concentration of total chlorophylls in the leaves or proportions of their respective types (*a* and *b*) varies with leaf age or position. Chlorophyll *a* is normally highest in the youngest leaves whereas chlorophyll *b* is highest in mature leaves. The latter is normally found in the reaction centres of photosystem I, II and in the pigment antenna system whereas the former is found only in the pigment antenna system (Lichtenthaler and Buschmann, 2001). In the current study, there was a general increase in the concentration of the chlorophyll *b* in the older leaves (Table 3). It is suggested that this is meant to maximize light capture because of the quaternary arrangement of leaves on the orthotropic stem, which dictates older leaves to receive less incident light than their younger counterparts (Sakiyama *et al.,* 2017). Chlorophylls normally are unable to utilize all the photosynthetically active radiation (PAR) and therefore plants have evolved mechanisms to avoid or detoxify ROS that result from excess excitation energy. In addition to energy evasion, by accumulating less amounts of chlorophylls (Revatipadale *et al.,* 2019), leaves contain

carotenoids which serves to protect the chlorophylls against oxidative stresses (Wangcharoen and Phimphilai, 2016). These pigments have also been reported to contribute to health benefits such as decreasing disease risk due to their high antioxidant activities when consumed (Wangcharoen and Phimphilai, 2016; Fiedor and Burda, 2014). Presence of high amounts of other phytochemicals such as alkaloids, phenolic compounds and sugars have also been reported in the coffee leaves (Talamond *et al.,* 2008; Campa *et al.,* 2012; Chen *et al.,* 2018). Our results also showed similar findings especially in the youngest leaves. Like carotenoids, these compounds protect the leaves against ROS that are by−products of aerobic metabolism more so in the young leaves (Mittler, 2002; Asada, 1999; Ende and Valluru; 2009). These compounds normally compliment the enzymatic defence system in detoxifying the ROS (Brewer, 2011). It has recently been shown that unlike the older counterparts, young coffee leaves have a poorly developed enzymatic antioxidant defence system and hence the reliance on oxidant scavenger compounds is inevitable (Das and Roychoudhury, 2014; Ramalho *et al.,* 2018). In addition to defence, some phytochemicals in the current study have other functions in coffee plants. Sucrose, a highly soluble disaccharide is synthesized in the leaf cytosol and hence its accumulation is directly related to photosynthesis (Lunn *et al.,* 2016). By virtue of their position, the youngest leaves accumulated the highest content of sucrose which reduced with leaf maturity (Table 3). It is also a storage reservoir molecule and a transportation solute which is readily broken down to provide energy for growth and other cellular functions (Hammond and White, 2008).

Biosynthesis of these phytochemicals is normally limited to specific organs. Phenolic compounds accumulation mainly occurs via the phenylpropanoid biosynthetic pathway (Sreekumar and Soniya, 2017). However, just like in Campa *et al.* (2012), this study found no correlation between chlorogenic acids (5–CQA) and mangiferin accumulation in the plant organs (Table 5). This is owed to the absence of metabolite competition for the two phenolic compounds and the silencing of the gene that encodes 3–ketoacyl–CoA thiolase (PhKAT1) protein which catalyses the committed step for benzoic acid production in the benzenoid biosynthetic pathway (Moerkercke *et al.,* 2009) from which mangiferin biosynthesis proceeds. Moreover, unlike chlorogenic acids that are distributed in all organs of the coffee plant (Aerts and Baumann, 1994), recent reports have only reported presence of mangiferin only in the photosynthetic tissues of the coffee leaves and the receptacle of the young fruits of arabica coffee, which is in agreement with our findings (Campa *et al.,* 2012).

Although it was earlier suggested that trigonelline too acts as a chemical defence against herbivory (Shimizu and Mazzafera, 2000), recent reports have suggested that trigonelline biosynthesis results from detoxification of excess nicotinic acid and therefore is reconverted to the required substrate whenever the need for NAD biosynthesis arises (Ashihara and Watanabe, 2014). Moreover, trigonelline accumulation was rather almost equally distributed in all the plant organs especially those with active meristems. Ours results agree with Ashihara and Watanabe (2014) who have also reported presence of trigonelline in all coffee plant organs with higher amounts especially in the upper stem and relatively lower amounts in the roots. Metabolism of the two alkaloid compounds occurs through two pathways; the *de novo* pathway and the salvage pathway. These two pathways for the alkaloids have been reported in the occur simultaneously in the youngest buds and expanding leaves hence resulting into high accumulation of alkaloids in such organs.

On the other hand, the mature leaves contain only the salvage pathway which is further constrained by reduced endogenous supply of the necessary substrates during biosynthesis (Zheng and Ashihara, 2004). Caffeine and trigonelline are degraded by demethylation into xanthine and nicotinic acid in mature plant organs. Our results suggest that caffeine degradation could be occurring at higher rates compared to that of trigonelline hence a higher degradation percentage in BL due to loss in biological value in dried leaves (Heaton and Marangoni, 1996; Zheng and Ashihara, 2004). The pattern of biosynthesis and accumulation of the two main alkaloid compounds; caffeine and trigonelline in coffee seeds especially the pericarp follows a similar trend (Koshiro *et al.,* 2006). It has been reported that, largely the two alkaloids are biosynthesized elsewhere and transported to the fruits and the seeds during maturation (Koshiro *et al.,* 2006). Therefore, the difference in caffeine and trigonelline content in the seeds corresponded with the difference in the youngest leaves which are the main sites of alkaloid biosynthesis.

Accumulation of the phytochemicals in coffee plants is highly dependent on the ambient environmental conditions that are influenced by diel or seasonal changes. The circadian clock regulates several physiological and metabolic plant processes through modulating the relative expression of associated genes (Johnson *et al.,* 2003; Shin *et al.*, 2017). Light generally switches on genes that regulate photosynthetic activity in chloroplasts as the main activity during daytime (Sulpice *et al.,* 2014; Moraes *et al.,* 2019). In the chloroplasts, photosynthesis is carried out by the two types of chlorophyll molecules *a* and *b* that are normally localized in

the photosystems in the thylakoid membrane (Porra *et al.,* 1989). Chlorophylls molecules execute the role of light capture and its subsequent utilization for starch metabolism in a process that result into photo–degradation of the pigments hence resulting into low chlorophyll concentration during the day (Rontani *et al.,* 1996; Lee *et al.,* 2014). However, leaves contain protective carotenoids which dissipate excess excitation during daylight thereby protecting the chlorophylls.

In many production areas, temperatures as high as 30$^{o}$C are not uncommon especially during summer (Damatta and Ramalho, 2006). Although high temperatures may translate in to rapid metabolism, prolonged exposure may result into uncoupling of several physiological and metabolic processes culminating into depressed growth (Awasthi *et al.,* 2015). In fact, it was earlier demonstrated that temperature above 24$^{o}$C may lead mesophyll resistance resulting in to diminished leaf conductance and photosynthetic productivity (Kumar and Tieszen, 1980). Moreover, extremely high temperatures in in coffee plants were associated with low photosynthetic rates especially in young leaves (Marias *et al.,* 2017; Rodrigues *et al.*, 2018). This is also caused by low photosynthetic efficiency which results in to production of ROS whose homeostatic regulation is crucial for survival under abiotic stress (Demmig−Adams and Adams, 2004). Summer conditions were associated with high pigments concentration which could have been an adaptive measure to support high metabolic rates (Table 6). Coffee plants exhibit some degree of plasticity and have been reported to adapt to relatively unfavourable environmental conditions (Damatta and Ramalho, 2006; Ramalho *et al.,* 2014). Higher temperatures in the summer also elicited high amounts the nonenzymatic antioxidant compounds especially 5–CQA resulting in to higher 5–CQA/caffeine ratio hence enhancing their ROS scavenging capacity (Ky *et al.*, 2001).

The month of October was associated with a remarkable drop in the ambient temperatures resulting into a typical autumn cool season through to mid−November after which temperature dropped further signifying the start of winter (Table 6 and 7). During cold temperature condition such as in winter coffee plants produce excessive amounts ROS due to leakage of electrons to oxygen molecule in the chloroplast and mitochondrial electron transport system (Mittler, 2002). During such abiotic stress conditions, the heightened increase in the production of these molecules increases the probability of erroneous energy transfers within the photosynthetic machinery leading to breaching of the pigments such as those in $P_{680}$ and D1 protein sub unit (Damatta and Ramalho, 2006). In the current study, this was evidenced by

rapid disintegration of the pigments in the winter months which were associated with low positive temperatures (Table 6). Furthermore, low concentration of photosynthetic pigments leads to increased production of ROS due to reduction in the efficiency of the photosynthetic machinery as observed by a decrease in the chlorophyll to carotenoid ratio (Porra *et al.,* 1989) similar to the observation in the current study (Table 7). Similarly, the content of antioxidant compounds especially 5–CQA which had reduced during autumn once again increased at the onset of colder conditions during winter as an adaptative measure against excessive ROS accumulation.

The photosynthetic apparatus in the leaves of arabica coffee plants is adapted to operate efficiently between photosynthetically active radiation (PAR) 300–700 $\mu mol^{-2}$ $s^{-1}$ (Clifford, 2012; Ramalho *et al., 2*014). High light intensity causes excess excitation energy resulting into inability of the photosynthetic apparatus to utilize all the incident PAR (Muller 2001). In the current study, sudden exposure of low light acclimated coffee plants to PAR 1800µmol $m^{-2}$ $s^{-1}$ was accompanied with progressive reduction in the concentration of chlorophyll molecules (Fig. 7). It has been suggested that this is an adaptive measure to reduce the amount of absorbed light energy hence reducing the likelihood of excess energy which would otherwise lead to accumulation of ROS and cause oxidative stress (Ruban, 2016). Nevertheless, the excess excitation energy in the current study caused damage to the photosynthetic apparatus which was also associated with increased production of the protective carotenoid molecules (Fig. 7) which is another photoprotective mechanism. In addition, higher amounts of antioxidant compounds were observed in sunlit plants compared to shaded counterparts (Fig. 9) for the same purpose (Das and Roychoudhury, 2014).

During winter, the coffee plants used for this experiment had developed visual symptoms of cold stress conditions as a result of low temperatures from October as indicated in the previous chapter (Fig. 5). Characteristically, the coffee plants had pale yellow leaves and stunted growth which is normally associated with low concentrations of chlorophyll pigments (Fig. 11). Foliar nitrogen sprays elevated the leaf nitrogen status to levels above 23 mg $DW^{-1}$ (Table 11), a threshold under which nitrogen deficiency symptoms are reported to occur in coffee plants (Pompelli *et al.,* 2010). The control treatment plants on the other hand, were still N deficient at the end of the experiment and consequently accumulated less amounts of chlorophylls and carotenoid pigments compared to the nitrogen supply treatments whose pigment concentration increased by 75, and 54%, respectively with increasing nitrogen concentration up to 20 mM

(Table 11). Low nitrogen nutrition generally results into low contents of both chlorophylls and carotenoids, with the pigments increasing by over 135 and 125%, respectively, when coffee plants were fertilized with 23 mM N twice every after 2 weeks (Pompelli *et al.,* 2010). Nevertheless, in this study, high nitrogen concentration in the 40 mM treatment was associated with a remarked reduction in the concentration of the pigments. This is plausibly due to the burning effect associated with high concentration of foliar urea sprays. The negative effects of high concentration urea application on plant metabolism has been reported to result from accumulation of urea in plant tissues to toxic levels rather its conversion to toxic substrates such as ammonia (Krogmeier *et al.,* 1989). Therefore, at 40 mM, urea application was above the threshold and therefore its toxicity supressed plant metabolism although no burns were visually observed. Nonetheless, nitrogen supply in an appropriate concentration from foliar urea can be readily assimilated through combining with carbonyl chains form carbohydrates to form proteins and other important molecules such as chlorophylls and carotenoids (Carelli *et al*, 2006; Pompelli *et al.,* 2010) similar to observations made in the current study.

The low contents of photosynthetic pigments in the control plants were associated with low rates of gas exchange (Fig. 12A–D). The seedlings used in the current study had been raised under a shaded vinyl house with irradiance levels similar to those used in the growth chamber (250 µmol $m^{-2}$ $s^{-1}$ PPFD). The current results agree with Praxedes *et al.* (2006) and Pompelli *et al.* (2010) who reported low net $CO_2$ assimilation in plants with low concentration nitrogen and photosynthetic pigments. Chlorophylls play a vital role in both absorption and conversion of sunlight energy into chemical energy. In the current study, a consistent increase in the chlorophyll concentration in the nitrogen treated plants was accompanied with high rates of $CO_2$ assimilation (Fig. 12A). Stomatal conductance also tended to be highest in plants with high $CO_2$ assimilation rates suggesting a positive effect of the improved water status in the coffee plants as a result of foliar nitrogen treatment on photosynthesis in general (Gimenez *et al.,* 2005). Moreover, compared to control plants, nitrogen treatment improved $WUE_i$ indicating a higher biomass gain per unit water loss that occurs through transpiration (Hartfield and Dold, 2019). Control plants also indicated the highest $C_i/C_a$ ratio compared to nitrogen treated plants (Fig. 12D). This was possibly due to the inability of the mesophyll cells to assimilate the absorbed $CO_2$ in the intercellular air spaces (Farquhar *et al.,* 1980). This phenomenon could have been induced by several biochemical limitations such as low photosynthetic enzymatic activities that were greatly improved by foliar nitrogen concentrations of up to 20 mM (Sun *et al.,* 2016). On the other hand, plants under 40 mM had

low rates of gas exchange (Fig. 12A–D), empathizing the negative effects caused by high nitrogen concentration in this treatment. Similar results were obtained in coffee plants under conventional nitrogen supply (Bote *et al.,* 2018) and in apple trees where plants grown under adequate nitrogen supply maintained higher photosynthetic rates even at low temperatures compared to those growing under soil nitrogen deficient conditions (Greer, 2008).

The trend in the $CO_2$ assimilation rate was further supported by corresponding variations in the PSII photochemistry (Table 12). Both $\Phi_{PSII}$ and ETR showed similar quadratic variation amongst the treatments being lower in the both control and 40 mM plants whereas low concentration nitrogen treated plants had moderate values. The current results suggest therefore, the PSII photochemical efficiency during steady−state photosynthesis tended to be downregulated in the photosynthetically depressed plants as a mechanism of photoprotection which would otherwise result into photoinhibition due to an inefficient photosynthetic apparatus (Maxwell and Johnson, 2000; Murchie and Lawson, 2013). Moreover, control plants had both the highest qP and NPQ with the two parameters reducing with increasing concentrations in the nitrogen treatments (Table 12). Nonetheless, a slight increase in the NPQ in plants under 40 mM indicated plausible damages to the photosynthetic apparatus resulting from the toxicity of the urea application in high concentrations. Moreover, plants under control and 40 mM treatments tended to close larger proportions of the PSII traps which otherwise could have caused photoinhibition due to biochemical limitations for high photosynthetic rates (Table 12). On the contrary, plants under moderate nitrogen concentration treatments had higher $q_L$ hence facilitating the flow of electrons from PSI to PSII, which resulted into higher photosynthetic rates (Murchie and Lawson, 2013).

The maximum quantum efficiency of PSII under both dark and light conditions was also lowest in the control and 40 mM treated plants (Fig. 13A–D). Both $F_v/F_m$ and $F_v/F_o$ for both the dark and the light−adapted states ($F_v'/F_m'$ and $F_v'/F_o'$) are indicators of the degree of photoinhibition in PSII (Adams *et al.,* 1995; Maxwell and Johnson, 2000; Demmig-Adams and Adams, 2004). The current results agree with Pompelli *et al.* (2010) who also reported lower $F_v/F_m$ ratio in low N coffee plants especially under high light conditions. Such low $F_v/F_m$ ratios have also been reported in coffee plants after exposure to cold stress conditions (Batista−Santos *et al.,* 2011; Guo and Cao, 2004). This decline in the maximum quantum efficiency is attributed to downregulation of PSII due to photoprotective energy dissipation which is associated with nocturnal retention and diurnal build−up of zeaxanthin in the xanthophyll cycle (Ramalho *et al.,* 2003; Partelli *et al.,* 2009; Pompelli *et al.,* 2010; Batista−Santos *et al.,* 2011). This defence

mechanism is however associated with inactive reaction centres and competes for energy with PSII photochemistry hence reducing its photochemical efficiency (Partelli *et al.,* 2009).

Coffee plants have an extensive mechanism of photoprotection which can be enhanced by adequate nutrient supply, particularly nitrogen (Pompelli *et al.,* 2010). Previous reports have indicated that this defence mechanism involves the development of an elaborated antioxidant system comprising of both enzymatic and nonenzymatic antioxidant components (Pompelli *et al.,* 2010; Ramalho *et al.,* 2014; Campa *et al.,* 2017; Ramalho *et al.,* 2018). The current results have shown that in absence of an efficient photosynthetic system such as in control treatment plants, coffee plants accumulate relatively higher amounts of the secondary metabolites except caffeine (Table 13). Coffee plants contain two main alkaloids compounds whose role in antioxidant defence are still well unknown (Ashihara, 2006). Accumulation of caffeine has been more associated with defence against herbivory, whereas trigonelline accumulates as a consequence of detoxification of nicotinic acid, a reservoir for biosynthesis of nicotinamide adenine dinucleotide (Shimizu and Mazzafera, 2000). High contents of trigonelline (up to 0.61 mg $gDW^{-1}$) in the control plants compared to other treatments were observed in the current study. This is in consistence with Garg (2006) who recently indicated that trigonelline may have antioxidant roles in plant cells.

The main hydroxycinnamic acid ester, 5−CQA (Campa *et al.,* 2012) and the xanthonoid, mangiferin were also highest in the control plants and decreasing in the nitrogen supply treatments (Table 13). The two phenolic compounds have been found to increase directly in response to oxidative stresses induced by high light intensities and cold stress conditions to which the coffee plant is not adapted to (Ramalho et al. 2014; Campa *et al.,* 2017). The current results indicated a higher ratio of 5−CQA to caffeine in the photosynthetically limited plants compared to those treated with foliar sprays of nitrogen (Table 13). Chlorogenic acids such as 5−CQA have a higher metabolic plasticity compared to caffeine and act as storage forms of cinnamic acid derivatives which are used for lignification during cell wall development (Aerts and Baumann, 1994). Therefore, the decline in the 5−CQA concentration could be attributed to a higher need for cell wall development than defence against ROS in plants whose photosynthetic apparatus had been recovered by foliar nitrogen treatment. Yildirim *et al.* (2007) also observed a decline in ascorbic acid, a well−known antioxidant compound in nitrogen treated broccoli plants compared to those under control conditions.

Abiotic stresses including cold stress reduce the $CO_2$ fixation capacity, which is normally associated with a low net $CO_2$ assimilation rate, stomatal conductance and transpiration rate in plants (Demmig-Adams and Adams 2004). Subjecting coffee plants to 12°C in the current study induced cold stress conditions which were associated with low gas exchanges, hence inducing diffusive limitations to photosynthetic productivity in coffee plants (Table 14). Similar effects of cold stress conditions on the photosynthetic physiology of coffee plants have been reported (Pompelli *et al.,* 2010; Ramalho *et al.,* 2014). These limitations are accompanied by a reduction in the PSII operating efficiency, resulting from the breakdown of the photosynthetic apparatus under cold stress conditions (Havaux *et al.,* 2000). This agrees with the observations made in the current study, where cold−stressed plants had the least values of $\Phi_{PSII}$, which was associated with low electron transport rates (Table 15). The reduction in the $CO_2$ fixation capacity, together with low PSII operating efficiency, result in an over production of reactive oxygen from any given incident light (Lima *et al.,* 2002). This normally results in a phenomenon known as photoinhibition, when excess amounts of ROS are produced in the antenna pigments of the chloroplasts by the Mehler reaction (Asada, 1999; Kovtun *et al.,* 2000). An overproduction of ROS in the photosynthetic apparatus typical under oxidative stress conditions causes a severe reduction in both the effective and maximum quantum yield of PSII (Verhoeven *et al.,* 1997). This agrees with the results of the current study, where cold−stressed plants had the low values of quantum yield in the dark and light−adapted chlorophyll states (Table 16). Nevertheless, the exogenous application of kinetin tended to modulate the dampening effects of cold stress on the PSII operating efficiency, hence resulting in improved gas exchange, which was accompanied with higher $\Phi_{PSII}$, ETR and an improved quantum efficiency in kinetin−treated plants compared to the non−treated plants under cold stress conditions (Tables 14, 15 and 16).

Taken together, our results agree with a number of studies which reported the positive effects of exogenous kinetin treatment on the photosynthetic machinery (Ahanger *et al.,* 2018; Moura *et al.,* 2018). Although the protective roles of kinetin are concentration dependent, similar to the concentration used in the current study, Moura *et al.* (2018) reported that 0.35 mM (75 mg $L^{-1}$) effectively improved the carboxylation efficiency, with corresponding effects on the photosynthetic apparatus. It has been reported that kinetin induces stomatal opening, hence encouraging gas exchange with associated net $CO_2$ assimilation under stress conditions (Pharmawati *et al.,* 1998). This is associated with both the protective effects of kinetin on the functioning of the photosynthetic light reaction and the functional enzymes of PSII and PSI,

such as the protein gradient regulation−5 (PGR−5) protein, as well as maintaining a pH gradient for a smooth electron flow from PSII to PSI (Tikkanen *et al.,* 2015).

Our results further indicated that exposure to cold stress conditions had a negative impact on the membrane stability in the leaves of the coffee plants (Fig. 14). This could be attributed to an increased production of ROS in the chloroplasts, as well as other organelles with an electron chain system, such as the mitochondria and peroxisomes (Del Rio *et al.,* 2002). ROS are normally produced by plants under optimal conditions, in this case acting as signaling and transduction molecules for normal cellular metabolism (Mittler, 2002; Mittler, 2017). However, their amplified production during oxidative stress conditions results in a disruption of the normal cellular homeostasis, leading to membrane lipid peroxidation characterized by the leakage of cellular electrolytes, protein oxidation and enzyme inhibition, as well as the disintegration of deoxyribonucleic acid and ribonucleic acid (Mittler, 2002; Gill and Tuteja, 2010). Cold stress conditions also resulted in the breakdown of chlorophyll molecules (Fig. 15). The accumulation of high amounts of ROS in the photosynthetic machinery particularly breaks down the D1 protein subunit of the PSII reaction center, leading to the oxidation and bleaching of chlorophyll molecules (Asada, 2006; Roach and Krieger-Liszkay, 2014).

Furthermore, in the current study, besides increasing carotenoid content under optimum conditions, kinetin application elicited contrasting results to the prior mentioned studies. Kinetin application tended to increase membrane damage, as well as the disintegration of chlorophyll molecules (Fig. 14 and 15, respectively). This could be related to the kinetin mechanism of action after exogenous application, through which it increases nicotinamide adenine dinucleotide activity, leading to the generation of $H_2O_2$, which acts as a signaling molecule for antioxidant defense (Gangwar *et al.,* 2014). Although the short−term increase in the content of the ROS is associated with increased cellular damage, it might substantially contribute to the overall enhancement of the antioxidant defense system, with improved tolerance to abiotic stress conditions in the long run. On the other hand, the up−regulatory effect of kinetin on carotenoids metabolism also coincides with the metabolism of polysaccharides and proteins of the photosynthetic apparatus, and this in fact causes the stimulation of the entire biogenesis of chloroplasts (Choudhury and Choe, 1996; Bris, 2017).

The kinetin–hydrogen peroxide−mediated reinforcement of the antioxidant system is supported by high amounts of antioxidant compounds analyzed in the current study (Fig. 16 – 18). The current results are in agreement with several reports were exogenous kinetin treatment

increased the tolerance of plants to abiotic stress conditions through the increased activities of antioxidant enzymes (Wang *et al.,* 2015; Ahanger *et al.,* 2018; Kaya *et al.,* 2018; Singh *et al.,* 2019), as well as the increased contents of several antioxidant compounds, such as flavonoids, gibberellins, salicylic acid, jasmonic acid and abscisic acid in salinity−stressed soybean plants (Hamayun *et al.,* 2015), proline in drought−stressed rice (Jalaluddin *et al.,* 2015), salvianolic acid and rosmarinic acid in *Dracocephalum forrestii* (Weremczuk-Jezyna *et al.,* 2018). Although the accumulation of several secondary metabolites in the leaves of coffee plants under oxidative stress conditions is a well−known phenomenon (Ramalho *et al.,* 2018; Campa *et al.,* 2017),

In addition, it has been reported that kinetin scavenges free oxygen radicals by directly neutralizing ROS using the hydrogen from the α−carbon of the amine bond of $N^6$−furfuryladenine. Kinetin also reacts with copper, forming a Cu (II)−kinetin complex, which encourages a faster dismutation of the radical oxygen species in solution (Barciszewski *et al.,* 1999), hence contributing to the antioxidative capacity of plants. Taken together, the exogenous foliar application of kinetin increased the ROS scavenging capacities in the treated plants (Table 17). This increase was more ostensibly indicated by the FRAP assay, whereas DPPH and ABTS, despite showing increment tendencies with kinetin treatment, were not affected significantly amongst the treatments. Although the assays employ different principles in the determination of radical scavenging values, they normally report consistent values (Chen *et al.,* 2018; Alvarez-Jubete *et al,* 2010), as was observed in the current study.

Similarly, exogenous application of salicylic acid has been reported to alleviate the dampening of a number of physiological and metabolic plant process resulting from abiotic stress (Rivas−San Vicente and Plasencia, 2011). In the current study, induction of cold temperatures resulted into significant membrane damages as assessed by the leakage of cellular electrolytes in both Cold and Cold + SA treatments plants (Fig. 19). Nevertheless, although non–significantly, exogenous application of salicylic acid tended to reduce the extent of membrane damage in the leaves of coffee plants by 3%. This could be as a result of accumulation of slightly less amounts of ROS in the salicylic acid treated plants under cold conditions hence causing less damage to cellular membrane and leakage of cellular components (Mittler, 2002; Shin *et al.,* 2008). Moreover, this protective effect of salicylic acid on cold stressed coffee plants was accompanied by significantly higher contents of nitrogen compared to cold treatment plants (Fig. 20). Application of exogenous salicylic acid has been reported to protect

the degradation of proteins through enhancement of transcription of psbA gene which encodes the D1 protein subunit of PSII complex (Wang *et al.,* 2014). Similarly, although cold stress induction resulted into disintegration of chlorophylls pigments, exogenous application of salicylic acid slowed down this breakdown (Fig. 21). The current results are consistent with findings in similar work where salicylic acid treated plants contained higher amounts of chlorophyll pigments compared to non–treated plants under abiotic stress conditions (Wang *et al.,* 2014; Tang *et al.,* 2017). The results of the current study indicated that exogenous salicylic acid under optimum conditions upregulated the metabolism of carotenoids molecules resulting into significantly highest content of these protective molecules.

In the current study, instigation of cold stress conditions resulted into repression of several gas exchange parameters including net carbon dioxide assimilation ($P_N$), stomatal conductance ($g_s$) and transpiration rate (Trmmol) as indicated in Fig. 22. This could be as a result of higher contents of ROS as also indicated by higher electrolyte index and disintegration of pigment molecules compared to plants under optimum conditions. Plants normally respond to abiotic stress conditions by modulating stomatal closure hence affecting gaseous exchange parameters (Daszkowska−Golec and Szarejko, 2013; Kollist *et al.,* 2019). Exogenous application of salicylic acid under optimum conditions was associated with depressed $P_N$, $g_s$ and Trmmol (Fig. 22). Similar findings have been reported regarding salicylic acid induced reduction in Pn, gs, E and Ci under optimum conditions which could be attributed to salicylic mediated stomatal closure (Tang *et al.,* 2017). Conversely, under cold stress conditions, salicylic acid improved net $CO_2$ assimilation with associated gas exchange parameters (Fig. 22). Therefore, it is suggested that the beneficial roles of salicylic acid are more evident under abiotic stress compared to optimum conditions. It has further been suggested that the improved photosynthetic activity resulting from salicylic acid under abiotic stress conditions is due to improved $CO_2$ uptake activity in the chloroplasts rather than mere modulation of stomatal opening (Tang *et al.,* 2017).

Reduction in photosynthetic activity due to abiotic stresses such as cold is associated with photoinhibition of PSII as a result of both diffusive and biochemical stomatal limitations (Pompelli *et al.,* 2010). In the current study, instigation of cold stress temperatures resulted into a reduction in both actual PSII operating efficiency ($\Phi_{PSII}$) as well as the maximum potential efficiency of PSII (Table 18 and Fig. 23, respectively). This indicates the occurrence of higher degrees of photoinhibition in the cold stressed plants compared to those under optimum

conditions. Although non–significantly, foliar sprays of salicylic acid tended to reduce the degree of photoinhibition especially during the light–adapted state as indicated by higher ratios of both $F_v'/F_o'$ and $F_v'/F_m'$. It has been suggested therefore, that exogenous application of salicylic acid can regulate chloroplast energy state hence resulting in to less accumulation of ROS (Tang *et al.,* 2017). Moreover, salicylic acid treated plants tended to have higher values of fluorescence quenching ($q_N$) than their un–treated counterparts although non–significantly (Table 18). These results suggest therefore that salicylic acid might alleviate photoinhibition resulting from cold stress conditions.

Salicylic acid treatment tended to increase the content of phenolic compounds especially 5–CQA under both optimum and cold stress conditions while having less effects on the alkaloids (Fig.24). This resulted into higher 5–CQA ratio and total content of the protective compounds in salicylic acid treated plants. Higher ratio of 5–CQA/caffeine is associated with higher ROS scavenging capacity due to potency of phenolic compounds over alkaloids (Ky *et al.,* 2001). Although the mechanism of stimulation of antioxidant compounds like 5–CQA cannot be confirmed at this point, it is suggested that salicylic acid differentially affect the expression of antioxidant transcripts resulting into modification of secondary composition in the cells (El−Esawi *et al.,* 2017). By reinforcing the antioxidant system, salicylic acid application therefore restores homeostatic conditions and hence minimize the deleterious effects of over accumulation of ROS.

However, melatonin, another phytochemical resulted into an increase in the extent of membrane damage (Fig. 25). This could be as a result of increased ROS resulting from exogenous melatonin application. It has been suggested that exogenous melatonin application generally reinforces the antioxidant system of plants through signal transduction by initiating the NADPH oxidase/ respiratory burst oxidative homolog (RBOH) a phenomenon known as ROS priming (Gong *et al.,* 2017). This mechanism involves an increase in the concentration of $H_2O_2$ which on one hand can result into formation of hydroxyl radicals ($OH^{\bullet-}$) via the Fenton reaction which causes severe damage to the cell membrane and increasing the leakage of cellular electrolytes (Mittler, 2017). Nevertheless, the perceived negative effect could be short lived since the increase in the ROS scavenging capacity resulting from elevated $H_2O_2$ levels via the cellular signalling pathway (Mittler, 2017) on another hand might counter the effects of cold stress in melatonin treated plants compared to untreated counter parts.

Both chlorophylls and carotenoids are susceptible to abiotic stress conditions (Ashraf and Harris, 2013). Under abiotic stress conditions, the reduction in the concentration of chlorophylls and carotenoid pigments results from both impaired pigment biosynthesis and accelerated rates of disintegration (Perveen *et al.,* 2010). In the current study, instigation of cold stress temperatures resulted into lower concentrations of chlorophylls and carotenoid pigments (Fig. 26). The increased concentration of ROS in the melatonin treatment also resulted into a decline in the concentration of especially chlorophyll b under optimum conditions and hence resulting into a lower ratio of total chlorophylls to carotenoids. The current results are in contrast to those where melatonin improved the concentration of chlorophyll pigments under abiotic stress conditions (Han *et al.,* 2017; Li *et al.*, 2017, Li *et al.,* 2018). This discrepancy could be as a result of the duration of evaluation of melatonin effects for in the above unlike in the previous studies, the current study evaluated the effects of melatonin application after 5 days of cold stress instigation. Therefore, it is plausible that melatonin effects are not only concentration dependent but are also affected by both the duration of treatment as well as time of evaluation.

The reduction in the concentrations of the photosynthetic pigments was associated with similar variation in the photosynthetic activity as shown by low gas exchange parameters such as $P_N$, $g_s$ and E (Fig. 27A−C, respectively). Low temperatures induce both stomatal and non−stomatal limitations to photosynthesis with the latter including stomata closure which concomitantly results diminished $P_N$, $g_s$ and E (Tissue *et al.,* 2005). An increase in the $C_i/C_a$ was also observed under cold stress conditions (Fig. 27D). This could be owed to the increase in the $C_i$ as a result of a reduction in the ability to reduce absorbed $CO_2$ due to cold induced reduction in the activities of both the light and the light independent photosynthetic cycles (Cen and Sage, 2005; Ensminger *et al.,* 2006). Photosynthesis is a highly regulated physiological process and immensely sensitive to variations in the environmental conditions. In the current study, exogenous melatonin treatment reduced $P_N$ under optimum conditions possibly due to initiation of RBOH and or melatonin induced stomatal closure (Wei *et al.,* 2018). Nevertheless, consistent but slightly higher gas exchange parameters and lower $C_i/C_a$ were observed in the melatonin treated plants under cold stress conditions signifying the possible protective role of melatonin under cold stress conditions.

The operating efficiency of PSII photochemistry which chiefly modulates the photosynthetic activity of plants was significantly affected by both cold stress conditions and exogenous melatonin treatment (Fig. 28 and Table 19). Both cold stress treatment and melatonin

application induced PSII photoinhibition as shown by reductions in the PSII maximum quantum efficiency ($F_v/F_m$ and $F_v/F_o$) during both dark and light adapted states. Such declines are associated with damages to the D1 protein subunit in the PSII photosystem which is highly sensitive to ROS accumulation induced by abiotic stress conditions including low temperatures (Murata *et al.,* 2007). The ability of the leaves to utilize the incident photosynthetically active radiation (PAR) is lowered abiotic stress conditions (Muller *et al.,* 2001). The resultant excess excitation energy from PAR translates into accumulation of ROS which directly inactivates the photochemical reaction centres of PSII (Murata, 2007). In the current study, this was evidenced by decline in the ETR, $\Phi_{PSII}$, $q_P$ as well as the closure of the PSII reaction centres under cold stress conditions (Table 19). Under optimum conditions, the initiation of RBOH by exogenous melatonin application also reduced the operating efficiency PSII photochemistry. However, after 5 days a slight improvement in the PSII operating efficiency was observed in the melatonin treated plants. Exogenous melatonin application has been shown to reduce the decline in the operating efficiency of PSII reaction centres (Han *et al.,* 2017). This could be attribute to the scavenging ability of both the melatonin molecule as well as its modulation effect in the cells' secondary metabolism (Debnah *et al.,* 2019).

The current results furthermore showed that the assessed compounds were generally increased by both melatonin and cold stress treatments (Fig. 29). This increment can be attributed to the ROS mediated signalling pathway which involves $H_2O_2$ mediated oxidation of cysteine residues on the protein resulting into changes in their structure and function (Mittler, 2017; Sies, 2017). In addition to this pathway melatonin has been shown to directly affect the cellular metabolism by affecting gene expression or suppression hence affecting metabolite accumulation in plants (Fan *et al.,* 2015).

The results of this current study also demonstrated the potential of foliar application of $TiO_2$ NPs in ameliorating the negative effects of cold temperatures on the photosynthetic physiology of coffee plants. Exogenous application of $TiO_2$ NPs on plant tissues causes their penetration into cellular components particularly into cell vacuoles and chloroplasts (Mohammadi *et al.,* 2013). This intake into plant tissues and subsequent localization is dependent on the size of the nanoparticles. The nanoparticles used in the current study were spherical−shaped and consisted of both anatase and rutile phases with an average diameter of about 21 nm (Fig. 30 and 31). Titanium oxide NPs are recognized amongst nanomaterials for special ability to speed−up photoreactions, a phenomenon referred to as photocatalysis (Jain and Vaya, 2017). In cold stressed plants, suppression of the photosynthetic efficiency associated with limited gas

exchanges and/or repressed carboxylic enzymes implies that any incident light on leaf tissues is potentially in excess and therefore is likely to result into excess excitation energy which lead to formation of toxic molecules such as ROS as well as charged states of chlorophyll molecules (Asada, 2006).

The current study also revealed that $TiO_2$ NPs treated plants tended to maintain higher concentrations of chlorophylls (Table 23). This result agrees with Singh *et al.* (2012). and Mohammadi *et al.* (2014) who reported significantly higher concentrations of the photosynthetic pigments in $TiO_2$ NPs treated plants under optimum and cold stress conditions. Such results could be attributed to the suppression of oxidative molecules as well as reinforcement of antioxidant defence mechanism (Mohammadi *et al.,* 2014). Moreover, $TiO_2$ NPs enhanced the metabolism of nitrogen which is an important component of amino acids and chlorophylls (Yang *et al.,* 2006). In additions, $TiO_2$ NPs treated plants tended to have higher concentrations of carotenoids which serve as a protective molecule against excess excitation energy which would otherwise degrade the photosynthetic pigments (Hong *et al.*, 2005).

The enhancement in the content of the photosynthetic pigments and the photocatalytic ability of the $TiO_2$ NPs led to improvement in the net photosynthesis on all the measurement days before and after instigation of cold temperatures. $TiO_2$ NPs have been reported to improve absorbance, acceleration and transformation of light energy through modifying the micro−environment of PSII leading to facilitation of energy transfer among amino acids within PSII protein complex and acceleration of energy transport from tyrosine to chlorophyll *a* (Mingyu *et al.,* 2007). Similar to what was observed in the current study, such effects lead to enhancement in the fluorescence quantum yield as well improved net $CO_2$ assimilation. $TiO_2$ NPs protect chloroplasts against aging hence prolonging their photosynthetic activity(Hong *et al.*, 2005) all which lead to sustained carbon assimilation during cold stress. In addition to getting involved in the electron transfer reactions in PSI and PSII, $TiO_2$ NPs cancels the effects of linolenic acids whose accumulation under abiotic stress conditions is associated with inhibition of photoreduction of the whole chain electron transport of the two photosystems and also the evolution of oxygen (Mingyu *et al.,* 2000).

Similar studies reported that $TiO_2$ NPs significantly improved the activity of ribulose 1, 5−bisphophate and phosphoenolpyruvate carboxylase through triggering the expression of both the small and large Rubisco subunits of the messenger RNA in the nano−anatase treated plants

(Xuming *et al.,* 2008; Hasanpour *et al.,* 2015). These enzymes catalyse the oxidation and oxygenation of ribulose 1, 5−bisphosphate which is the first committed step in the competitive metabolic pathways of photorespiration and photosynthetic $CO_2$ fixation in higher plants (Mauser *et al.,* 2001).

It is further suggested that, $TiO_2$ NPs improved tolerance of the treated plants to cold stress by stimulating the nonenzymatic antioxidant compounds evaluated in this study. Instigation of cold stress conditions stimulated the content of anthocyanins in coffee leaves (Table 24). However, $TiO_2$ NPs downregulated the contents of these flavonoid compounds possibly due to enhancement of other protection mechanisms during cold stress. On the other hand, $TiO_2$ NPs tended to enhance the metabolism of 5–caffeoylquinic acid while supressing that of caffeine resulting into an increase in the ratio of 5–CQA/caffeine with increasing $TiO_2$ NPs concentration. 5 – caffeoylquinic acid is the main chlorogenic acid in coffee plants representing over 80% of the phenolic compound with a very high potency in scavenging the ROS (Campa *et al.,* 2017).

By combining the results of chapter 4 where foliar nitrogen recovered the photosynthetic apparatus and chapter 5 in which the foliar application of the elicitor compounds (such as melatonin) reinforced the nonenzymatic antioxidant system, the results of chapter six indicated that, nitrogen treated plants maintained the highest maximum rates of both net and gross $CO_2$ assimilation in repose to both $C_i$ and PPFD compared to those with no foliar nitrogen treatment (Fig. 35A–D and Fig. 36, respectively). Melatonin application under optimum conditions caused a decline in the maximum net and gross $CO_2$ assimilation in response to both $C_i$ and PPFD (Fig. 35A–D and Fig. 36). Exogenous melatonin is generally known for its improvement in the photosynthetic activity of many plants (Li *et al.,* 2018; Zhang *et al.,* 2017; Yu *et al.,* 2018). However, its mechanism of action involves initiation of RBOH which increase the concentration of ROS hence reducing the photochemical efficiency of PSII (Gong *et al.,* 2017; Chen *et al.,* 2018).

Furthermore, the $A/C_i$ curve analysis indicated a decline in the maximum rate of $CO_2$ assimilation under cold stress conditions in response to controlled increase in $C_i$ (Fig. 35A and B). A similar decline was observed in the light−response curve analysis which also indicated saturation below 1000 μmol $m^{-2}$ $s^{-1}$ under both cold and optimum conditions (Fig. 35C and D, respectively). This decline in the maximum rate of $CO_2$ assimilation is normally associated with both diffusivity and biochemical constraints emanating from low temperature stress

(Pompelli *et al.,* 2010). Moreover, while accounting for both photorespiration and dark respiration, GA which also represents true photosynthesis or carboxylation followed a typical trend, being higher under optimum conditions in all the foliar treatments (Fig. 36A and B, respectively). Similar impacts of low temperature conditions on gross $CO_2$ assimilation and $O_2$ evolution have been reported in several plants (Flexas *et al.,* 1999; Mishra *et al.,* 2019). In addition to lowering the stomatal conductivity, cold stress conditions cause substantial decline in the PSII photochemical efficiency, thylakoid electron transport, enzyme activity and the metabolism of both carbon and nitrogen (Partelli *et al.,* 2009). In the current study, nonetheless, the photochemical yield showed less variation amongst the temperature treatments in contrast to the previous studies (Fig. 37A–D). This could however be attributed to the recovery in the PSII photochemistry as has been reported to occur in some varieties of arabica with better cold tolerance (Partelli *et al.,* 2009).

Moreover, cold−stressed plants had a lower photorespiratory $CO_2$ release and diminished values of RuBisCO oxygenase to carboxylase which is normally associated with low photosynthetic rates (Fig. 38). This decline in the maximum $CO_2$ assimilation could be attributed more to the cold−induced thermodynamic reduction in the Calvin−Benson cycle enzymes thus limiting the supply of $NADP^+$ and ADP for phosphorylation (Rasool *et al.*, 2014). Moreover, the capacities of RuBP carboxylation and regeneration ($V_{c,max}$ and $J_{max}$, respectively) together with the triose phosphate utilization rate (TPU) which represent the main 3 limitations to photosynthesis (Fabre *et al.*, 2019) were all dampened by cold stress conditions (Fig. 39). The sensitivity of these parameters to cold stress has been indicated in several plants (McClain and Sharkey, 2018). TPU is situated at the interface between photosynthetic production and consumption, therefore photosynthesis can only be as fast the plants can remove the resources away from the chloroplasts (Sharkey *et al.,* 2019).

The decline in the photosynthetic activity of the coffee plants under cold stress conditions was more prominent in the plants under nitrogen treatment. Under optimum conditions, the role of nitrogen in photosynthetic regulation is an undisputed phenomenon. However, in a previous study, we reported a decline in the content of nonenzymatic antioxidant compounds in the nitrogen treated plants compared to those under control conditions (see chapter 4). This was attributed to the improvement in the efficiency of the photosynthetic apparatus in the nitrogen treated plants thereby reducing the need for an elaborated antioxidant system due to low levels of ROS in the photosynthetic apparatus. Subjection of such plants to cold stress conditions as in this study showed a substantial decline in the maximum $CO_2$ assimilation. On the other hand,

melatonin improved the photosynthetic activity of coffee plants under cold stress conditions. The current results concur with a number of reports that have indicated positive effects on the photosynthetic physiology of exogenous melatonin in several plants (Szafranska *et al.,* 2017; Zhang *et al.,* 2017; Yu *et. al.,* 2018). Similar to the above studies, exogenous melatonin improved the maximum $CO_2$ assimilation via enhancement of the quantum yield of PSII photochemistry. Melatonin priming has also been shown to upregulate the gene expressions of enzymes responsible for ATP, NADPH, RuBisCO activase as well directly improving the activity of RuBisCO and hence facilitating photosynthetic activity during abiotic stress conditions (Erdal, 2019).

Membrane damage analysis expressed as electrolyte leakage index indicated a surge in ROS on exposure of coffee plants to cold stress conditions especially in plants under control treatment (Fig. 40). A corresponding decline in the content of the photosynthetic pigment was in the cold stressed plants compared to their optimum counterparts (Fig. 41A−F). The photosynthetic apparatus of coffee plants is highly dependent on nitrogen metabolism and therefore the risk of photoinhibition is highest in nitrogen deficient plants (see chapter 4). This reduction in the photosynthetic capacity is further worsened by exposure to cold stress conditions which result into heightened production of ROS which cause cell damage and disintegration of chlorophyll pigments while increasing the photoprotective molecules such as carotenoids (Sharma *et al.,* 2012; Tripathy and Oelmuller, 2012). In addition to improving photosynthetic activity during cold stress conditions, melatonin is an antioxidant with ROS scavenging abilities (Arnao and Hernandez−Ruiz, 2006). These protective role of melatonin on the damage to photosynthetic apparatus were more apparent in the nitrogen treated plants which tended to maintain a higher chlorophyll/carotenoid ratio (Fig. 41F).

In the current study, the content of anthocyanins only declined in plants under Melatonin treatment while no significant variation was observed amongst other treatments under the two temperature conditions (Fig. 42A and B). Despite showing no significant variation amongst the treatments, flavonoids and flavonols tended to be highest in the plants under Nitrogen which also had the highest photosynthetic rate under optimum conditions (Fig. 42C and D). Its plausible therefore, that during high rates of photosynthetic activity, coffee plants upregulate the synthesis of flavonoids for ROS scavenging. Flavonoids and flavonols have been shown to surge under oxidative stress conditions (Sarmadi *et al.,* 2018).

HPLC analysis of the selected metabolites also indicated highest accumulation of trigonelline in plants treated with nitrogen (Fig. 43A). Trigonelline (1−methylpyridinium−3−carboxylate) a pyridine alkaloid and therefore contains nitrogen and directly benefits from nitrogen metabolism. This compound is NAD reservoir and therefore its accumulation might have a direct effect on the content of NADP, a key intermediate in the $CO_2$ fixation pathway (Perchat *et al.,* 2018). Trigonelline accumulation in the coffee plants was however dampened by low temperature due to a general decline in general plant metabolism under cold stress conditions (Hussain *et al.,* 2018). Mangiferin ((1S)−1,5−Anhydro −1−(1,3,6,7 – tetrahydroxy – 9 – oxo − 9H – xanthen – 2 − yl) – D −glucitol) and 5−caffeoylquinic acid as well as the total of the HPLC metabolites were also generally highest in the nitrogen treated plants although unlike trigonelline, the two phenolic compounds were slightly increased by cold stress conditions (Fig. 43B–D). The current results agree those observed in chapter 4 where a dramatic increase of these compounds in coffee plants during oxidative stress conditions.

Total phenolic content (TPC) analysis generally showed no significant differences in the content of TPC amongst the treatments except in plants under Melatonin treatment during optimum conditions (Fig. 44). Nevertheless, on the basis of the anthocyanins, flavonoids, flavonols, mangiferin and 5−cafeeoylqunic acid, subjection of cold of plants to cold stress conditions elevated accumulation of these phenolic compounds. It has recently become clear that these components are modulated by ROS whose production is exacerbated by oxidative stress conditions and or by the RBOHs initiated by melatonin burst (Kreslavski *et al.,* 2012; Baxter *et al.,* 2014; Mittler, 2017; Sies, 2017). Antioxidant analysis indicated an increase in the ROS scavenging capacity of the coffee plants after subjection to cold stress conditions (Fig. 45). The current results agree with those in chapter 4 who reported a positive relationship between accumulation of phenolic compounds during cols stress conditions and the ability to neutralise ROS. Phenolic compounds possess electrophilic glycosylic linkages and hydroxyl components that scavenge ROS (Das and Roychoudhury, 2014). Moreover, this ROS scavenging ability was highest in plants under Nit + Mel during cold stress conditions. This suggests an elevated ability to quench the ROS compared to their Nitrogen only treated counterparts.

ROS have become an integral part of many living organisms due to the abundance of $O_2$ in the atmosphere. Although produced under normal cellular metabolism, excessive production of ROS occurs during abiotic stress conditions such as low temperature often resulting into a condition known as oxidative stress (Mittler, 2002). For coffee plants, cold stress conditions

are prompted when the ambient temperature conditions fall below 18°C (Ramalho *et al.,* 2014). Subjection of the coffee plants to 12/12°C therefore, increased the cellular ROS by reducing the photosynthetic capacity causing a mismatch between energy capture and C–assimilation (Asada, 2006). This led to erroneous energy transfer to chlorophylls triggering their excitation into $^{3}Chl^{*}$ and $^{1}Chl$ as well as formation of $^{1}O_2$ and $O_2^{\bullet-}$ in the PSI and PSII (Khorobrykh *et al.,* 2020). Moreover, ROS are also generated at other sites such as mitochondria and peroxisomes due to presence of several electron transport reactions (Lismont *et al.,* 2015). By SOD, the formed $O_2^{\bullet-}$ dismutases to $H_2O_2$ which is partly neutralised to $H_2O$ by APX and CAT. However, via the Fenton reaction, the remaining $H_2O_2$ forms the highly toxic $OH^{\bullet-}$ which despite its short life span, reacts readily with cellular membrane casing their rapid disintegration. The results of the current study indicated an increase in ELP within 4 days after instigation of cold stress conditions (Fig. 46). This agrees with previous results were subjection to cold stress conditions caused a spike in the ELP in the leaves of the coffee plants.

However, after reaching a maximum by day 6 of cold stress conditions, ELP indicated a plateau despite a further progression of cold stress conditions. This coincided with elevated amounts of antioxidant compounds especially ascorbic acid (Fig. 47) and to a less extent 5–caffeoylquinic acid while generally, the other assayed compounds showed nonsignificant response (Fig. 48). Ascorbic acid a powerful antioxidant compound with very high free radical scavenging activity and therefore forms the most immediate response during oxidative stress conditions (Wang *et al.,* 2019). On the other hand, phenolic compounds like 5–caffeoylquinic acids, mangiferin, flavonoids and flavonols constitute the secondary response given their slow accumulation during oxidative conditions (Fig. 48, 49 and 50, respectively).

Nevertheless, despite the reinforced nonenzymatic antioxidant system, cold stress conditions slowed down the metabolism of nitrogen which was evidenced by a consistent decline in the nitrogenous compound such as N−methyl nicotinate (trigonelline) (Perchat, 2018). The results of the current study therefore suggest that, the $H_2O_2$ formed by ROH and RBOH and its subsequent involvement in the signal transduction pathway causes various effects on coffee plants secondary metabolism (Mittler, 2017). While the metabolism of antioxidant compounds such as ascorbic acid and phenolic compounds are promoted under such conditions, the $H_2O_2$ induced change in the protein structures might slow down the metabolism of some compounds such as trigonelline as indicated in Table 26.

# Chapter nine

# General conclusion and future research

## 9.1 General conclusion

The current study has revealed that coffee plants accumulate high amounts of phytochemicals especially in their leaves. These include alkaloids, phenolics, carotenoids and vitamins among others. These compounds confer a high ROS scavenging capacity to this organ. Accumulation of these phytochemicals is dramatically influenced by changes in the ambient environmental dynamics. During a 24–hr period circadian rhythm, changes in the concentration of chlorophylls and carotenoids may not vary significantly although the latter tends to increase under daylight conditions hence causing a high ratio of chlorophylls to carotenoids by the end of the day. In addition, daylight conditions initiate high amounts of antioxidant compounds especially 5–CQA. On the hand, both summer and winter conditions elicit higher amounts of the protective antioxidant compounds following a remarkable damage to the photosynthetic apparatus especially during winter. Similarly, exposure of coffee plants to excess excitation energy causes a decline in the contents of the photosynthetic pigments and damage to the photosynthetic apparatus as indicated by low chlorophyll to carotenoid ratio. This is accompanied by rapid accumulation of antioxidant compounds especially phenolics compared to alkaloids.

In order to recover this photosynthetic apparatus of the previously cold-stressed plants, foliar urea supply containing 10 mM nitrogen was found adequate although it is associated with a decline in the nonenzymatic antioxidant system. On the other hand, the exogenous foliar application of kinetin, salicylic acid, melatonin and $TiO_2$ $NP_S$ improved the antioxidative capacity of the coffee plants by upregulating the metabolism of the nonenzymatic antioxidant compounds. This was associated with increased reactive species scavenging capacity in the treated plants. Exogenous application of the substances slightly increased the photochemical and mesophyll efficiency for $CO_2$ fixation in addition to maintaining somewhat higher gas exchanges parameters under cold stress conditions compared to the nontreated plants. Therefore, on the basis of the results presented in this chapter current study, it is suggested that exogenous application of elicitor compounds has a potential to modulate the growth of the coffee plants under cold stress conditions, and therefore more studies should be conducted to explore its efficacy under field conditions. Exploring cold mitigation or tolerance enhancement is not only useful in the face of unpredictable climatic changes, but also is likely to expand coffee

cultivation to new production areas, whose environmental conditions are currently unsuitable for coffee production.

Therefore, the combined effects of nitrogen and melatonin indicate a possible remedy for mitigating cold−induced decline in the photosynthetic physiology of coffee plants with the former improving the efficiency of the photosynthetic apparatus while the latter reinforcing the nonenzymatic antioxidant system of the coffee plants. Moreover, this reinforcement of the nonenzymatic antioxidant system is associated with initiation of RBOH that are involved in the $H_2O_2$ signalling and although might negatively impact the photosynthetic efficiency in the short term, the reinforced antioxidant system in the longer run certainly confers tolerance of coffee plants to cold stress conditions.

### 9.2 Future research

The current study has demonstrated the role of phytochemicals which compose the nonenzymatic antioxidant system in protection against oxidative stress conditions in coffee plants. Evaluations in the study focused on metabolome analysis, leakage of cellular electrolytes, disintegration of photosynthetic pigments, antioxidant assays and assessment of the photosynthetic physiology under optimum and abiotic stress conditions such as low temperatures and high light intensity.

However, to fully understand the mechanism underlying cold tolerance in coffee plants, proteomic and transcriptomic studies still need to be done. Moreover, such studies will likely enable development of transgenic plants that are better suited to survive the increasingly challenging environmental conditions in addition to maintaining or increasing productivity of the coffee plants.

Furthermore, the studies undertaken here were done under semi or fully controlled environmental conditions in a vinyl-house or growth chamber, respectively. However, the results need to be tested under field conditions in order to obtain the appropriate efficacy levels of the treatment measures. In addition, long-term evaluations are still necessary to determine the applicability of the treatments in view of sustaining or increasing crop yields irrespective of the fluctuations in the ambient environmental conditions.

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## Summary

This study aimed at profiling and reinforcing the nonenzymatic antioxidant system in young coffee plants through exogenous applications of several elicitor compounds to enhance coffee cultivation under cold stress conditions.

**In chapter two,** the phytochemical composition of coffee plant organs and their corresponding antioxidant capacities compared to green and roasted coffee beans was investigated. HPLC analysis indicated that the investigated compounds were present in all organs, except mangiferin which was absent in roots, stems and seeds and caffeine which was absent in stems and roots. Total phytochemicals were highest in the green beans (GB) at 9.70 mg $gDW^{-1}$, while roasting caused a 66% decline in the roasted beans (RB). This decline resulted more from 5–CQA and sucrose decomposition by 68 and 97% respectively, while caffeine and trigonelline were not significantly thermally affected. Roasting increased the total phenolic content (TPC) by 20.8% which was associated with an increase of 68.8, 47.5 and 13.4% in the antioxidant capacity (TEAC) determined by DPPH, ABTS and FRAP assays respectively. Amongst the leaves, the youngest (L1) contained the highest content at 8.23 mg g $DW^{-1}$, which gradually reduced with leaf age to 5.57 mg $gDW^{-1}$ in the oldest (L6). Leaves also contained the highest TPC (over 60 mg GAE $gDW^{-1}$) and exhibited high TEAC, the latter being highest in L1 at 328.0, 345.7 and 1097.4 and least in L6 at 304.6, 294.5 and 755.1 µmol Trolox $gDW^{-1}$ for the respective assays. Phytochemical accumulation, TPC and TEAC was least in woody stem (WS) at 1.42 mg $gDW^{-1}$, 8.7 mg GAE $gDW^{-1}$, 21.9, 24.9 and 110.0 µmol Trolox $gDW^{-1}$ while herbaceous stem (HS) contained up to 4.37 mg g $DW^{-1}$, 27.8 mg GAE $gDW^{-1}$, 110.9, 124.8 and 469.7 µmol Trolox $gDW^{-1}$, respectively. Roots contained up to 1.85 mg g $DW^{-1}$, 15.8 mg GAE $gDW^{-1}$ and TEAC of 36.8, 41.5 and 156.7 µmol Trolox $gDW^{-1}$. Amongst the organs therefore, coffee leaves possessed higher values than roasted beans on the basis of phytochemicals, TPC and TEAC. Leaves also contain carotenoids and chlorophylls pigments with potent health benefits. With appropriate processing methods, a beverage prepared from leaves (coffee leaf tea) could be a rich source of phytochemicals and antioxidants with therapeutic and pharmacological values for human health.

**In chapter three,** the response of chlorophyll and carotenoid pigments together with oscillations in the contents of the phytochemicals during; (i) 24hr cycle; (ii) summer to winter seasons and (iii) in response to high light intensity was investigated. Diel fluctuations in the environmental conditions caused no significant changes in the contents of pigments and

antioxidant compounds. Nevertheless, daylight conditions tended to elicit higher concentrations of carotenoids causing a significantly least ratio of chlorophylls to carotenoid by evening. This was accompanied by higher concentrations of non–enzymatic compounds such as 5–caffeoylquinic acid (5–CQA) especially at midday. On the other hand, both chlorophyll and carotenoid content were significantly highest during summer season. This was followed by a remarkable reduction in their content with reduction in the ambient temperatures from the onset of winter at the beginning of November. This resulted into a reduction in the ratio of chlorophylls to carotenoid. The content of the antioxidant compounds and the ratio of 5–CQA/caffeine were generally least during autumn conditions while both summer and winter conditions elicited higher amounts of these protective compounds. Similarly, exposure of previously low light acclimated coffee plants to high sunlight intensity caused remarkable reductions in both chlorophylls. Extreme sunlight conditions elicited higher concentration of carotenoid pigments resulting into lower chlorophyll to carotenoid ratio. In addition, direct sunlight conditions caused rapid accumulation of antioxidant compounds especially 5–CQA and mangiferin while caffeine and trigonelline were not significantly affected. This, resulted into a higher ratio of 5–CQA/caffeine and total HPLC metabolite content in sunlit plants compared to the shaded counterparts. The results presented in the current study demonstrate the protective role of antioxidant compounds especially 5–CQA, mangiferin and carotenoids against reactive oxygen species that reduce chlorophyll efficiency during abiotic stress conditions.

**In chapter four,** due to cold−stressed leaves possessing an elevated risk of photodamage because of an inefficient photosynthetic apparatus during winter conditions, recovery of cold−stressed coffee seedlings treated with different concentrations of nitrogen applied as foliar urea sprays; Control (0), 5, 10, 20 and 40 mM, was investigated under optimum growth chamber conditions (25/20°C) for 3 months. Concentrations of nitrogen and photosynthetic pigments in the leaves increased with increasing concentration of the foliar sprays up to 20mM. This was accompanied with a recovery of the photosynthetic apparatus and increased net carbon assimilation rate. In addition, 10 mM and 20 mM treated plants also had the highest photosystem II maximum efficiency compared to their lower or higher nitrogen concentration treated counterparts. Conversely, these plants contained lower concentrations of 5−caffeoylquinic acid, mangiferin, trigonelline and caffeine than control plants. It was concluded therefore, that foliar sprays of appropriate nitrogen concentration were adequate for

recuperating the photosynthetic apparatus and improved the photosynthetic performance of the cold−stressed coffee seedlings.

**In chapter five,** evaluation of several elicitor compounds on reinforcement of the nonenzymatic antioxidant system of coffee plants with concomitant effects on the photosynthetic physiology during cold stress conditions was evaluated. Exogenous foliar application of kinetin, salicylic acid, melatonin and $TiO_2$ $NP_S$ improved the antioxidative capacity of the coffee plants by upregulating the metabolism of the nonenzymatic antioxidant compounds. This was associated with increased reactive species scavenging capacity in the treated plants. Exogenous application of the substances slightly increased the photochemical and mesophyll efficiency for $CO_2$ fixation in addition to maintaining somewhat higher gas exchanges parameters under cold stress conditions compared to the nontreated plants. Therefore, on the basis of the results presented in this chapter, it is suggested that exogenous application of elicitor compounds has a potential to modulate the growth of the coffee plants under cold stress conditions, and therefore more studies should be conducted to explore its efficacy under field conditions. Exploring cold mitigation or tolerance enhancement is not only useful in the face of unpredictable climatic changes, but also is likely to expand coffee cultivation to new production areas, whose environmental conditions are currently unsuitable for coffee production.

**In chapter six,** by combining the results of chapter 4 and 5, this chapter evaluated the effects of nitrogen and melatonin on improvement of cold tolerance of coffee plants. The results indicated that nitrogen improved the photosynthetic physiology of the coffee plants by enhancing both the net and gross maximum $CO_2$ assimilation rates in response to increasing $C_i$ and PPFD compared to non−nitrogen treated plants. This was associated with a higher nitrogen partitioning for both the metabolism of photosynthetic pigments and the RuBisCO enzymes and other nitrogenous compounds such as trigonelline. Moreover, plants treated the foliar nitrogen exhibited a high capacity for RuBP carboxylation and regeneration under optimum conditions. Nevertheless, cold stress conditions caused strong reductions in the photosynthetic physiology of coffee plants which caused a tremendous damage to the cell membranes. This damage was however less prominent in melatonin treated plants which also had high contents of phenolic nonenzymatic antioxidant compounds such as anthocyanins, flavonoids, chlorogenic acids, mangiferin. Accumulation of these compounds caused a higher ROS scavenging capacity in coffee plants especially those treated with melatonin. Therefore, the

combined effects of nitrogen and melatonin indicate a possible remedy for mitigating cold−induced decline in the photosynthetic physiology of coffee plants.

**In chapter seven,** the mechanism through which the nonenzymatic antioxidant system is modulated during cold stress conditions by either oxidative stress or RBOH induced ROS in coffee plants was evaluated. The results indicated that excessive production of ROS instigated by cold stress conditions caused membrane damaged and supressed the metabolism of nitrogen containing compounds especially trigonelline. However, accumulation of ROS initiated by both cold stress and RBOH also triggered nonenzymatic antioxidant protective mechanism which led to increase in the levels of especially ascorbic acid and 5–caffeoylquinic acid which restored ROS to homeostatic levels and thereby preventing further oxidation of the cellular membrane.

**Finally** , this study therefore concludes that, phytochemical play an important role in protection of the photosynthetic apparatus during oxidative stress conditions. Thus, for successful cultivation of coffee plants during abiotic stress conditions, the combined effects of nitrogen and elicitor compounds such as melatonin indicate a possible remedy for mitigating cold−induced decline in the photosynthetic physiology of coffee plants with the former improving the efficiency of the photosynthetic apparatus while the latter reinforcing the nonenzymatic antioxidant system of the coffee plants.

## Summary in Japanese（要約）

本研究はコーヒーの豆，器官別の抗酸化活性能力を調査するとともに，酸化ストレス条件下におけるコーヒー幼苗自体の応答およびエリシター化合物の施用による幼苗木の非酵素的抗酸化物質の強化によるストレスからの回避戦略を解明し，新たな知見を得た.

第 2 章では，コーヒーの根，葉，茎およびコーヒー豆の生豆と焙煎豆の抗酸化能力を調査した．さらにコーヒー葉の下位葉から上位葉および茎の形態別における抗酸化能力も同時に調査した． 分析した抗酸化物質のうち，マンギフェリンは葉のみ，そしてカフェインは葉および豆にのみ存在することが明らかとなった．またコーヒーの代表的な抗酸化物質総含量は，生豆が 9.70 mg $gDW^{-1}$ で最も高く，焙煎豆の総含量は生豆に比べて 66%減少していた．葉中の総抗酸化物質含量は，上位葉（L1）で 8.23 mg g $DW^{-1}$ と最も高く、下位葉が 5.57 mg $gDW^{-1}$ と最も低い値となり，また葉の総ポリフェノール含量は他の器官に比べ最も高く(60 mg GAE $gDW^{-1}$ 以上)，抗酸化能力の指標である DPPH，ABTS および FRAP においても上位葉は下位葉に比べて高い値を示した.

第 3 章では，環境条件の変化；（i）24 時間サイクル，（ii）夏から冬の季節，（iii）遮光率の違いにおける葉中の抗酸化能力への応答について調査した．日中は，カロテノイド， 5–カフェオイルキナ酸（5–CQA）が高まる傾向を示した．一方，抗酸化物質の含有量と 5–CQA /カフェインの比率は、夏と冬で増加傾向を示し，秋で最も少なくなる傾向を示した．さらに，遮光率の違う条件では直射日光を直接コーヒーの葉が受けると，抗酸化化合物の一種である 5–CQA とマンギフェリンが急速に蓄積したが，カフェインとトリゴネリンには大きな影響はなかった．このように，コーヒーの葉に含まれる 5–CQA，マンギフェリンおよびカロテノイドの増加は，環境ストレスに対して保護的な役割をしていることが明らかとなった.

第 4 章では，酸化ストレスの 1 つである低温ストレスは，コーヒーの生育にとって回避させなければいけない環境条件の 1 つであり，回避策はまだ明らかになっていなかった．そこで低温ストレス回避のために窒素の葉面散布効果を検討した．この結果，20mM までの窒素（尿素）は，対照区に比べ葉中の窒素と葉色を改善させ，10～20mM 窒素はコーヒーの葉の光化学系Ⅱ効率を高めた．さらに窒素葉面散布は，5-カフェオイルキナ酸、マンギフェリン、トリゴネリンおよびカフェインを低濃度であるが増加傾向を示した．したがって，適切な窒素濃度の葉面散布は光合成機能を回復させるのに十分であり，低温ストレスを受けたコーヒー苗の光合成性能を改善したと結論付けた.

第 5 章では，低温ストレス条件化におけるコーヒー自体の非酵素的抗酸化システムの強化に関するいくつかのエリシター化合物の効果を光合成生理学に評価した． エリシター化合物としてキネチン，サリチル酸，メラトニンおよび TiO2 NPS の計 4 種とした．この結果，これらのエリシター化合物の葉面散布は，低温ストレスで発生する活性酸素を消去する抗酸化機能の増加を示した．特に，光合成関連では低温ストレス条件化においても高いガス交換を示し， CO2 固定の光化学効率を向上させた． したがって，この章で示した結果に基づいて、エリシター化合物の葉面散布は，低温ストレス条件下でコーヒー植物の成長を調節する可能性を示唆した.

第 6 章では，3 と 4 の結果から，低温ストレスに対する窒素とメラトニンの混合効果を評価した．この結果，窒素はコーヒーの純および最大同化率を高め，光合成に関連するクロロフィル，RuBisCO 酵素およびトリゴネリンなどの他の窒素化合物の代謝を高めた．一方，メラトニンは，アントシアニン，フラボノイド，クロロゲン酸，マンギフェリンなどのフェノール系非酵素的抗酸化化合物も増加させる効果を示した．したがって，窒素とメラトニンの複

合散布効果は，低温ストレスにおけるコーヒーの光合成生理機能低下を緩和させる手法の 1 つであることが明らかとなった.

第 7 章では，低温ストレス条件下におけるコーヒー生長障害は，低温によって発生した ROS の過剰生産が細胞膜の損傷を引き起こし，窒素の代謝を抑制したことであると考えられた. ただし，発生した ROS 蓄積は、同時に葉中の非酵素的抗酸化物質，特にアスコルビン酸と 5-カフェオイルキナ酸のレベルを上昇させ， これらの物質によって ROS を消去し，値を通常レベルに戻すことにより，細胞膜のさらなる酸化を防ぐのに役立っていることを明らかにした.

以上のことから，コーヒーは焙煎豆よりも生豆の方が，抗酸化能力が高く，さらに器官別では，特に葉の若葉に多くの機能性成分が含まれていることが明らかとなり，蓄積する抗酸化物質の量も明らかにした. さらに低温ストレスのような酸化ストレス条件下でのコーヒーの光合成機能を回復させるための抗酸化物質の上昇に誘導させるエリシター化合物の役割を明らかにした. したがって，冬季の低温ストレスのような非生物的ストレス条件下でもコーヒー栽培を成功させるために，窒素とメラトニンなどのエリシター化合物の複合散布効果は，コーヒーの光合成生理学の低温による誘発する活性酸素種を消去させる非酵素的抗酸化システムを強化させ，低温による光合成効率を改善させることが明らかとなった.

## Scientific publications

1. **Acidri, R.; S**awai, Y.; Sugimoto, Y.; Handa, T.; Sasagawa, D.; Masunaga, T.; Yamamoto, S.; Nishihara, E. Phytochemical Profile and Antioxidant Capacity of Coffee Plant Organs Compared to Green and Roasted Coffee Beans. *Antioxidants* **2020,** *9*(2): 93 (doi: 10.3390/antiox9020093).

   - **Chapter 2**

2. **Acidri, R.;** Sawai, Y.; Sugimoto, Y.; Sasagawa, D.; Masunaga, T.; Yamamoto, S.; Nishihara, E. Foliar nitrogen supply enhances the recovery of photosynthetic performance of cold-stressed coffee (*Coffea arabica* L.) seedlings. *Photosynthetica* **2020,** *58*(4): 951–960 (doi: 10.32615/ps.2020.047).

   - **Chapter 4**

3. **Acidri, R.;** Sawai, Y.; Sugimoto, Y.; Handa, T.; Sasagawa, D.; Masunaga, T.; Yamamoto, S.; Nishihara, E. Exogenous Kinetin Promotes the Nonenzymatic Antioxidant System and Photosynthetic Activity of Coffee (*Coffea arabica* L.) Plants Under Cold Stress Conditions. *Plants* **2020**, *9*(2): 281 (doi: 10.3390/plants9020281).

   - **Chapter 5 (in part)**

## Conference proceedings abstract

1. **Acidri** R., E. Nishihara. Exogenous kinetin promotes nonenzymatic antioxidant defense system in young *Coffea arabica* L. plants subjected to cold stress conditions. Poster. International conference on integrative plant physiology. Barcelona, Spain (October, 2019).

2. **Acidri R.**, D. Sasagawa, C. Wacal, D. Basalirwa, Y. Sawai and Nishihara E. Photosynthetic and metabolic response of cold stressed coffee (*Coffea arabica* L.) seedlings to foliar nitrogen supply. Oral. The 33rd Horticultural conference of Japanese Society of Horticultural Sciences. Tokyo, Japan (March 2019).

3. **Acidri R.**, D. Sasagawa, C. Wacal, D. Basalirwa, Y. Sawai and Nishihara E. Phytochemical profile of coffee (*Coffea arabica* L.) leaves as an alternative source of dietary antioxidants for human consumption. Oral – The 32nd Horticultural conference of Japanese Society of Horticultural Sciences. Kagoshima, Japan (September, 2018).

4. **Acidri R.**, T. Ishigaki, D. Sasagawa, C. Wacal, D. Basalirwa, Y. Sawai, Ohmi K. and Nishihara E. Amelioration of cold induced stress in coffee (Coffea arabica L.) seedlings by $TiO_2$ nanoparticles. Poster – The 27th Biennial conference for Association for Science and Information on Coffee. Portland, United States (September, 2018).

5. **Acidri R.**, D. Sasagawa, C. Wacal, D. Basalirwa, Y. Sawai and Nishihara E. Inorganic nitrogen-source preference of coffee (*Coffea arabica* L.) plants in hydroponic culture under low irradiance. Oral Presentation – The 31st Horticultural conference of Japanese Society of Horticultural Sciences. Nara, Japan (March, 2018).

6. **Acidri R.**, Y. Nakamura, D. Sasagawa, C. Wacal, D. Basalirwa, M. Kato, Y. Sawai and Nishihara E. Light-emitting diode (LED) optimization for growth and leaf antioxidant content of coffee (*Coffea arabica* L.). Oral Presentation – The 30th Horticultural conference of Japanese Society of Horticultural Sciences. Hokkaido, Japan (September, 2017).

# Appendices

## Appendix 1

1 *Coffea abbayesii* J. -F. Leroy
2 *Coffea affinis* De Wild
3 *Coffea alleizettii* Dubard
4 *Coffea ambanjensis* J. -F. Leroy
5 *Coffea ambongesis* J. -F. Leroy ex A. P. Davis
6 *Coffea andrambovatensis* J. -F. Leroy
7 *Coffea ankaranensis* J. -F. Leroy ex A. P. Davis
8 *Coffea anthonyi* Stoff. & F. Anthony
9 *Coffea arabica* L.
10 *Coffea arenesiana* J. -F. Leroy
11 *Coffea augagneurii* Dubard
12 *Coffea bakossii* Cheek & Bridson
13 *Coffea benghalensis* B. Heyne ex Shult
14 *Coffea bertandii* A. Chev.
15 *Coffea betamponensis* Porteres & J. -F. Leroy
16 *Coffea bissetiae* A. P. Davis and Rakotonas.
17 *Coffea boinensis* A. P. Davis & Rakotonas.
18 *Coffea boiviniana* A. P. Davis & Rakotonas.
19 *Coffea bonnieri* Dubard
20 *Coffea brassii* (J. -F. Leroy) A. P. Davis
21 *Coffea brevipes* Hiern
22 *Coffea bridsoniae* A. P. Davis & Mvungi
23 *Coffea buxifolia* A. Chev.
24 *Coffea canephora* Pierre ex A. Froehner
25 *Coffea carrisoi* A. Chev.
26 *Coffea charrieriana* Stoff. & F. Anthony
27 *Coffea cochinchinensis* Pierre ex Pit.
28 *Coffea commersoniana* (Baill.) A. Chev.
29 *Coffea congensis* A. Froehner
30 *Coffea constatifructa* Bridson
31 *Coffea coursiana* J. -F. Leroy
32 *Coffea dactylifera* Robbr & Stoff.
33 *Coffea decaryana* J. -F. Leroy
34 *Coffea dubardii* Jum.
35 *Coffea ebracteolata* (Hiern) Brenan
36 *Coffea eugenioides* S. Moore
37 *Coffea fadenii* Bridson
38 *Coffea furafanganensis* J. -F. Leroy
39 *Coffea floresiana* Boerl.
40 *Coffea fotsoana* Stoff. & Sonke
41 *Coffea fragilis* J. -F. Leroy
83 *Coffea montekupensis* Stoff.
84 *Coffea montis-sacri* A. P. Davis
85 *Coffea fragrans* wall. ex Hook. f.
86 *Coffea gallienii* Dubard
87 *Coffea moratii* J. -F. Leroy ex A. P. Davis & Rakotonas.
88 *Coffea mufindiensis* Hutch. ex Bridson
89 *Coffea myrtifolia* (A. Rich. ex DC) J. -F. Leroy
90 *Coffea namorokensis* A. P. Davis & Rakotonas.
91 *Coffea neobridsoniae* A. P. Davis
92 *Coffea neoleroyi* A. P. Davis
42 *Coffea gravei* Drake ex A. Chev.
43 *Coffea heimii* J. -F. Leroy
44 *Coffea homollei* J. -F. Leroy
45 *Coffea horsfieldana* Miq.
46 *Coffea humbertii* J. -F. Leroy
47 *Coffea humblotiana* Baill
48 *Coffea humilis* A. Chev.
49 *Coffea jumellei* J. -F. Leroy
50 *Coffea kapakata* (A. Chev.) Bridson
51 *Coffea kianjavatensis* J. -F. Leroy
52 *Coffea kihansiensis* A. P. Davis & Mvungi
53 *Coffea Kimbozensis* Bridson
54 *Coffea Kivuensis* Lebrun
55 *Coffea labatii* A. P. Davis & Rakotona
56 *Coffea lancifolia* A. Chev.
57 *Coffea lebruniana* Germ & Kester
58 *Coffea leonimontana* Stoff.
59 *Coffea leroyi* A. P. Davis
60 *Coffea liaudii* J. -F. Leroy ex A. P. Davis
61 *Coffea liberica* Hiern
62 *Coffea ligustroides* S. Moore
63 *Coffea littoralis* A. P. Davis & Rakotonas.
64 *Coffea lulandoensis* Bridson
65 *Coffea mabesae* (Elmer) J. -F. Leroy
66 *Coffea macrocarpa* A. Rich
67 *Coffea madurensis* Teijsm. & Binn. Ex Koord.
68 Coffea magnistipula Stoff. & Robbr.
69 *Coffea malabarica* (Sivar., Biju & P. mathew) A. P. Davis
70 Coffea mangoroensis Porteres
71 *Coffea manni* (Hook. f.) A. P. Davis
72 *Coffea manombesis* A. P. Davis
73 *Coffea mapiana* Sonke, Nguembou & A. P. Davis
74 *Coffea mauritana* Lam
75 *Coffea mayombensis*
76 *Coffea mcphersonii* A. P. Davis & Rakotonas.
77 *Coffea melanocarpa* welw. ex Hiern
78 *Coffea merguensis* Ridl.
79 *Coffea millotii* J. -F. Leroy
80 *Coffea minutiflora* A. P. Davis & Rakotonas
81 *Coffea mogenetti* Dubard
82 *Coffea mongensis* Bridson
104 *Coffea sahafaryensis* J. -F. Leroy
105 *Coffea sakaraliae* J. -F. Leroy
106 *Coffea salvatrix* swynn. & philipson
107 *Coffea sambavensis* J. -F. Leroy ex A. P. Davis & Rakotonas.
108 *Coffea sapinii* (De wild.) A. P. Davis
109 *Coffea schliebenii* Bridson
110 *Coffea semsei* (Bridson) A. P. Davis
111 *Coffea sessiliflora* Bridson
112 *Coffea stenophylla* G. Don
113 *Coffea tetragona* Jum. & H. Perrier

**93** *Coffea perrieri* Drake ex Jum. & H. Perrier
**94** *Coffea pervilleana* (Baill.) Drake
**95** *Coffea pocsii* Bridson
**96** *Coffea pseudozanguebariae* Bridson
**97** *Coffea pterocarpa* A. P. Davis & Rakotonas.
**98** *Coffea racemosa* Lour.
**99** *Coffea rakotonasoloi* A. P. Davis
**100** *Coffea ratsimamangae* J. -F. Leroy ex A. P. Davis & Rakotonas.
**101** Coffea resinosa (Hook. f.) Radlk.
**102** *Coffea rhamnifolia* (Chiov.) Bridson
**103** *Coffea richardii* J. -F. Leroy
**114** *Coffea togoensis* A. Chev.
**115** *Coffea toshii* A. P. Davis & Rakotonas.
**116** *Coffea travancorensis* wight & Arn.
**117** *Coffea tricalysioides* J. -F. Leroy
**118** *Coffea tsirananae* J. -F. Leroy
**119** Coffea vatovavyensis J. -F. Leroy
**120** *Coffea vavateninesis* J. -F. Leroy
**121** *Coffea vianneyi* J. -F. Leroy
**122** *Coffea vohemarensis* A. P. Davis & Rakotonas.
**123** *Coffea wightiana* wall. ex Wight & Arn
**124** *Coffea zanguebariae* Lour.