Biochemical Analyses of Functional Metabolites in *Allium*: Prospective Strategies for Improving Crop Stress Tolerance

ネギ属における機能性代謝物の生化学分析：作物ストレス耐性の改変に関する将来戦略

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By

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CHAPTER I. GNERAL INTRODUCTION

Onions (*Allium cepa* L.) are the third most economically important crop worldwide, with a total production of 85 million tons per year (FAOSTAT, 2013). Its consumption as a food or ethnomedicine is mainly attributed, respectively, to its nutritional and functional properties, including antiasthmatic, antichlolesterolemic, and antimicrobial properties (Mostafa et al., 2013; Abdelrahman et al., 2014; Caruso et al., 2014). The Sumerians were the first to write about onions, dating back to 3500–3200 BC. In the Papyrus Ebers, which is based on old Egyptian tomb wall art, inscriptions, and carvings, we learn that onions and garlic played an important role in the Old Kingdom of ancient Egypt. They were used medicinally for indigestion and as diuretics, spasmolytics, and aphrodisiacs.

The genus *Allium* is an enormous genus (850 species) that is spread broadly across the northern hemisphere from the dry subtropics to the boreal zone; the one exception is *A. dregeanum*, which is located in South Africa (Kamenetsky and Rabinowitch, 2006) (Fig. 1). A region of exceptionally high species diversity stretches from the Mediterranean Basin to Central Asia, and a second, less sound, center of species diversity occurs in western North America (Kamenetsky and Rabinowitch, 2006). *Allium* species have adapted to diverse ecological niches, leading to the development of an astonishing number of different morphotypes. This is the main reason for the widely recognized difficulties in taxonomy and classification of *Allium* (Gregory et al., 1998). Many plants of this genus have high economic significance, including vegetables [*A. cepa* (bulb onion and shallot), *A. sativum* (garlic), *A. fistulosum* (Japanese bunching onion), *A. ampeloprasum* (leek, kurrat, great-headed garlic, and pearl onion), *A. schoenoprasum* (chives), *A. tuberosum* (Chinese chives)] and ornamentals [(*A. aflatunense*, *A. giganteum*, *A. karataviense*)]

(Kamenetsky and Rabinowitch, 2006). In addition, about two dozen other *Allium* species are locally cultivated or collected as highly valued vegetables, seasonings, and/or medicinal plants (Hanelt, 2001; Fritsch and Friesen, 2002). However, knowledge about these species is incomplete. The genus *Allium* is divided into various subgenera. The following figure (Fig. 2) shows the phylogenetic division of the genus *Allium*, according to Fritsch et al. (2010). The listed subgenera are also divided into numerous sections. Similar to many other plant crops, the possibility of gene introgressions from both parental species and close relatives is significantly reduced the farther they are from the center of evolution. Additionally, since their initial domestication, many immediate ancestors have either been lost or changed beyond recognition. Genetic shifts and drastic, unbalanced selection pressure by growers and breeders resulted in the loss of many traits important for modern agriculture; therefore genes of potentially useful characteristics were lost or are not readily available for crop improvement (Kamenetsky and Rabinowitch, 2006). The most characteristic constituents in *Allium* plants are organosulfur compounds, which are the most important substances in terms of both their chemotaxonomic value and biological activity (Rose et al., 2005). Organosulfur compounds are associated with a hot pungent taste and strong sulfur smell, and many health benefits of *Allium* species are attributed to these compounds (Fritsch and Keusgen, 2006). These substances are physiologically active and are used as antibiotic and antitumor agents (Kusterer and Keusgen, 2010). High levels of cysteine sulfoxides have also been shown to have antibacterial and antifungal properties that are probably beneficial for onions during extreme environmental conditions (Fritsch and Keusgen, 2006). The most important sulfur-containing substances are the amino acid cysteine and its derivatives, especially the S-substituted cysteine sulfoxides and the γ-glutamyl peptides.

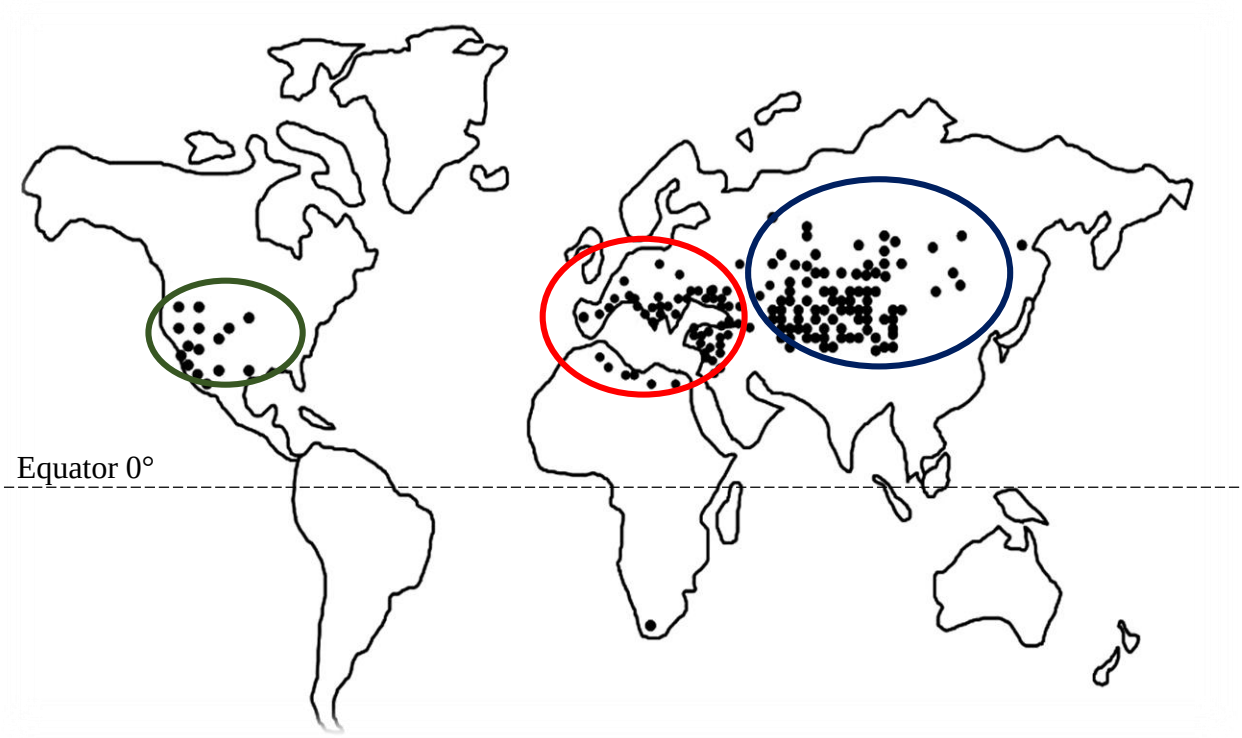


Fig. 1 Scheme of geographical distribution of bulbous *Allium* species, based on floristic literature from several different regions according to Kamenetsky and Rabinowitch, (2006)

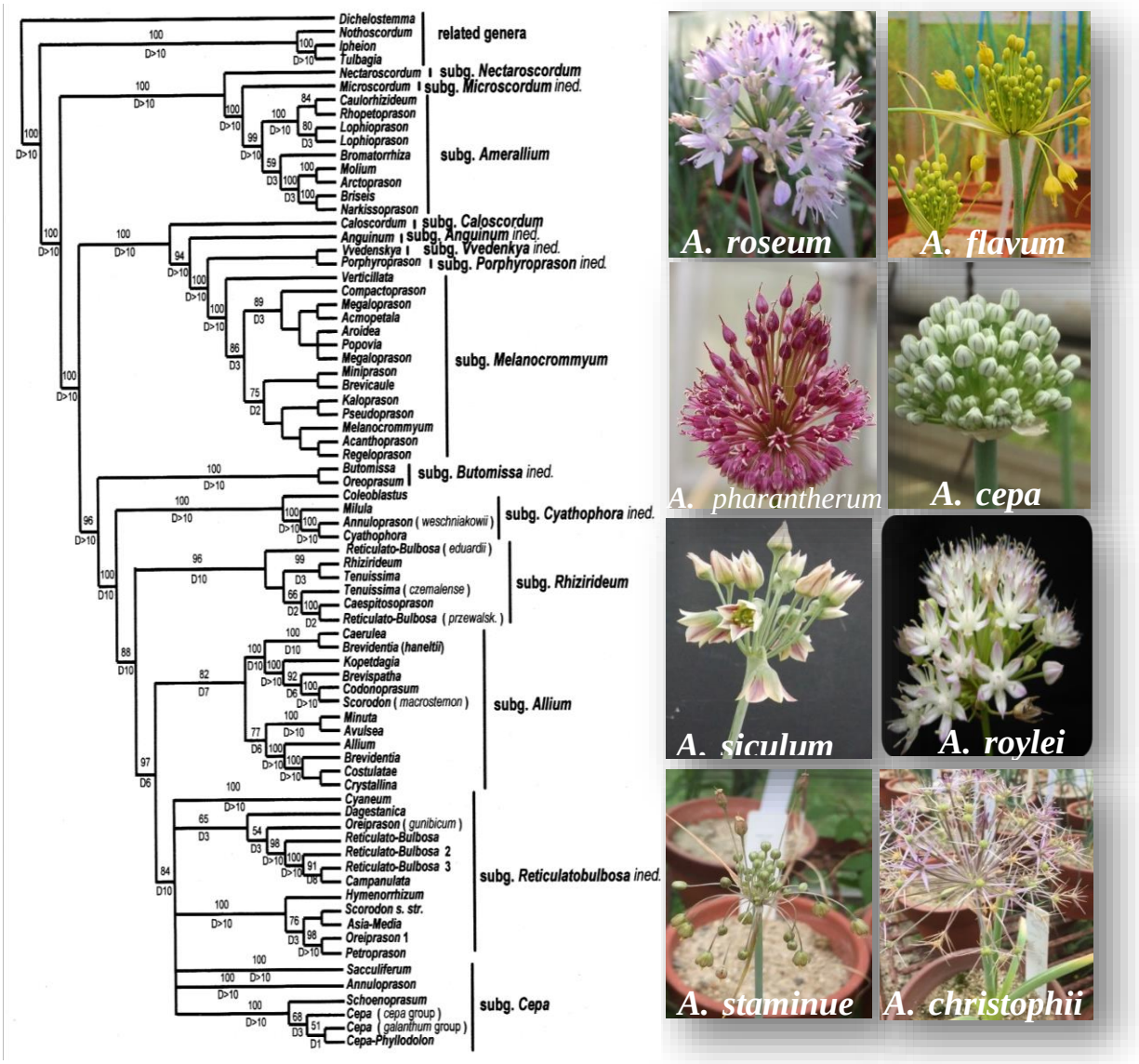
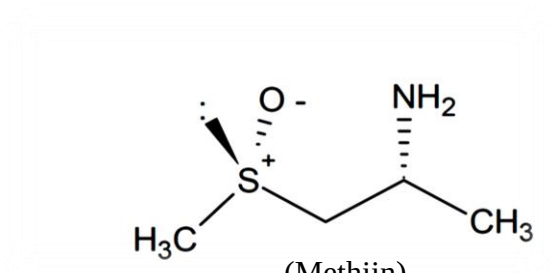


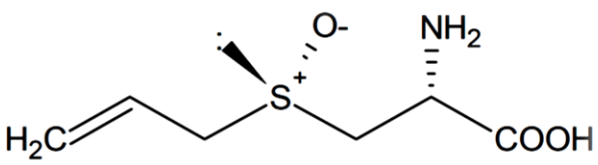
Fig. 2 Phylogenetic tree of *Allium* species according to Fritsch et al. (2010) (left side) and inflorescences photos of different *Allium* species grown at Yamaguchi University greenhouse (right side)

There are four basic representatives of cysteine sulfoxides: (+)-S-methyl-L-cysteine sulfoxide, (+)-Spropyl-L-cysteine sulfoxide, (+)-S-(2-propenyl)-L-cysteine sulfoxide, and (+)-S-(1-propenyl)-L-cysteine sulfoxide (Freeman and Whenham, 1975; Hashimoto et al., 1984). (Fig. 3). Differences between species and cultivars in flavour characteristics probably arise from variability in sulfur uptake and in its metabolism through the flavour biosynthetic pathway. Some *Allium* species exhibits a characteristic pattern of cysteine sulfoxide considerably different from that of garlic or onion, and their total amount may be higher that 1% of the bulb fresh weight (Fritsch, 2001).

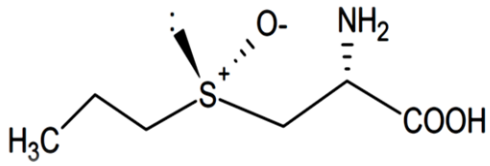
Chemotaxonomy of more than 40 *Allium* species from various subgenera revealed at least seven different chemotypes of the aroma profiles and showed specific arrays of volatile sulfur compounds in the rhizomatous species (Storsberg et al., 2003; Kamenetsky and Rabinowitch, 2006). This classification can contribute to a better selection of wild species for breeding experiments aimed at purposeful improvement of aroma, taste, and pharmacological properties of interspecific *Allium* hybrids. However, the proposed classification into chemotypes does not agree with taxonomical or bio-morphological divisions within the genus (Kamenetsky and Rabinowitch, 2006). On the other hand, various researchers tend to attribute the potential pharmacological benefits of *Allium* plants to constituents other than sulfur compounds, such as steroidal saponins and polyphenolic compounds, especially flavonoids, as well as fructans, N-cynnamic amides, and antioxidative enzymes, considered to be equally important (Matsuura, 2001; Lanzotti, 2005; Stajner et al., 2006; Lanzotti, 2012b).



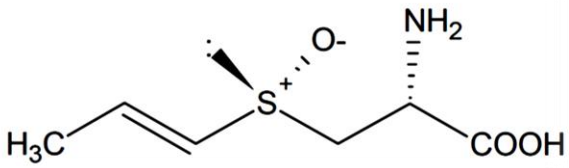
(Methiin)
(+)-S-methyl-L-cysteine sulfoxide



(Alliin)
(+)-S-(2-propenyl)-L-cysteine sulfoxide



(Propiin)
(+)-S-(propyl)-L-cysteine sulfoxide



(Isoalliin)
(+)-S-(1-propenyl)-L-cysteine sulfoxide

Fig. 3 Chemical structure of the four major representative cysteine sulfoxides compounds in *Allium* species

Allium species are a rich source of steroidal saponins with potential antifungal activity (Adao et al., 2011; Mostafa et al., 2013; Abdelrahman et al., 2014). Apart from the Amaryllidaceae family, steroidal saponins are widely distributed in other monocot families: Asparagaceae (*Agave*, *Asparagus*, *Convallaria*, *Hosta*, *Nolina*, *Ornithogalum*, *Polygonatum*, *Sansevieria*, *Yucca*), Costaceae (*Costus*), Dioscoreaceae (*Dioscorea*), Liliaceae (*Lilium*), Melanthiaceae (*Paris*), and Smilacaceae (*Smilax*). Interestingly, these compounds have been reported in some dicotyledonous angiosperms as well: Zygophyllaceae (*Tribulus, Zygophyllum*), Solanaceae (*Solanum*, *Lycopersicon*, *Capsicum*), Plantaginaceae (*Digitalis*), and Fabaceae (*Trigonella*) (Sobolewska et al., 2014). Several reports refer to pharmacological activities of steroidal saponins. Some of them showed promising antifungal, cytotoxic, anti-inflammatory, antithrombotic, and hypocholesterolemic effects (Sparg et al., 2004; Lanzotti, 2005). Steroidal sapogenins and saponins have been identified in more than 40 different *Allium* species so far. The earliest reports regarding *Allium* saponins date back to the 1970s and dealt with the identification of diosgenin in *A. albidum* (Kereselidze et al., 1970). Additional studies performed worldwide in the following years led to the isolation of a large number of new compounds (Sobolewska et al., 2014). Despite much research that has addressed the role of saponins as remarkable antifungal metabolites against different pathogens, few investigations have considered characterizing the distribution of total saponins within the different organs in *Allium* species (Abdelrahman et al., 2014). This information is very important, especially in vegetable crop research, for obtaining the most valuable plant material with optimum nutritional components for proper harvesting or breeding strategies. The prospects of saponin compounds as chemical markers for fungal disease resistance genotype

selection would be an interesting point for future onion genetic research (Abdelrahman et al., 2014).

With the development of genomic initiatives to outline genome and gene expression in the context of plant-environment interaction, metabolic profiling is required to better understand plant response mechanisms against various environmental stresses (Peremarti et al., 2014). MS-based metabolomics allow concomitant detection of several hundred metabolites as a snapshot of metabolomic phenomena (Sawada et al., 2009). Integrated metabolomics, genomics, and transcriptomics—referred to as *omics technologies*—play an important role in phytochemical genomics and crop breeding in sequenced plants (Saito and Matsuda, 2010).

In the present study, an approach for profiling targeted metabolites and RNAseq genotyping was adopted with a strong focus on saponin compounds and its biosynthesis genes as prospective chemically based genetic markers to 1) explore intraspecific variation within *Allium* species subg. Melanocrommyum and Nectaroscordum; 2) isolate, purify, and evaluate potential saponin compounds related to *Fusarium* disease resistance from *A. nigrum*; 3) isolate and purify several saponin compounds from shallots and their application as chemical markers for screening of *Fusarium* disease resistance candidates within different hybrid populations of shallots-bulb onions, F_1 and F_2 progeny; 4) explore the transcriptomic regulation of saponin biosynthesis genes and transcriptional factors by using a complete set of *A. fistulosum*-shallot monosomic additional lines; 5) evaluate the involvements of *A. roylei* metabolites in the phenotypic expression for disease resistance; and 6) investigate the transcriptome and target metabolome variability in doubled haploids of *A. cepa* for prospective stress responsiveness.

CHAPTER II. METABOLITE PROFILING OF ORANMENTAL *ALLIUM* SPECIES SUBG. MELANOCROMMYUM AND NECTAROSCORDUM USING CHEMICAL AND ANTIMICROBIAL ASSAYS

Introduction

The genus *Allium* L., comprising over 800-850 species (Hanelt et al., 1992; Fritsch et al., 2010). One of the large subgenera with in the genus is Melanocrommyum, which includes about 150 species distributed mainly in the Oriental-Turanic region, especially in the Turkestanic province (Fritsch, 1992a; Khassanov and Fritsch, 1994).The subgenus consists mostly of diploid perennial species with an extremely short developmental period (Fritsch, 1992a). This subgenus consists of xerophilous and heliophilous plants. Their preferred habitats are dry steppes, semi-desert and deserts and stony slops (Fritsch, 1993; Hanelt et al., 1992). Karyological analyses revealed uniform karyotypes without any clear species-specific or section-specific characteristics (Fritsch and Astanova, 1998). Despite the uniform karyotypes, genome size in Melanocrommyum is quite variable ranges from 26 to 50 pg for 2C DNA content (Ohri et al., 1998; Gurushidze et al., 2008). Due to their large and often dense inflorescences and peculiar flower colors, some members of the subgenus are cultivated as ornamentals, including hybrids derived from a few wild species such as *A. christophii*, *A. Karataviense*, *A. stipitatum* and *A. hollandicum* (Friesen et al., 1997). These non-edible *Allium* species may represent a good source for many natural compounds with therapeutic qualities (Kamenetsky & Rabinowitch, 2006).

Cysteine sulfoxides are one of the major metabolites that characterize *Allium* genus and play a critical role in determining the smell and taste of these plants (Keusgen, 1999). A previous

phytochemical analyses for Menlanocrommyum subgenera revealed that these plants species have a low ACSO contents (Fritsch and Keusgen, 2006), and this explain the odorless smell for most of the plants belongs to this subgenera. Polyphenols, anthocyanin, flavonoids, quercetin, kaempferol and their glycosides have been reported in *Allium* genus (Crozier et al., 1997; Fossen et al., 1997). These active metabolites have been reported to have antimicrobial and antioxidant properties, which is highly potential for the human health as well as for plant protection (Challier et al., 1998; Yin and Cheng, 1998; Stajner and Varga, 2003). Triterpene saponins are widely distributed in nature and typical constituents of dicotyledonous, while steroidal saponins are less distributed and usually found in many monocotyledonous, especially Dioscoreaceae, Agavaceae and Liliaceae. *Allium* plants belong to this last family and are known to possess steroidal saponins (Lanzotti, 2006). Saponins are a major family of secondary metabolites generally considered to be produced by plants to counteract pathogens (Osbourn, 1996; Francis et al., 2002; Sparg et al., 2004). Besides their role in plant defense, saponins are of growing interest for drug research as they are active constituents of several folk medicines and provide valuable pharmacological properties (Augustin et al., 2011). Saponins antifungal activity is attributed to their ability to form complexes with sterols and results in an increase of fungal membrane permeability and leakage of cell contents (Morrissey and Osbourn, 1999). Saponin accumulation is also known to be influenced by several environmental factors such as nutrient and water availability (Szakiel et al., 2011). Saponins are valuable compounds to humans due to their use in pharmacy industry, cosmetics, agriculture and food market (Tanaka et al., 1996; Cheeke 1998; Skene and Sutton, 2006; Sun et al., 2009). Therefore, significant efforts have been made in the qualitative and quantitative analyses of saponins in different plant species (Sen et al., 1998; Ward, 2000; Pecetti et al., 2006). In this chapter we describe the biochemical characterization of some bioactive compounds such as

S-alk(en)yl-L-cysteine sulfoxide (CSO), polyphenol , total sugar content and crude saponin content in the fresh bulbs of different *Allium* species from Melanocrommyum and Nectaroscordeum subgenera. Moreover, antimicrobial bioassay for the crude saponin extract against wide range of fungal pathogen was conducted.

Materials and methods

Plant material

Fresh bulbs from different *Allium* species subgenus Melanocrommyum and Nectaroscordum from Netherlands (Baltus) were used in this study (Fig. 4).

Determination of S-alk (en)yl-L-cysteine sulfoxide

Bulb fresh weight (5 g) was measured and microwaved for two minutes around 500W. The cooked tissue was gently weighted after heating to calculate the amount of water lost by heating (Y gm), (fresh weight X + water lost by heat Y) for the same amount of tissue dry weight was homogenized in a mortar pestle tissue. The homogenate was transferred to a centrifuge tube, and centrifuged at 4000 rpm for 10 minutes at room temperature. The supernatant was collected using micropipette and keep at -20 ºC. 300 µl was collected in Eppendorf tubes and centrifuged at 15000 rpm for 2 minutes. Supernatant was diluted 10X using 0.005% TFA buffer (Trifluroacetic acid) and filtrated by using 0.45 µm syringe-type filter (HCL-Disk3, Kanto Chemical Co., Inc., Tokyo, Japan). A 60 µl filtered sample was injected into a high performance liquid chromatography (HPLC) system and quantified. The HPLC system include pump, a Degasser, a Column oven, a diode array detector set to 220 nm, a data collection system (EZchrom Elite TM , Hitachi high-Technologies Corporation, Tokyo, Japan) SS-1251 column (4.6 mm i.d. x 250 mm long, Senshu Scientific Co.,

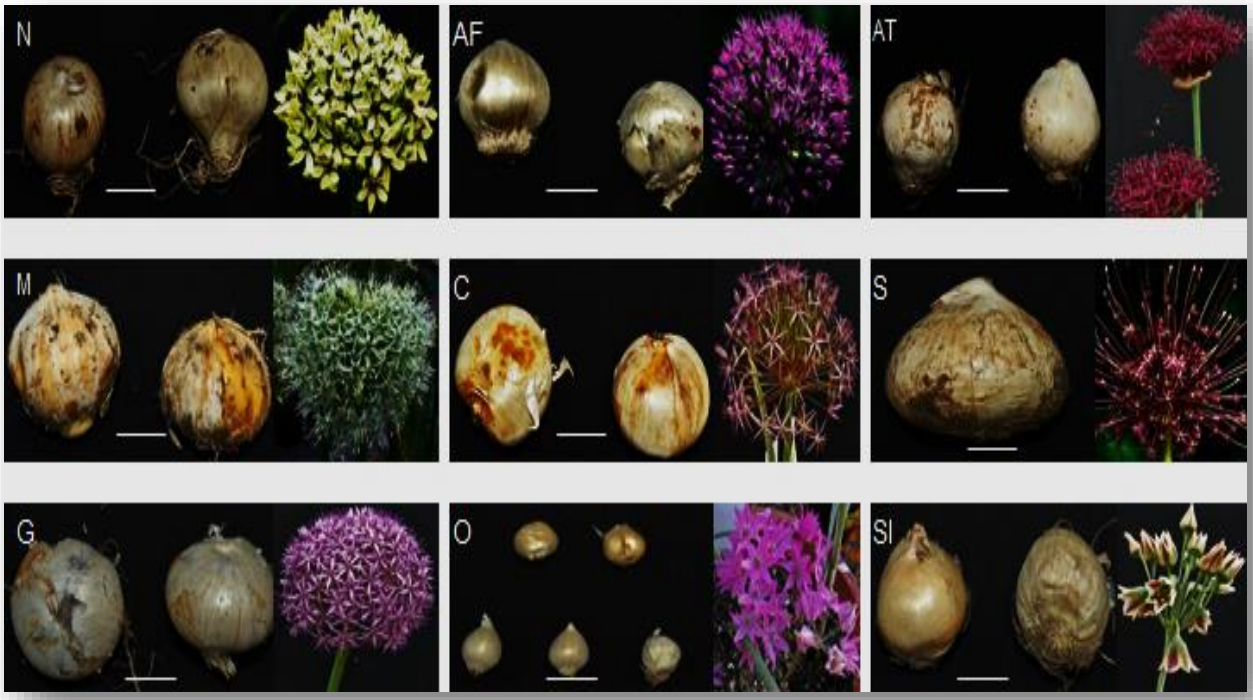


Fig. 4 Bulbs (left) and inflorescences (right) of *A. nigrum* (N), *A.aflatunense* (AF), *A. atropurpureum* (AT), *A. Mount Everest* (M), *A. christophii* (C), *A. schubertii* (S), *A. gladiator* (G), *A. oreophilum* (O) and *A. siculum* (SI). Scale bar = 1.5 cm

Ltd., Japan). The solvent was 0.005% TFA and flowed for 15 minutes at a flow rate 0.6 ml/min. Standard compounds were synthesized at Somatech Center (House Food Corporation, Japan). A series of standard were dissolved in distilled water and analyzed as described above.

Determination of total polyphenol

Total polyphenol measurement was performed according to the Folin-Denis colorimetric method (Folin and Denis, 1915). Two ml of sample Ethanol 70% extraction was first mixed with 5 volumes of water. Then one ml of this extract was mixed with one ml of Folin-Ciocalteau's reagent. After 10 min, one ml of 10% saturated sodium bicarbonate was added and mixed well. After one hour the absorbance of the mixed samples was measured by a spectrophotometer at 530 nm. Catechin was used as the standard to which the samples were compared.

Determination of total reduced sugar content

Sugar from fresh bulbs (5g) was extracted with hot 74% ethanol (final concentration 70%) for 15 minutes. The extract was filtered through filter paper 90 mm (ADVANCTEC). Five ml of the extract was filtered by a Sep-Pac C18 cartridge (waters) to remove pigments, the first two ml is eliminated and the other three ml is filtered with 0.45µm filter (ADVANCETEC). The filtrate was analyzed by HPLC analysis according to Yamashita et al. (1993). For HPLC analysis; a Hitachi Model L-2130 pump and L-7490 RI detector were used. Sugars were separated on LiChrospher NH2 column (MERCK), 4X250 mm using Acetonitrile:water solvent (85:15 v/v). The flow rate was 0.8 ml/min and the injection volume 20 µl and analysis time 30 minutes. Identification of fructose, sucrose and glucose was based on retention time using standards (0.5% fructose, sucrose, and glucose).

Saponin extraction

Bulbs were hand-cut, air-dried at room temperature without exposure to light giving a dry weight (*A. christophii*, *A. schubertii*, *A. mount everes*t, *A. aflatunense*, *A. atropurpureum*, *A. gladiator*, *A. nigrum*, *A. siculum*, *A. oreophilum* 21.0, 11.5, 20.0, 8.7, 10.0, 13.5, 8.3, 4.3, and 2.5 g, respectively). The dry bulbs were exhaustively extracted, at room temperature, with the following solvents in this order (100 ml *n*-Hexane, the mixture was sonicated for 15 minutes and keep in room temperature for one day. The defatted bulbs materials were extracted with 100 ml 100 % methanol (MeOH), the mixture was sonicated for 15 minutes and keep in room temperature for one day, each step was repeated three times and filtered. The MeOH extract was partitioned using 100 ml *n*-Butanol and H_2O (1:1, v/v) in order to remove sugar compounds using separation funnel this step was repeated three times. The butanol organic layer was filtered and then concentrated under vacuum dried giving a crude extract.

Determination of total saponin content

Total saponin content in the all species under study was determined spectrophotometrically according to (Ebrahimzadeh and Niknam, 1998). 500 µl of crude saponin extract solution was diluted with 500 µl of H_2O. one ml of the diluted sample was mixed with one ml of (0.7% Vaniline-60% H_2SO_4, v/v) reagent. After that mixture was incubated in water path at 60ºC for one hour it was followed by ice cooling for 10 minutes, the color will be changed into brown and sample is ready to measure spectrophotometrically at 473 nm using Hitachi, Model U-2001. The same process was submitted for control preparation and saponin standard (Disogenin, Merck). Determination of saponin concentration was based on average value of absorbance at each concentration of standard.

Crude saponin polymorphism using thin layer chromatography

A common simple, inexpensive and fast method for the separation of saponin compounds from mixture is thin layer chromatography (TLC). A small amount of the extract (10-20 µl) is applied approximately at 1.5 cm from the bottom of a thin layer of the TLC plate. The TLC plate placed in a glass container with solvent $CHCl_3$: MeOH: H_2O (7:3:1, v/v/v) filled to approximately one cm from the bottom. The solvent will move to the top of the TLC plate as result of capillary action. Since each compound of saponin extract in the matrix will have a unique way of interacting with the matrix and the solvent, some saponin compounds will move faster on the top of the TLC than others.

Antifungal activity

Antifungal activity of the crude saponin extract from different ornamental Allium species were tested on soil-borne pathogens (*Fusarium oxysporum* f. sp. *cepae*, *F. oxysporum* f. sp. *spinaciae*, *F. oxysporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *fragariae*, *F. oxysporum* f. sp. *lagenariae*, *F. oxysporum* f. sp. *lycopersici* and *F. verticillioides*) and air-borne pathogen *Colletotrichum graminicola* and *Colletotrichum gloeosporioides*). Antifungal activity was assessed by the disc diffusion method. Plates of 9 cm were filled with 20 ml of potato dextrose agar media (PDA), after solidification a 5 mm disc of selected agar fungal growth was inoculated in the center of the plate media surface. A 5 mm disc of agar was removed by sterilized cork-porer at 1.5 cm from the Center of the fungal inoculation and three different concentrations 500, 1000 and 1500 µg/ml of crude saponin solution were added to the wells. Plates were incubated at 25°C and after 96 h the inhibition zone was measured in compare with control.

Results and discussion

S-alk(en)yl-L-cysteine sulfoxides (ACSOs) content in ornamental *Allium* species

The quantitative and qualitative differences in flavor precursor content result in the different flavors of many of the *Allium* spp., particularly the presence or absence of PeCSO which is responsible for the lachrymatory effect of onions and AlCSO which produces the characteristic taste of garlic (Lancaster and Shaw 1989). The examined plants in this study are belonging to subgenus Melanocrommyum except for *A. siculum* which belongs to *Nectaroscordum* section, according to the modern *Allium* taxonomy (Gregory et al., 1998) and two ornamental species *A. Mount Everest* and *A. glaidator*. The S-alk(en)yl-L-cysteine sulfoxides were separated with baseline resolution in this method. MeCSO was the first separated (retention time 6.4 min), followed by AlCSO (7.4 min) and PeCSO (9.4 min). Among the all plants examined, MeCSO was the most abundant organo-sulfur compound. The highest MeCSO content was recorded in *A. gladiator* (1.73 mg/g FW), *A. siculum* (1.77 mg/g FW) and *A. Mount Everest* (0.31 mg/g FW). Only very low traces of AlCSO and PeCSO were detected in the all tested plants except for *A. siculum* which show a detectable amount of AlCSO (0.7 mg/g FW) and PeCSO (0.1 mg/g FW) (Figs. 5 and 6). These findings are in accordance with previous investigations (Freeman and Whenham, 1975; Fritsch and Keusgen, 2006) who found that cysteine sulfoxides content were below 0.1% in the species of bulbous subgenera Melanocrommyum, where most of the plants materials in this study belong. Furthermore, the detectable amount of cysteine sulfoxides in *A. siculum* was previously reported by (Lancaster and Shaw 1989) who found that *A. siculum* contains MeCSO as the major flavor precursor with minor amounts of PeCSO. *A. gladiator* and *A. Mount Everest* which show a high MeCSO content are commercial ornamental plant originated from *A.*

stipitatum and this later one have been reported to have a strong unpleasant smell, and its MeCSO content was high (Fritsch and Keusgen, 2006). Moreover, most of these plants have no special smell except for *A. siculum* which make this plant species is potential to be involved in the *Allium* breeding programs to improve the aroma and taste of the hybrid *Allium.*

Total polyphenol content

Phytochemicals, particularly polyphenols from natural sources such as vegetables and fruits have gained popularity due to their protective properties against several chronic diseases such as cancer and cardiovascular diseases (Temple, 2000). Most of the polyphenol studies on *Allium* have been focused on edible *Allium* species such as garlic (*A. staivum*), onion (*A. cepa*), shallot (*A. cepa* L. Aggregatum group) and leek (*A. porrum*). However, data on the polyphenol content in other *Allium* species are certainly lacking (Dziri et al., 2012). Total phenol compounds (TP), as determined by Folin Ciocalteu method, are reported as Catechol equivalents by reference to standard curve. There was no much variance in the TP content among the plants examined in this study. The total phenolic contents ranged from 28 to 41.6 mg/100g FW (Fig. 7). *A. oreophilum* showed the highest TP content (41.6 mg/100g FW) and *A. nigrum* was the lowest (28 mg/100g FW). The TP content of these ornamental *Allium* species expressed in equivalent catechol was in general lower than garlic (61.8 mg/100 g FW) and shallot who has the highest phenolic content (114.7 mg/100 g) among the bulb onion verities tested by (Lanzotti, 2006), and higher than that of onion (31.0 mg/100 g FW) as reported by (Kaur and Kapoor, 2002). Previous reports regarding the quantification of TP in other *Allium* species showed quantitative differences that seem to depend on plant species/cultivar or variety origin and plant organ (Yang et al., 2004; Prakash et al., 2007).

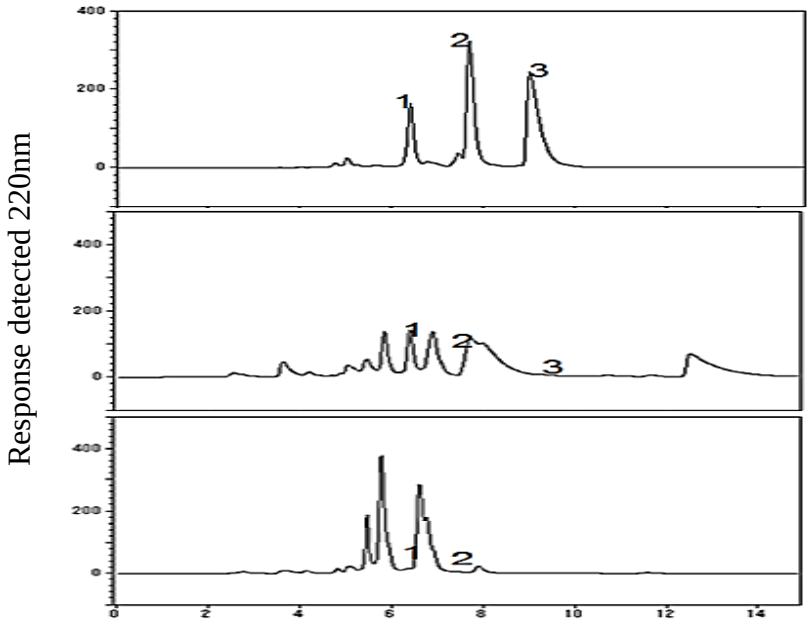


Fig. 5 Representative chromatograms of MeCSO (1), AlCSO (2) and PeCSO (3) in: (A) standard, (B) *A. siculum* and (C) *A. nigrum*

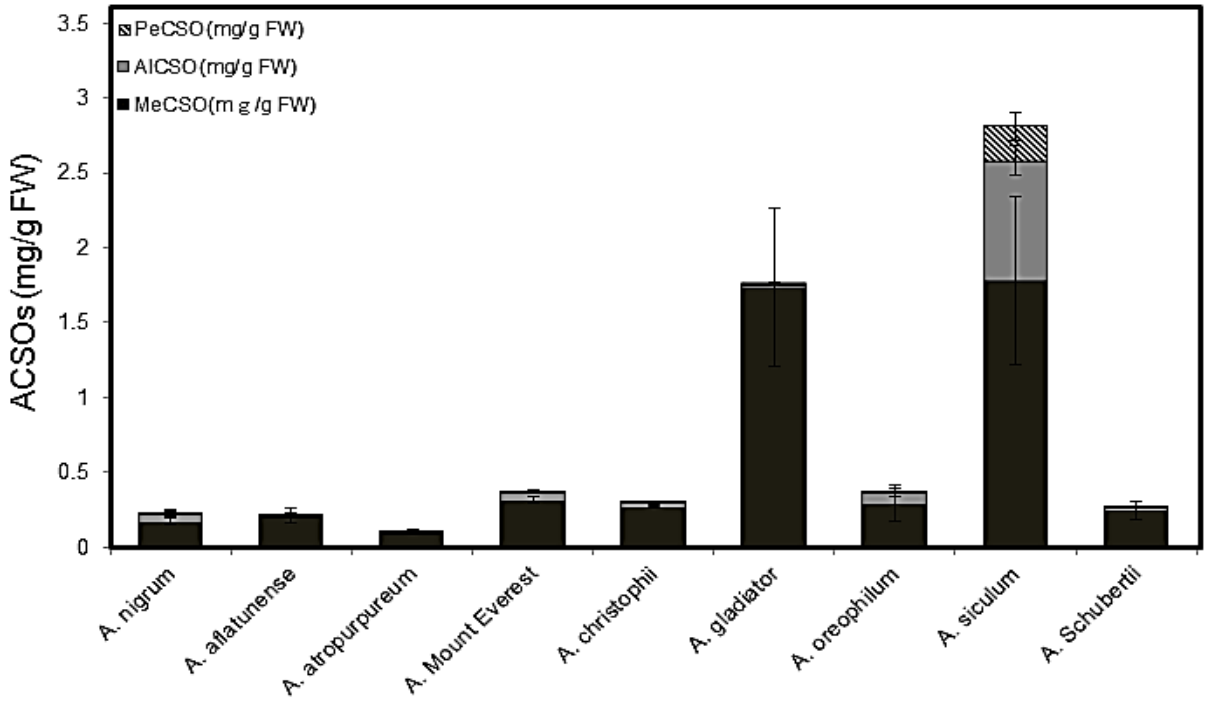


Fig. 6 Total content of MeCSO, AlCSO and PeCSO in different ornamental *Allium* species. Values are means ± SE (*n*=2)

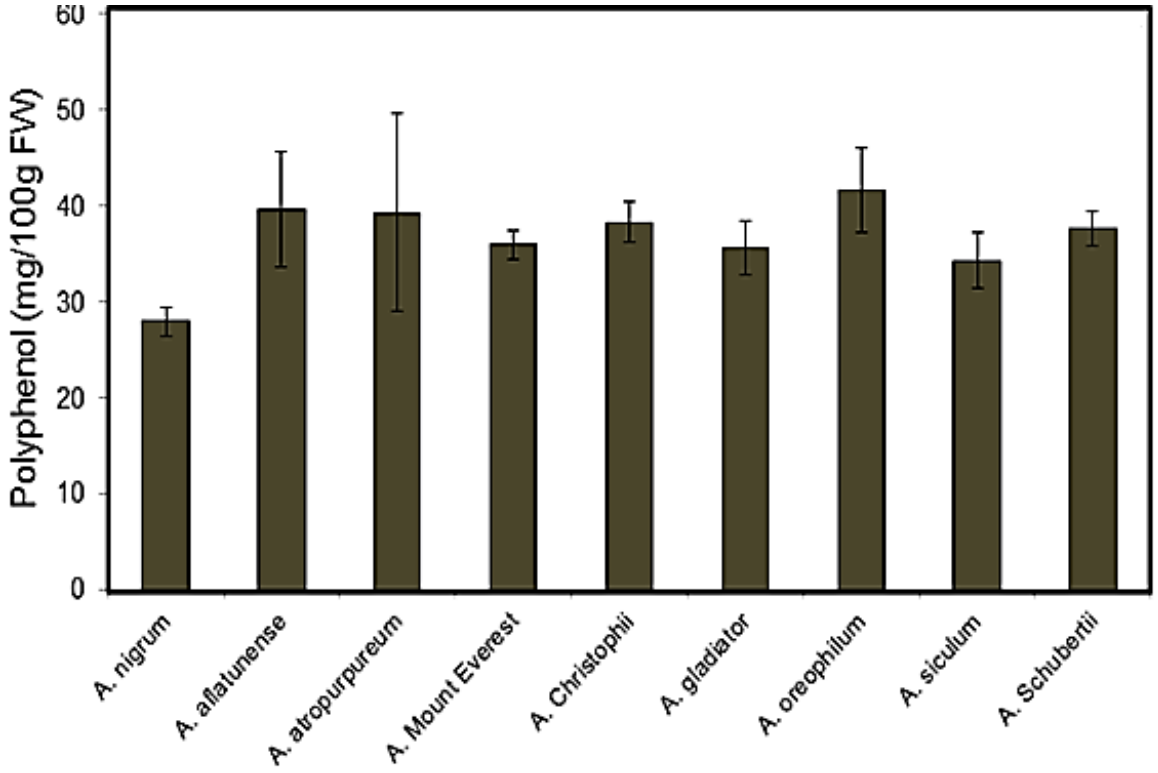


Fig. 7 Total polyphenol content mg/100g FW in different ornamental *Allium* species. Values are means ± SE (*n*=2)

Total reduced sugar content

Carbohydrates are the most abundant class of chemical compounds in *Allium* species and include glucose, fructose and sucrose, together with a series of oligosaccharides, the fructans (Darbyshire and Steer, 1990; Kamenetsky and Rabinowitch, 2006). The water soluble carbohydrates content in the examined *Allium* species ranged from 7.5 to 19 mg/g FW (Fig. 8). Only sucrose was represented on the chromatogram however, glucose and fructose was not detected. The highest sucrose content was determined in *A. oreophilum* (19 mg/g FW) and the lowest content was determined in *A. schubertii* (7.5 mg/g FW). The total reduced sugars contents in the investigated plants showed low sugars content in compare with the edible one. Total sugar content in *A. cepa* was ranged from 13.5% to 71.8% in Nigerian cultivars (Ketiku, 1975). A previous record for total reduced sugars content in *A. cepa* was 55 mg/g DW according to (Abrameto et al., 2010). Three sugars (glucose, fructose and sucrose) were identified in all the onion samples examined by (Galdon et al., 2009), and the total sugar content ranged from 2.3 to 3.79%. These findings are higher than the total sugar in our data. The wide range variability of the carbohydrates content with in the different *Allium* species could be influenced by the differentiation of the genomic levels. A previous study by (McCallum et al., 2006), revealed a one significant QTL in chromosome 8 of intraspecific mapping population 'BYG15-23' x 'AC43'using a complete molecular marker map and this locus, provisionally named Frc, may account for the major phenotypic differences in bulb carbohydrate content.

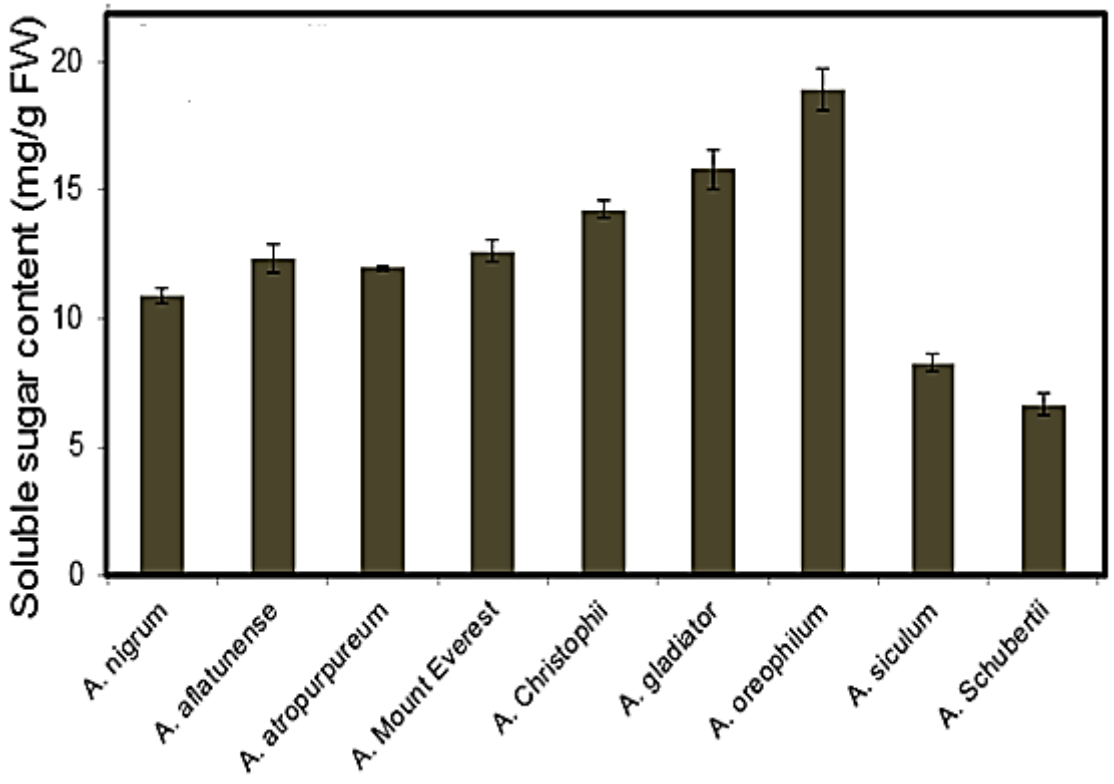


Fig. 8 Total reducing sugars content (mg/g FW) in different ornamental *Allium* species. Values are means ± SE (*n*=2)

Total saponin content

Saponins are a large group of glycosides, widely distributed in the plant kingdom. They are believed to form the main constituents of many plant drugs and folk medicines (Estrada et al., 2000). The content of saponins with in plants is quite variable and it's mainly reflects the physiological status of the plant, which mainly depend on the stage of the growth and development (Szakiel et al., 2011). Total saponin content among the examined plants revealed variable qualitative and quantitative saponin content (Fig. 9). TLC analysis of the crude saponin from *A. schubertii* showed a remarkable saponin profile in compare with other TLC profiles from other extracts. Moreover, the quantitative spectral analysis of the total saponin content was almost in correlation with the TLC figure where high saponin content was detected in *A. schubertii* and lowest content was detected in *A. Mount Everest*. The overall conclusion from these outputs, that each plant has its unique saponin profile which can be translated into a specific bioactivity and this will be precious tools for better selection of wild *Allium* species to be used in the breeding program to improve disease resistance in edible *Allium*. Furthermore, previous reports have showed that even though among the same species the saponin content could be variable and this variability of saponin content could be influenced by the surrounding environment. The local geoclimate, seasonal changes, external conditions such as light, temperature, humidity and soil fertility as well as cultivation techniques affect both quantitative amount and qualitative composition of saponins (Augustin et al., 2011; Szakiel et al., 2011). Significant quantitative and qualitative differences in saponin content depending on the site of growth have been reported (Lim et al., 2005).

Crude Saponin assay

Antifungal activity of the crude saponin extract (500, 1000 and 1500 ppm) from each plant was tested on nine different fungal pathogens. Seven soil-born pathogen (*Fusarium oxysporum* f.sp. *cepae, F. oxysporum* f. sp. *spinaciae, F. oxysporum* f. sp. *radicis-lycopersici, F. oxysporum* f. sp. *fragariae, F. oxysporum* f. sp. *lagenariae, F. oxysporum* f. sp. *lycopersici* and *F. verticillioides*), and two air pathogen *Colletotrichum graminicola* and *Colletotrichum gloeosporioides*). All the crude saponin extracts significantly showed concentration-dependent antifungal activity except for the crude saponin from *A. siculum* which doesn't revealed a strong antifungal activity (Fig. 11). Relatively higher antifungal activities against all fungus strains were observed in *A. schubertii*, *A. atropurpurm*, and *A. nigrum* in compare with other plants extracts. *C. graminicola* and *C. gloeosporioides* were highly sensitive to the crude saponin in compare with *Fusarium* strains. The high antifungal activity of the crude saponin from the ornamental *Allium* species suggested that these bioactive metabolites may act as chemical barriers to protect the plant against pathogen attack. Our finding is matching with many previous reports about the antifungal activity of saponin compounds (Zimmer et al., 1967; Osbourn, 1996; Nicol et al., 2002; Lanzotti et al., 2012a and b). Moreover, the different *Fusarium* sensitivity to the variant crude saponin may be related to the different nature of the aglycone in each extract. The correlation coefficient between the total saponin content and antifungal activity revealed a positive but week correlation (Fig. 11). The scatter plot of the metabolite signature in each genotype was illustrated by principal components analyses (Fig. 10). PC1 show the highest significant of variance with 45.87%, mainly dominated by ACSO and saponin, and PC2 18.06 % with sucrose and saponin as a major component. Further biochemical analysis will be conducted to isolate and identify the pure compounds responsible for this bioactivity.

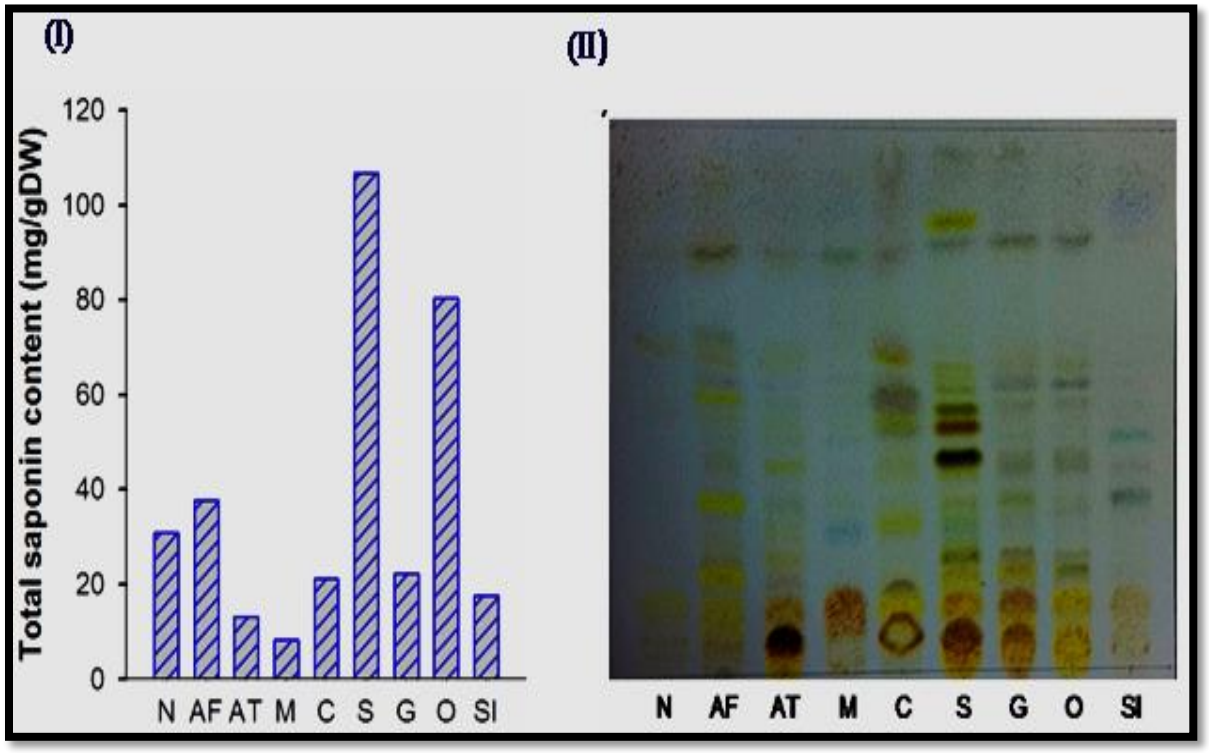


Fig. 9 Total saponin contents (I) and TLC profiles (II) of crude extracts in *A. nigrum* (N), *A. aflatunense* (AF), *A. atropurpureum* (AT), *A. Mount Everest* (M), *A. christophii* (C), *A. schubertii* (S), *A. gladiator* (G), *A. oreophilum* (O) and *A. siculum* (SI).

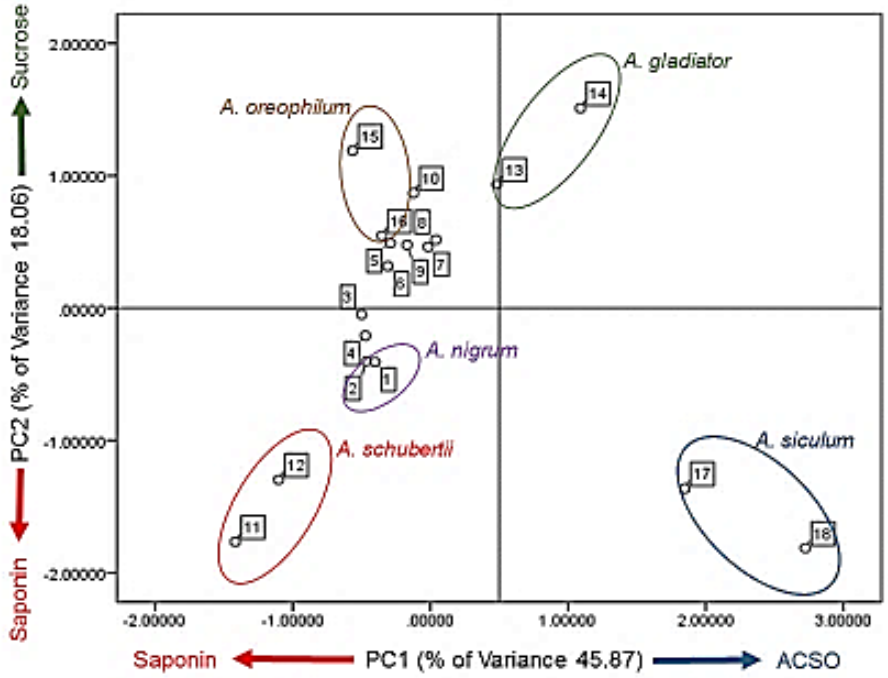


Fig. 10 PCA of the metabolite profile in different ornamental *Allium* species

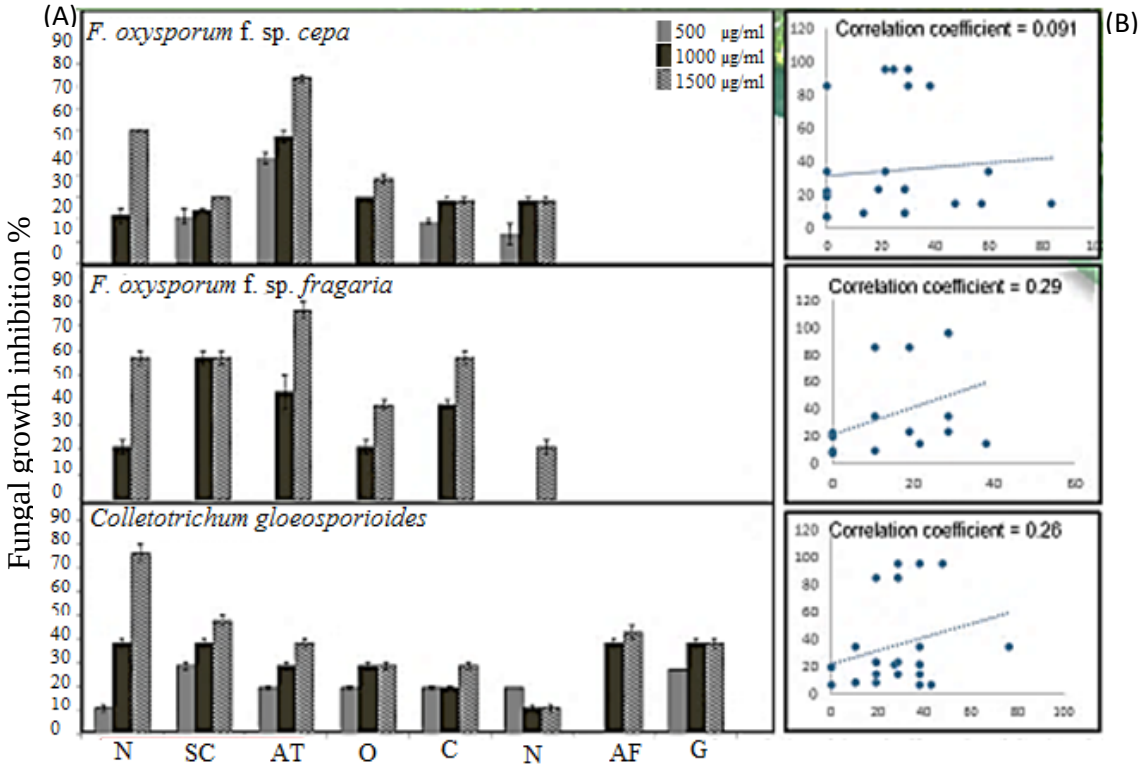


Fig 11 Percentages of antifungal inhibition at 500, 1000 and 1500 µg/ml of the crude saponins against different fungal strains (A) and correlation coefficient between total saponin content (x-axis) and antifungal activity (y-axis) (B). Values are means ± SE (n=3)

CHAPTER III. AGINOSIDE SAPONIN, A POTENT ANTIFUNGAL COMPOUND, AND SECONDARY METABOLITE ANALYSES FROM *ALLIUM NIGRUM* L.

Introduction

Plants during their life cycles produce a diverse array of secondary bioactive metabolites, many of which are involved in plant defense (Dixon, 2001). Based on this concept, the phytochemical diversity of antimicrobial compounds includes saponins, phenolics, and indoles. Saponins are a major family of glycosides with triterpenoid or steroidal aglycone and show remarkable antifungal activities widespread among many plant species (Osbourn, 1996; Barile et al., 2007; Lanzotti et al., 2012a). Triterpene saponins are the most common saponins, occurring mainly in dicotyledonous angiosperms, while steroidal saponins are less widely distributed and are present exclusively in monocotyledonous families (Bruneton, 1995). *Allium* spp. possess antibacterial and antifungal activities and contain powerful sulfur and other phenolic compounds which hold potential for future research (Griffiths et al., 2002). *Allium* plants represent the most economically important and representative genus of the Alliaceae. They are also a rich source of steroidal saponins with potential antifungal activity (Adao et al., 2011). Different *Allium* species, such as bulb onion (*Allium cepa* L.), garlic (*Allium sativum* L.), shallot (*A. cepa* L. Aggregatum group), chive (*Allium schoenoprasum* L.), and leek (*Allium porrum* L.), have been used in folk medicine and food for a long time (Fattorusso et al., 2000). *Allium nigrum* L. is an ornamental plant of the Alliaceae family subgenera Melanocrommyum, first described by Linnaeus in 1762. According to an ethnobotanical reference in Sicily, this species was used as a food spice (Lentini and Venza, 2007). Aginoside, a spirostane saponin, was first isolated from bulbs of *Allium giganteum*

(Kawashima et al., 1993) and recently was reported in *A. nigrum* (Jabrane et al., 2011). To date, most pharmacological and phytochemical studies regarding aginoside have focused on clinical pathogens and cancer cell lines. However, the antifungal activity of the aginoside compound against phytopathogens has not been investigated. In this study, a phytochemical analysis of *A. nigrum* was carried out to evaluate the potential of *A. nigrum* for use in plant breeding programs to improve disease resistance and health properties in other *Allium* species. In addition, *A. nigrum* is a natural source of agrochemicals that can be used to manage pests and fungi. We determined the content of cysteine sulfoxides, total polyphenols, and total saponins in different organs of *A. nigrum* by using high-performance liquid chromatography (HPLC) and spectral techniques. Furthermore, we isolated, quantified, and assessed the antifungal activity of the aginoside compound in a wide range of phytopathogens

Materials and Methods

General experimental procedures

Optical rotations were taken with a JASCO DIP-1000 automatic digital polarimeter. 1H and 13C NMR spectra were recorded in a pyridine-d5 solution on a JEOL ECA 500 (500 and 125 MHz, respectively) spectrometer. Chemical shifts are reported in parts per million (ppm) and J values in Hz using pyridine-d5 for 1H NMR (7.20 ppm) and 13C NMR (123.5 ppm) as an internal standard. The HR-ESI-MS was recorded with a JEOL JMS-T100LP spectrometer (Tokyo, Japan). Isolation of the compound was carried out using glass column chromatography (3 cm x 60 cm) on silica gel C-300 Wakogel (Wako, Tokyo, Japan). Thin-layer chromatography (TLC) was performed on

precoated silica gel plates (60 F254; Merck, KgaA, Darmstadt, Germany). The saponin profile was detected by spraying with a 5 ml *p*-anisaldehyde reagent.

Plant materials

Bulbs of wild *A. nigrum* L. were obtained from the Netherlands (Baltus Co., Vaassen). They were grown in a greenhouse (23 $\pm$ 2°C) at Yamaguchi University, Japan, from October 2010 and harvested in May 2011.

Extraction and isolation

Fresh root–bulb basal stem of *A. nigrum* (80 g) was hand-cut and air-dried at room temperature (22°C), and the final dry weight (30 g) was obtained and used for this study. The dry weight was exhaustively extracted at room temperature with the following solvents: *n*-Hexane and 70% MeOH. Each solvent extraction step was conducted for 1 day and repeated three times with 30 min of sonication and filtering. The MeOH extract was dried in a rotary evaporator with a vacuum pump (v-700; BUCHI, Rotavapor R-3) and then partitioned between BuOH and H_2O (1:1, v/v). The BuOH layer was filtered and then concentrated under vacuum, giving a saponins crude extract (1.69 g). The aliquot of the crude extract was chromatographed by C300 silica gel column chromatography (3 cm X 60 cm; AG Tokyo, Japan). The column was developed using a gradient solvent system starting with $CHCl_3$, $CHCl_3$–MeOH (9:1–1:9), MeOH, and MeOH–H_2O (9:1–7:3) as eluents to give 10 fractions after the evaporation of solvents (AN1–AN10). Fractions were re-chromatographed using TLC silica gel plates (60 F254; Merck KgaA, Darmstadt, Germany). The chromatogram was developed with chloroform:methanol:water (30:15:2.5, v/v/v). In a preliminary NMR study of the eluted fractions, AN8 gave a pure compound (13 mg).

Determination of aginoside content in different organs

Different concentrations (6, 12, 30, and 36 mg) of purified aginoside compound isolated from the root–bulb basal stem and crude saponin extract from the root, bulb, and leaves were subjected to TLC, as mentioned earlier. Quantification of the TLC band intensity of the purified aginoside compound and crude extract was detected using Image J version 1.42Q/Java 1.6.0_10 image analysis. The aginoside content in the crude extract of each organ was calculated by comparison to the purified aginoside calibration curve.

Extraction and analysis of S-alk(en)yl-L-cysteine sulfoxides

The *A. nigrum* bulb, leaf, and root fresh weight (2.5 g) were water extracted according to the method of Masamura et al. (2011). The extract was diluted 10 times using a 0.005% trifluroacetic acid buffer (TFA) and filtrated using a 0.45 mm syringe-type filter (HCL-Disk3; Kanto Chemical Co., Inc., Tokyo, Japan). A 60 ml filtered sample was injected into a high performance liquid chromatography (HPLC) system and quantified. The HPLC system includes a pump, a degasser, a column oven, a diode array detector set to 220 nm, a data collection system (EZchrom Elite TM; Hitachi High-Technologies Corporation, Tokyo, Japan), and an AQUASIL SS-1251-120 column (4.6 mm i.d. 250 mm long; Senshu Scientific Co., Ltd., Japan). The solvent, 0.005% TFA, flowed for 15 min at a flow rate of 0.6 ml/min. Standard compounds were synthesized at the Somatech Center (House Food Corporation, Japan).

Determination of total phenol content

Two grams of leaf, bulb, and root tissue was extracted by 70% EtOH according to the method of Hang et al. (2004). The extracted materials were adequately diluted with water, and the total phenolic (TP) contents were determined by the Folin–Ciocalteau method (Folin and Denis, 1915).

The polyphenol contents were quantified spectrophotometrically at 530 nm (Hitachi, Model U-2001, Tokyo, Japan).

Determination of total saponin content

Dried root, bulb, and leaf powder (160 mg) of *A. nigrum* was extracted with 70% MeOH, as previously described. The total saponin content was determined spectrophotometrically at 473 nm in the crude extract from each organ according to Ebrahimzadeh and Niknam (1998) using 0.7% Vanillin-60% H_2SO_4 reagent and a saponin standard serial dilution (Disogenin, Merck, Germany).

Biological assays

The antifungal activity of crude saponins, CSO, and TP was tested on seven soil-borne pathogens (*F. oxysporum* f. sp. *cepae*, *F. oxysporum* f. sp. *spinaciae*, *F. oxysporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *fragariae*, *F. oxysporum* f. sp. *lagenariae*, *F. oxysporum* f. sp. *lycopersici*, and *F. verticillioides*) and one air-borne pathogen (*C. gloeosporioides*). Pathogens were obtained from the Laboratory of Plant Molecular Pathology, Faculty of Agriculture, Yamaguchi University, Japan. The antifungal activity was evaluated with the agar plate diffusion method using 3.2 cm Perspex plates of potato dextrose agar (PDA). Crude saponin, CSO, and TP were added to obtain three final concentrations (500, 1000, and 1500 ppm), and the plates were inoculated with a 5 mm plug containing the fungi grown on PDA for five days. The plates were incubated at 25°C, and the fungal radical growth was measured after one week. The antifungal activity of the aginoside compound was assessed against three soil-borne pathogens (*F. oxysporum* f. sp. *cepae*, *F. oxysporum* f. sp. *radicis-lycopersici*, and *F. verticillioides*) and two airborne pathogens (*B. squamosa* and *C. gloeosporioides*). Plates of 3.2 cm of PDA added to the aginoside at four final concentrations (10, 100, 200, and 400 ppm) were inoculated with a 5 mm plug containing the

fungi grown on PDA for five days. The plates were incubated at 25°C, and the fungal radial growth was measured after one week. The antifungal activity was assessed by the *in vitro* spore germination test as described by Lorito et al. (1996). A suspension of 10^3 spores of *B. cinerea* and *C. gloeosporioides* was prepared in 100 ml of potato dextrose broth (PDB) separately. Aginoside was added to obtain three final concentrations (10, 50, and 100 ppm). One hundred microliters of each solution was placed in an ELISA 96-well plate (Nunclon, Denmark) and incubated at 25°C. The number of germinated spores and the hyphal length were measured after 24 h using ELISA (Emax, S/N 9807, USA) at 620 nm. Tomato and strawberry leaves were detached from 4- to 6-week-old greenhouse plants and sprayed with aginoside at three concentrations (10, 50, and 100 ppm). Treated and non-treated tomato and strawberry leaves were inoculated with 5 mm mycelial agar discs of *B. cinerea* and *C. gloeosporioides*, respectively. A disc was placed in the middle of each leaflet, which was then placed in a closed plastic container on wet filter paper to maintain 70% humidity. The containers were placed in an incubator at 22°C for 10 days. Lesion development was recorded daily.

Statistical analysis

All experiments consisted of three replications. Values are expressed as the mean $\pm$ standard error (SE) and analysis of variance (ANOVA) calculated using SPSS, Inc. 16.0. The significant effects of treatments were determined by the magnitude of the P-value ($P < 0.05$). Treatment means were separated by the Tukey's Honestly Significant Differences (HSD) test.

Results and discussion

S-alk(en)yl-L-cysteine sulfoxides

Methiin (MeCSO) was first separated at a retention time of 6.4 min, followed by alliin (AlCSO) at 7.4 min and isoalliin (PeCSO) at 9.4 min, as shown in a previous study (Masamura et al., 2011). Only a small amount of total cysteine sulfoxide (0.367 mg/g FW) was detected in *A. nigrum* (Fig. 12A). These findings are in agreement with previous investigations in which the total cysteine sulfoxide contents were below 0.1% in proportion to the fresh weight in the examined *Allium* species of bulbous subgenera Melanocrommyum (Fritsch and Keusgen, 2006). Among different plant organs, the highest MeCSO content was detected in the bulb (0.17 mg/g FW), whereas a trace amount of MeCSO was detected in the leaf (0.10 mg/g FW) and root (0.08 mg/g FW). Our data agree with those obtained by Kusterer et al. (2011), who reported methiin as the predominant CSO in all investigated samples of a wild *Allium* species subgen. Melanocrommyum, with the highest amount found in *Allium suworowii* (0.6% FW) and the lowest in *Allium rosenorum* (0.01% FW). AlCSO was detected only in the bulb (0.011 mg/g FW). On the other hand, this compound was not detected in the leaf or root. PeCSO was not found in any plant organs. These findings contradict the previous reports by Fritsch and Keusgen (2006), who found detectable amounts of PeCSO in all investigated *Allium* species subgen. Melanocrommyum. The low level of cysteine sulfoxides in *A. nigrum* is the reason many *Allium* species among the Melanocrommyum lack a specific smell (Rabinowitch and Currah, 2002).

Total polyphenol

The total phenolic contents were highest in the leaf organ (116.05 mg CE/100 g FW), followed by the root (65.09 mg CE/100 g FW). The lowest amount of TP (33.29 mg CE/100 g FW) was detected in the bulb (Fig. 12B). This organ-dependent compartmentation of TP was recently reported for *A. roseum* (736.65 and 749.54 mg CE/100 g of DW) in flowers and leaves, respectively, the richest organs in terms of TP (Najjaa et al., 2011). Nencini et al. (2011) reported higher TP contents (1.23, 1.22, and 1.36 mg GAE/g) in the leaves than in the bulbs (0.25, 0.39, and 0.73 mg GAE/g) of *A. neapolitanum* Cyr., *A. subhirsutum* L., and *A. sativum* L., respectively. From a quantitative viewpoint, it is difficult to compare our findings with those of previous reports because of the variations in the conditions, extraction solvent used, and TP assays. Most reports regarding the quantification of TP in other *Allium* species show quantitative differences that seem to depend on the plant species/ cultivar or variety origin and plant organ (Prakash et al., 2007; Dziri et al., 2012). Exposure to UV-B radiation and light intensity could be a reason for the higher accumulation of polyphenol compounds in the leaves than in the bulbs, which, from a biological viewpoint, have a protective role against environmental abiotic stress (Wang and Feri, 2011). The interesting finding in our results is the detectable amount of polyphenols in the root organ. The antimicrobial activity of the polyphenol compounds to protect a plant against pathogens could explain this accumulation. Many previous studies have reported the antimicrobial activity of polyphenols as efficient protection against pathogens and predators (Popa et al., 2002; Wang and Feri, 2011).

Total saponin content

Saponins accumulated in the vegetative organs of *A. nigrum*, with a higher content in the roots (19.38 mg/g DW) and lower contents in the bulbs (15.65 mg/g DW) and leaves (10.48 mg/g DW). These results show a dynamic variance trend of total saponins among different organs (Fig. 12C). Such organ repartition, with higher saponin accumulation in the root organs than in the aerial parts (stem and leaves), was previously reported by Teng et al. (2009). Szakiel et al. (2011) reported that the saponin content of plants is variable and may be affected by the surrounding environment, growth, and developmental stage. In addition, the biological activities of saponins (Sparg et al., 2004) could be one of the parameters influencing saponin distribution among different organs in *A. nigrum*.

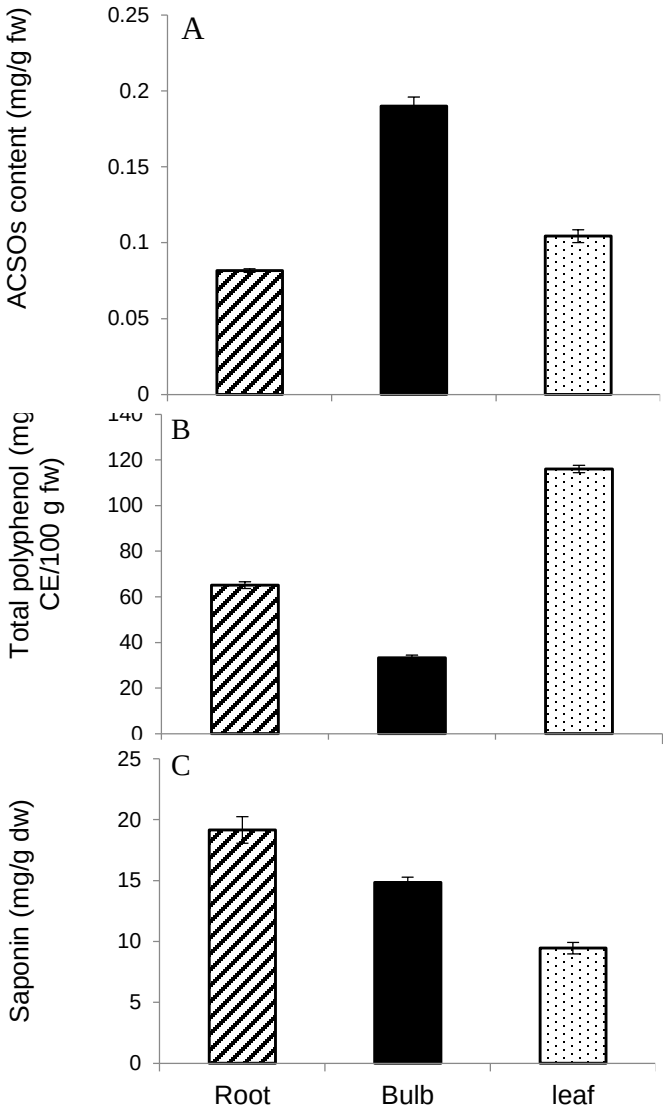


Fig. 12 Total ACSOs (A), polyphenol (B), and total saponins content (C) in root, leaf and bulb organs of *A. nigrum*. [a] Values are means of three independent replicates. [b] $P < 0.05$, bars represent standard errors. Means followed by the same letter(s) within the column are not significantly different according to Tukey's HSD

Characterization of the isolated compound

The BuOH root–bulb basal stem extract was subjected to TLC ($CHCl_3$/MeOH/H_2O, 30:15:2.5, v/v/v) and was shown to contain saponins. It was initially separated by column chromatography with a gradient solvent system starting with $CHCl_3$, $CHCl_3$/MeOH (9:1–1:9), MeOH, and MeOH/H_2O (9:1–7:3), yielding a partially purified fraction AN8 (20 mg). This fraction was further purified by TLC, affording a pure aginoside compound (13 mg), which was recently isolated from *A. nigrum* (Jabrane et al., 2011). The structure of the aginoside (AN8) was elucidated by 600 MHz NMR analyses, including 2D NMR experiments, and compared with literature data (Fig. 13). The aginoside compound, isolated as an amorphous white solid power in high yield, showed a molecular formula of $C_{50}H_{82}O_{24}$, deduced by high-resolution FABMS measurements and confirmed by 13C NMR data: HR-ESI-MS (positive-ion mode): m/z 1089.5027 [M+Na]+ (calcd. for 1089.5094): 1H NMR (pyridine-d5, 500 MHz) d: 5.54 (1H, d, J = 8.0 Hz, terminal-Glc-H-1), 5.14 (1H, d, J = 7.5 Hz, Xyl-H-1), 5.10 (1H, d, J = 8.0 Hz, inner-Glc-H-1), 4.93 (1H, d, J = 8.0 Hz, Gal-H-1), 3.97 (1/2H, m, H-26(S)), 3.54 (1/2H, br d, J = 8.1 Hz, H- 26(R)), 3.44 (1/2H, t, J = 11.5 Hz, H-26(R)), 3.32 (1/2H, d, J = 10.9 Hz, H-26(S)), 1.16 (3H, s, H-19), 1.07 (3H, d, J = 6.9 Hz, H-21), 0.99 (3/2H, d, J = 6.9 Hz, H-27(S)), 0.75 (3/2H, s, H-18(R)), 0.74 (3/2H, s, H-18(S)), 0.62 (3/2H, d, J = 5.7 Hz,H-27(R)); 13C NMR (pyridine-d5, 125 MHz) data (Table 1). The aginoside content has a variable distribution within different organs in *A. nigrum* (Fig. 14).The highest accumulation (2.9 mg/g DW) was detected in the root, followed by the bulb (2.34 mg/g DW), and the lowest was recorded in the leaves (1.57 mg/g DW). Huhman et al. (2005) similarly reported that soyasapogenol was mainly accumulated in the root organ (>32% of the total saponin content) compared with the leaf and stem of *Medicago truncatula*.

Tabel 1. 13 C NMR spectroscopic data of the aglycone and sugar moieties of aginoside (*S*/*R*) in pyridione-d, in ppm

Pos.	AN8 (S) Turoside	AN8® Aginoside	Pos.	
C-1	46.3	46.3	gal-1	102.2
2	69.9	69.9	2	71.8
3	83.6	83.6	3	74.6
4	31.5	31.5	4	78.8
5	47.0	47.0	5	75.0
6	69.3	69.3	6	60.3
7	39.4	39.4	i-glc-1	103.6
8	29.3	29.3	2	80.2
9	53.7	53.7	3	86.4
10	36.3	36.3	4	69.3
11	20.7	20.7	5	76.7
12	39.8	39.8	6	61.9
13	40.2	40.2	t-glc-1	103.7
14	55.5	55.5	2	75.0
15	31.1	31.1	3	77.2
16	80.6	80.6	4	69.0
17	62.2	62.2	5	77.6
18	15.9	15.9	6	62.0
19	16.4	16.4	Xyl-1	104.0
20	41.8	41.8	2	74.2
21	14.2	14.4	3	77.6
22	108.8	109.3	4	70.6
23	25.6	30.9	5	66.4
24	25.4	28.5		
25	26.8	29.9		
26	64.5	66.4		
27	15.6	16.7		

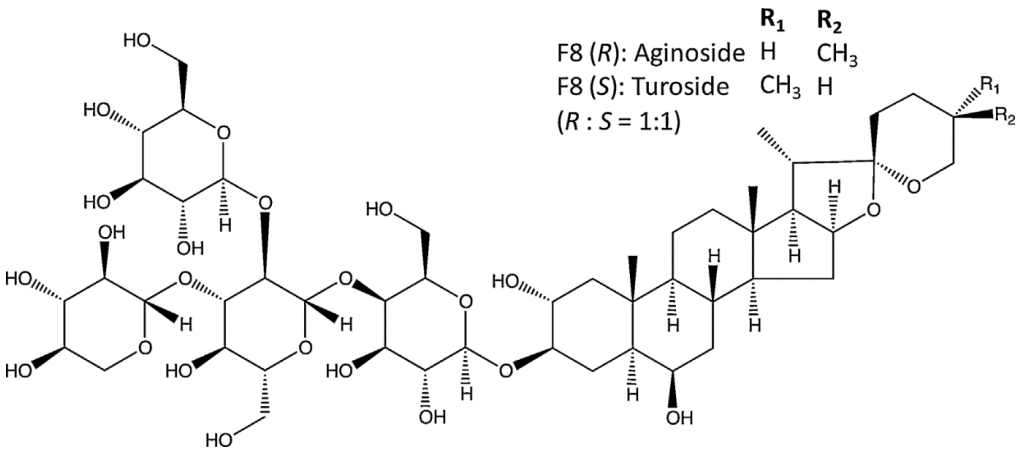


Fig. 13 Chemical structure of aginoside

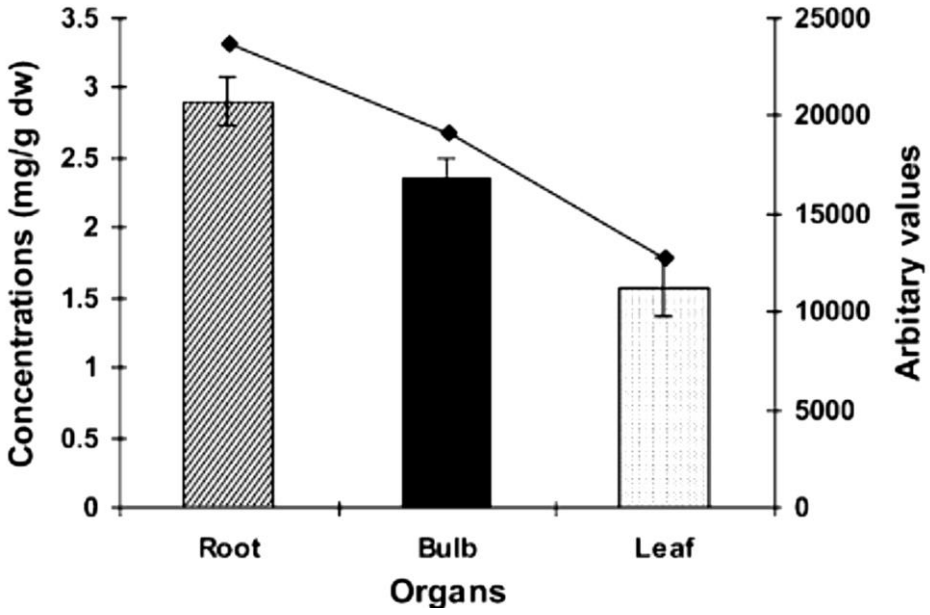


Fig. 14 Aginoside content detected in different plant organs. Quantification of TLC band intensity of purified aginoside compound and crude extract was detected using Image J version 1.42Q/Java 1.6.0_10 image analysis. The right y-axis represents mean relative hybridization intensity of three independent replicates expressed in arbitrary unit. Left y-axis represents concentration (mg/g DW)

Phytochemical bioactivity

The crude saponins showed the following rank of antifungal activity depending on the applied concentration and the tested fungal species (Fig. 15). *Fusarium oxysporum* f. sp. *lycopersici* had the highest resistance to the crude saponin of all tested concentrations, whereas *Colletotrichum gloeosporioides* was the most susceptible pathogen. The potential antifungal activity of the crude saponins in the root organs of *A. nigrum* supports our hypothesis that this bioactive metabolite plays a major role in the plant's defense mechanism, which explains the high accumulation of saponins in the root organs. The antifungal activity of the three dominant CSO compounds, MeCSO, AlCSO, and PeCSO, at different concentrations (500, 1000, and 1500 ppm) revealed no inhibition for MeCSO and PeCSO, whereas AlCSO at 1500 ppm recorded a maximum inhibition of 16% against *C. gloeosporioides* and *F. oxysporum* strains (10%). These results are in agreement with those of Coley-Smith et al. (1987), who reported that a high CSO content is directly associated with a high disease incidence in *Allium* species. Yamada and Azuma (1977) confirmed that the antifungal activity of thiosulfinates was linked to allicin, which was effective against *C. cryptococcus*; however, allicin showed less inhibition against *Aspergillus*. Furthermore, Yoshida et al. (1998) suggested that the antimicrobial activity of the cystine sulfoxide group could be ranked as follows: AlCSO > MeCSO > PrCSO. A fair to moderate fungal inhibition of phenolic extract from the leaves was noticed among tested concentrations (500, 1000, and 1500 ppm) against phytopathogens; the highest inhibition of 35% was recorded in *C. gloeosporioides* at 1500 ppm. Similar findings of concentrations dependent on the antifungal activity of phenolic compounds were reported in onion and garlic extracts (Benkeblia et al., 2005). Various phenolic compounds are known to exhibit antifungal activity, as reviewed by Cushnie and Lamb (2005).

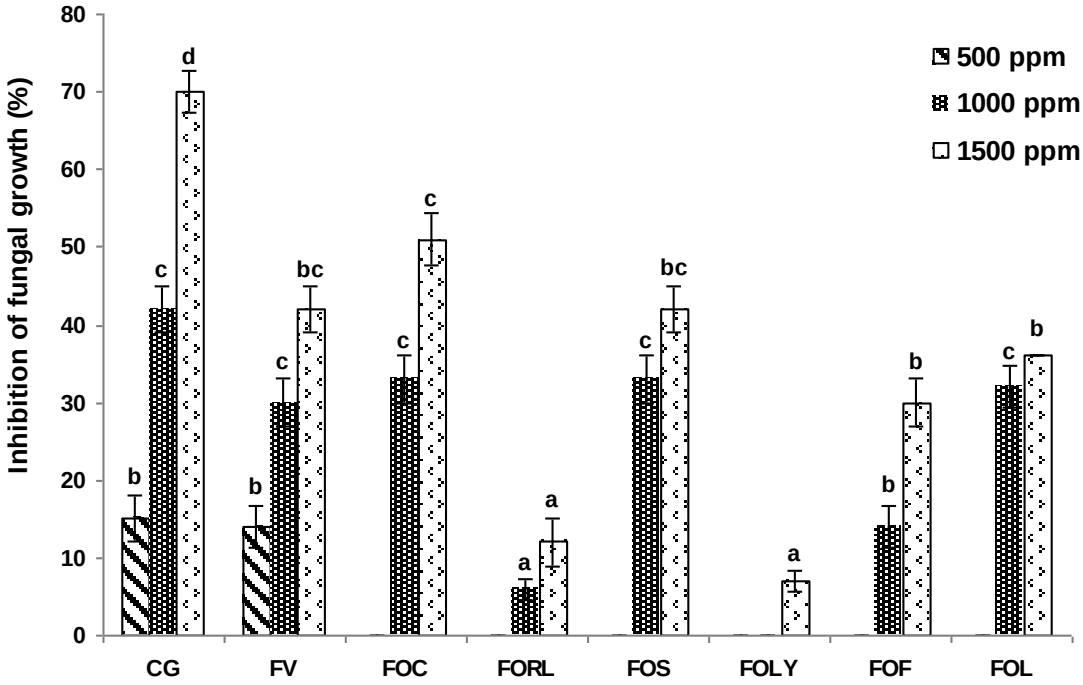


Fig. 15 Antifungal activity against FOC (*Fusarium oxysporum* f. sp. *cepae*), FOS (*F. oxysporum* f. sp. *spinaciae*), FORL (*F. oxysporum* f. sp. *radicis-lycopersici*), FOF (*F. oxysporum* f. sp. *fragariae*), FOL (*F. oxysporum* f. sp. *lagenariae*), FOLY (*F. oxysporum* f. sp. *lycopersici*), FV (*Fusarium verticillioides*) and CG (*Colletotrichum gloeosporioides*) of crude saponins isolated from *A. nigrum* at three final concentrations (500, 1000 and 1500 ppm). Data are inhibition of fungal growth in percentage compared to control (= 0). [a] Values are means of three independent replicates. [b] $P < 0.05$, bars represent standard errors. Means followed by the same letter(s) within the column are not significantly different according to Tukey's HSD.

Further investigation may be necessary to examine the presence of organo sulfur and phenolic compounds in other *Allium* plants and to clarify the contribution of these two metabolites to antifungal activity. Aginoside compounds showed significant ($P < 0.05$) antifungal activity (Fig. 16). The 400 ppm of aginoside completely inhibited the fungal growth of *C. gloeosporioides*, *F. verticillioides*, and *B. squamosa* and partially suppressed that of *F. oxysporum* f. sp. *cepae* and *F. oxysporum* f. sp. *radicis-lycopersici*. The potent antifungal activity displayed by aginoside suggests that this compound, either alone or in addition to others, may act as a chemical barrier to protect plants against fungal attacks. Our results are in agreement with those of Jabrane et al. (2011), who reported aginoside to be the most active spirostane saponin against colon cancer cell lines in comparison with other steroidal saponins from *A. nigrum*. In other research, Sata et al. (1998) stated that aginoside displayed *in vitro* cytotoxicity against P388 leukemic cells at 2.1 mg/ ml and antifungal activity against *Mortierella ramanniana* at 10 mg/disk. Moreover, Coleman et al. (2010) reported the significant antifungal activity of aginoside against the clinical fungal pathogen *Candida albicans* at MIC (47.0 mg/ml).

Our results regarding the antifungal activity of aginoside against *F. oxysporum* f. sp. *Lycopersici* are consistent with the data obtained by Lanzotti et al. (2012b), who found high resistance of FOL against three identified saponin compounds , cepasoide A, B and C isolated from the bulbs of *A. cepa* at three different concentrations (10, 50, and 200 ppm). Many fungi attack plants by producing enzymes that degrade saponins into non-toxic molecules (Morrissey and Osbourn, 1999). Most probably, the relatively high tolerance of *Fusarium oxysporum* to aginoside could be explained by this suggestion. The *in vitro* spore germination test, together with different aginoside concentrations (10, 50, 100 ppm), showed a high inhibition of spore germination in all tested pathogens (*B. cinerea* and *C. goleosporioides*). A relatively high

suppression of spore germination (< 40%) was detected at two different concentrations, 50 and 100 ppm (Fig. 17). The results for *B. cinerea* and *C. goleosporioides* are in agreement with those of the *in vitro* assay (Fig. 18). Fungal infection expansion and black necrotic lesions were observed in the control (untreated leaves). In contrast, lesion expansion was totally inhibited in infected leaves treated with a concentration of 100 ppm.

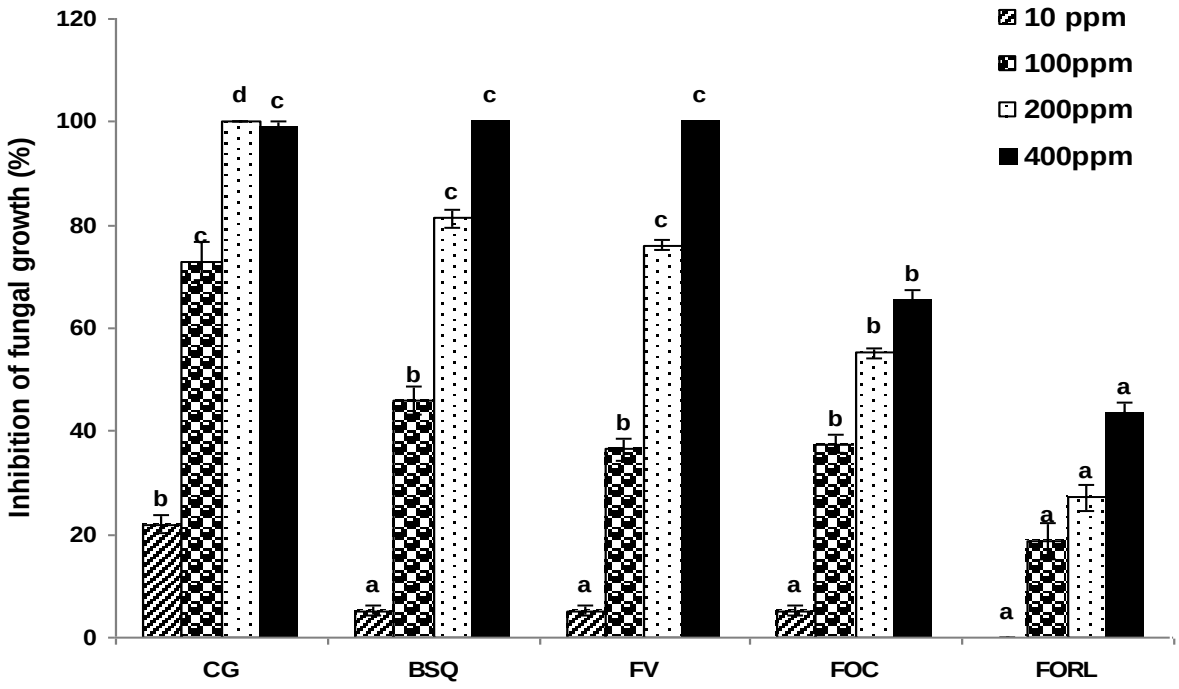


Fig. 16 Examination of aginoside-antifungal activities against *C. gloeosporioides* (CG), *B. squamosa* (BSQ), *F. verticillioides* (FV), *F. oxysporum* f. sp. *cepae* (FOC), *F. oxysporum* f. sp. *radices lycopersici* (FORL), at four final concentrations (10, 100, 200 and 400 ppm). Data are inhibition of fungal growth in percentage compared to control (= 0). [a] Values are means of three independent replicates. [b] $P < 0.05$; bars represent standard errors. Means followed by the same letter(s) within the column are not significantly different according to Tukey's HSD

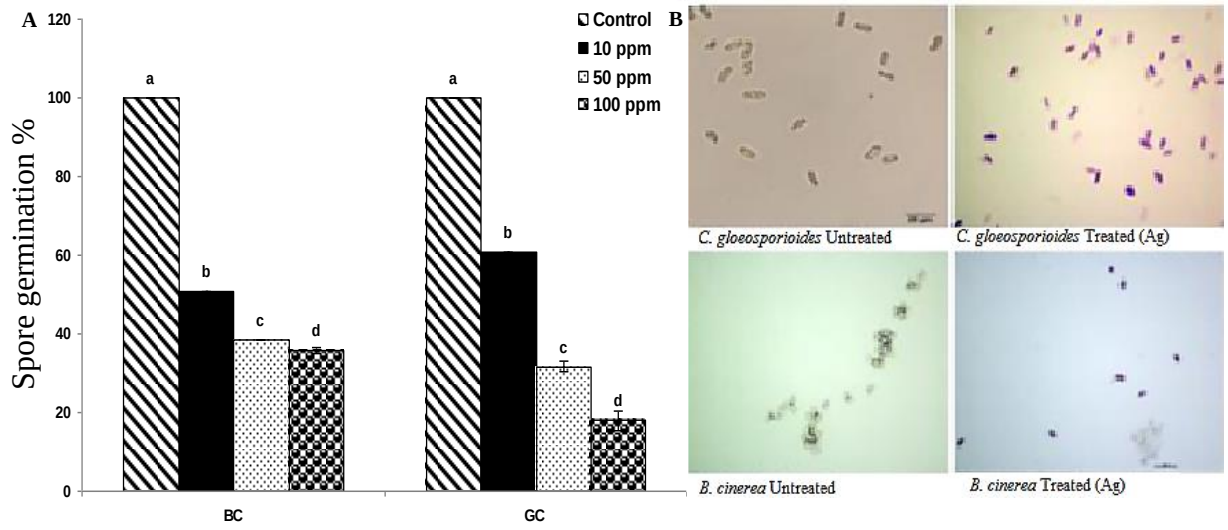


Fig. 17 Examination of Aginoside activity against spore germination of *B. cinerea* and *C. gloeosporioides* at three different concentrations 10, 50 and 100 ppm (**A**). Light microscopic examination of aginoside (Ag) treated and untreated on spore inhibition of *C. gloeosporioides* and *B. cinerea* at 200 µg (**B**). Spore staining using Evan blue. [a] Values are means of three independent replicates. [b] $P < 0.05$; bars represent standard errors. Means followed by the same letter(s) within the column are not significantly different according to Tukey's HSD

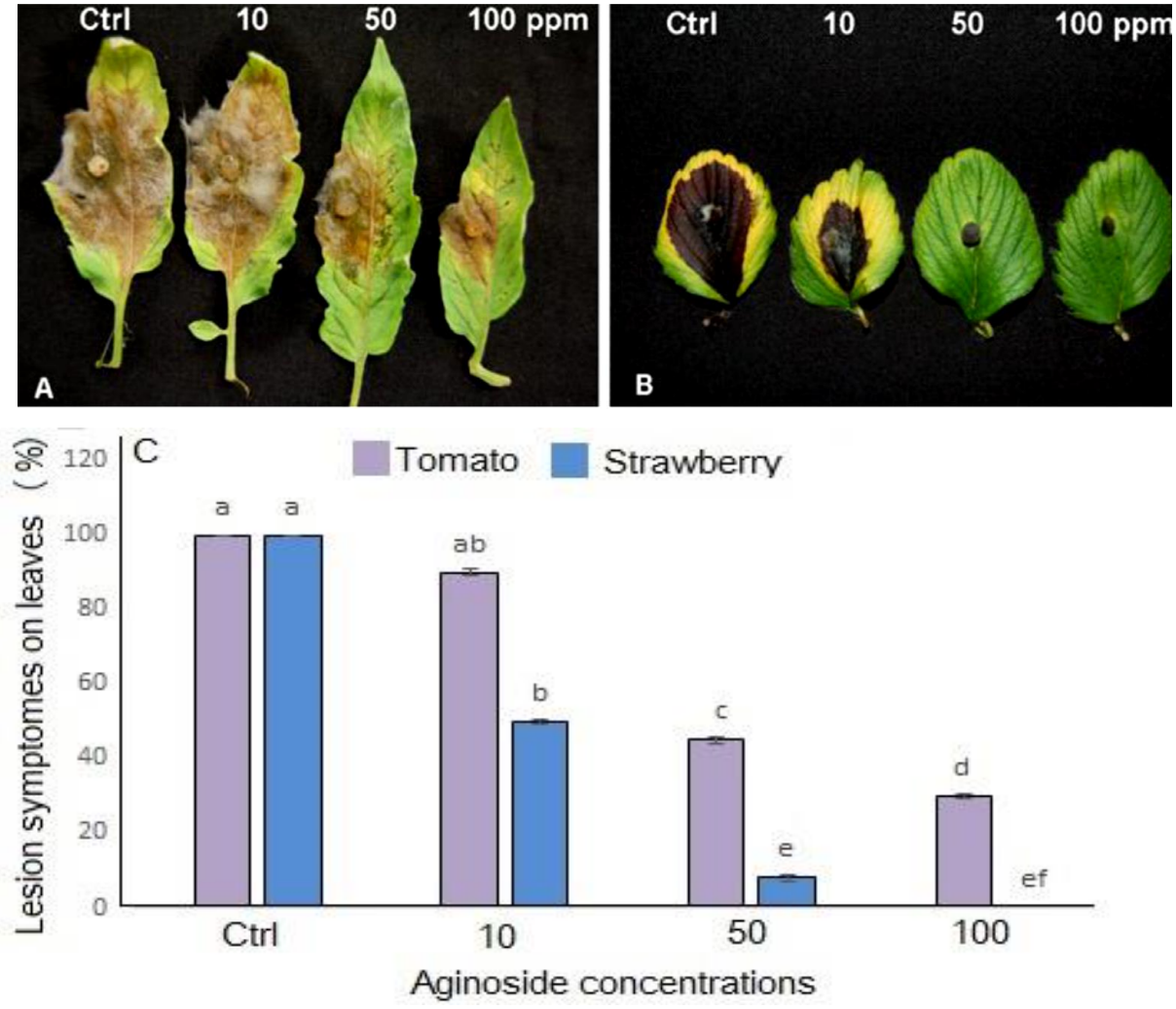


Fig. 18 Antifungal activities of the Aginoside (10, 50 and 100 ppm) upon artificial infection of *B. cinerea* on tomato leaves (A) and *C. gloeosporioides* on strawberry leaves (B) for 7 days after infection. (C) Leaf lesion symptoms (%)

CHAPTER IV. COMPARATIVE SAPONIN PROFILES IN DIFFERENT *ALLIUM* GENOTYPES AND THEIR PROSPECTIVE APPLICATIONS AS CHEMICAL MARKERS FOR FUSARIUM BASAL ROT DISEASE RESISTANCE

Introduction

Steroidal saponins are broadly disseminated among monocots, including the Amaryllidaceae family in which the *Allium* genus is currently classified. Apart from sulfur compounds, these are important biologically active compounds that are considered to be responsible for the observed activity of *Allium* species (Sobolewska et al., 2014). Numerous reports refer to the pharmacological activities of steroidal saponins. Some showed promising antifungal, cytotoxic, anti-inflammatory, antithrombotic, and hypocholesterolemic effects (Sparg et al., 2004; Lanzotti, 2005; Sobolewska et al., 2014). The first chemical survey of saponins from the genus *Allium* was published by Kravets in 1990; this was followed by an update by Lanzotti in 2005 (Kravets et al., 1990; Lanzotti, 2005). The approach for structurally elucidating saponins is mainly based on the use of spectroscopic techniques, including HRFABMS and advanced 2D NMR experiments, thus keeping the material for further biological assays (Mostaf et al., 2013). Chemical methods are only applied for the stereostructure elucidation of sugar moieties. 2D NMR methods have the enormous advantage in overcoming the overlapping of a large number of signals in the 1D NMR spectra of saponins and in determining: the aglycone structure, the number of sugar residues (including their nature and their configuration), the glycosylation site, and the interglycosidic linkages (Lanzotti, 2005, 2012a). Surprisingly only a few studies have investigated

the antimicrobial properties of *Allium* saponins. Among these, Barile et al. (2007) reported three saponins, named minutosides A–C, and tested against several fungal plant pathogens, including *Botrytis cinerea*, *Fusarium oxysporum*, and *Rhizoctonia solani*. In particular, minutoside B was the major saponin plant tissue (83.5 mg kg^{-1}) and also showed the highest activity in inhibiting spore germination and hyphal growth of the tested fungi. In addition, it has been shown that the antifungal activity of ceposides isolated from *A. cepa* against the plant pathogen *B. cinerea* and the biocontrol agent *Trichoderma harzianum* can be synergistically enhanced when they are supplied in combination (Lanzotti et al., 2012a).

No research has addressed the application of these saponin compounds as chemical markers for disease resistance against fungal pathogens, which would be an interesting point of research for *Allium* genetics. Therefore, in the present study, the saponin-TLC profiling approach was selected to discriminate the saponin compound signatures within different *Allium* genotypes, including the double haploid (DH) parent lines (*A. cepa*, *A. fistulosum*, and *A. roylei*), introgressed F_1 hybrids, and allotriploid and amphidiploid lines of these genotypes. Further, promising saponin compounds were isolated and identified using column chromatography combined with 2D NMR. The *in vitro* antifungal activity of these compounds was carried out. Finally, saponin pattern changes within different resistant and susceptible genotypes before and after pathogen inoculation were conducted.

Materials and methods

Plant materials

Shallot (*A. ceap* L. Aggregatum group) dry root was used to isolate and identify potent saponin compounds. For comparative TLC profiling, crude saponin was extracted from the dry root materials of shallot, *A. roylei,* F_1 hybrids, allotriploid and, amphidiploid lines, and a triploid line of the parent material (Fig. 19 and Table 2).

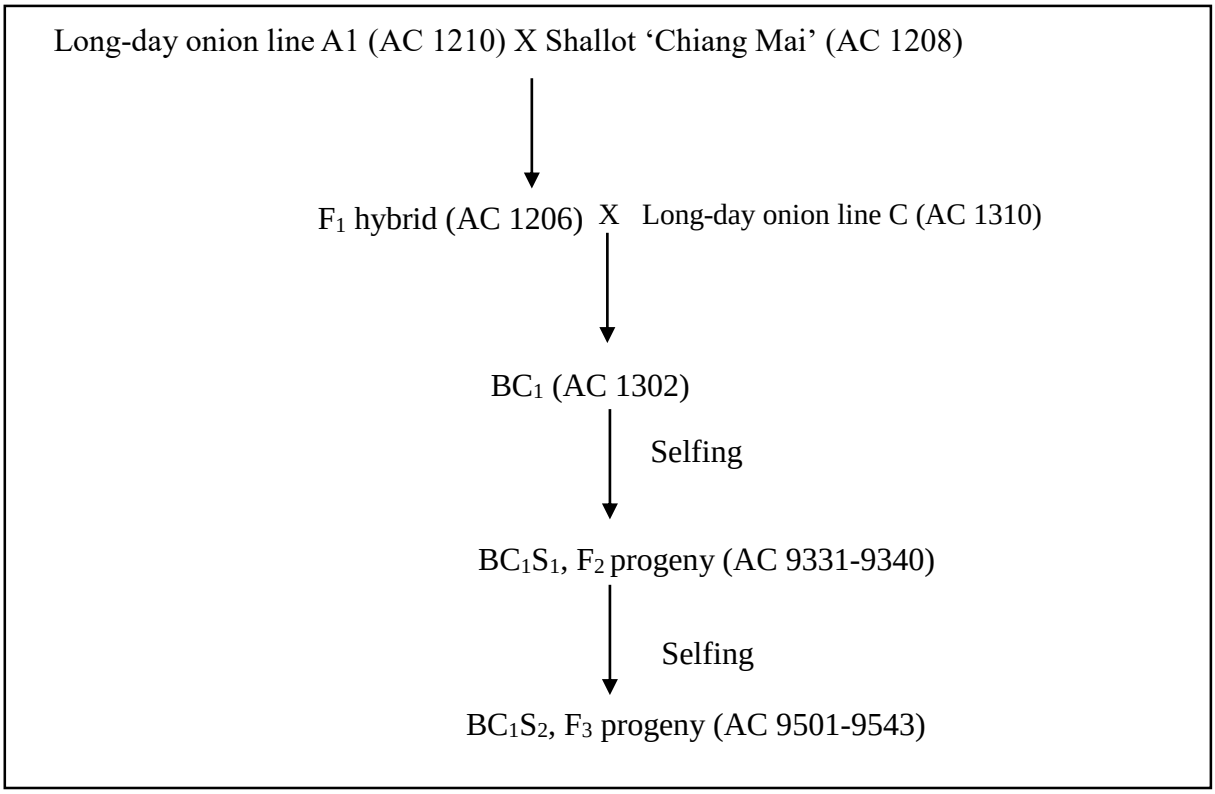


Fig. 19 Pedigree of the BC_1, BC_1S_1, and BC_1S_2 production method using the long-day onion A1 line and the 'Chaing Mai' shallot

Table 2 Plant material accession numbers, cultivar names, and disease tolerance levels

Accession Number	Line/cultivar name	Disease tolerance level in the field
1201	'Higuma'	Susceptible
1202	'Kamui'	Moderately Susceptible
1203	'Brown-bear'	Resistant
1204	'Kitamemiji-2000'	Resistant
1206	Long-day onion line A X shallot 'Chaing Mai'	Resistant
1207	Long-day onion line B X shallot 'Chaing Mai'	Resistant
1208	Shallot 'Chaing mai'	Resistant
1210	Long-day onion line A2	Resistant
1211	Long-day onion line A3	Resistant
1302	Long-day onion line A X shallot 'Chaing Mai' X long-day onion line C (BC_1)	Resistant
1303	Long-day onion line B X shallot 'Chaing Mai' X long-day onion line C (BC_1)	Resistant
1310	Long-day onion line C	Resistant
9331-9340	1302 F_2 (BC_1S_1)	Resistant and susceptible
9341-9350	1303 F_2 (BC_1S_1)	Resistant and susceptible
9501-9543	1302 F_3 (BC_1S_2)	Resistant and susceptible
9544-9586	1303 F_3(BC_1S_2)	Resistant and susceptible

Saponin extraction

Saponin compounds were extracted in accordance with the method described by Mostafa et al. (2013). Freeze dried roots were first extracted with hexane, and the defatted material was extracted with 80% methanol. The methanol extract was further dried and partitioned with butanol and water (1:1, v/v). The final crude saponin was subjected to TLC profiling using $CHCL_3$:MeOH:H_2O (30:15:2.5, v/v/v) solvent system. For saponin compounds, a visualization anisaldehyde reagent was applied and heated in an oven at 120°C. The crude saponin extract was subjected to column chromatography with a gradient solvent system changing from 100% $CHCL_3$ to 100% MeOH. The obtained fractions were analyzed by TLC to check the purity of the fractions. Finally, isolated pure compound structure elucidation was achieved by 2D NMR

Antifungal activity

In vitro antifungal activity of the crude saponin and pure saponin compounds was carried out using the agar diffusion method against different strains of *Fusarium oxysporum* f. sp. *cepa*, as the major threat pathogen for *Allium* crop production, and *Colletotrichum gloeosporioides*, in accordance with Mostafa et al. (2013).

Results and discussion

Isolation, identification and antifungal activity of Alliospiroside A and Alliospiroside B isolated from the shallot

Phytochemical analysis of the crude saponin extract from the dried root tissue of the shallot using column chromatography combined with TLC followed by anisaldehyde reagent spray gave three major fractions (cepa1, cepa2, and cepa3). The *in vitro* antifungal activity of these three fractions was examined against different soil-borne pathogen strains of *Fusarium oxysporum* f. sp. *cepa* (FOC Takii, AF22, AF60, and AC214) and the airborne pathogen *Colletotrichum gloeosporioides* (CG) (Figs. 20 and 21). The cepa2 fraction showed the highest antifungal activity against all *Fusarium* pathogens. Therefore, this fraction was selected for further fractionation and purification by TLC, leading to the isolation of two saponin compounds. The pure compounds were subjected to 2D NMR for structure elucidation. The compounds obtained were spirostanol saponins, Alliospiroside A and Alliospiroside B (Fig. 22). The antifungal activity of Alliospiroside A and B alone and in combination was carried out (Fig. 23).The obtained results showed that Alliospiroside A was a more powerful antifungal compound than either Alliospiroside B or crude saponins. This result suggested that Alliospiroside A could be a prospective chemical marker for *Fusarium* disease resistance. Our results were in line with recent studies revealing that Alliospiroside A was a powerful antifungal compound against different phytopathogens (Teshima et al., 2014). We hypothesize that onion genotypes that can accumulate a high amount of Alliospiroside A will be potential genetic material for an onion-breeding program to improve *Fusraium* basal rot disease resistance.

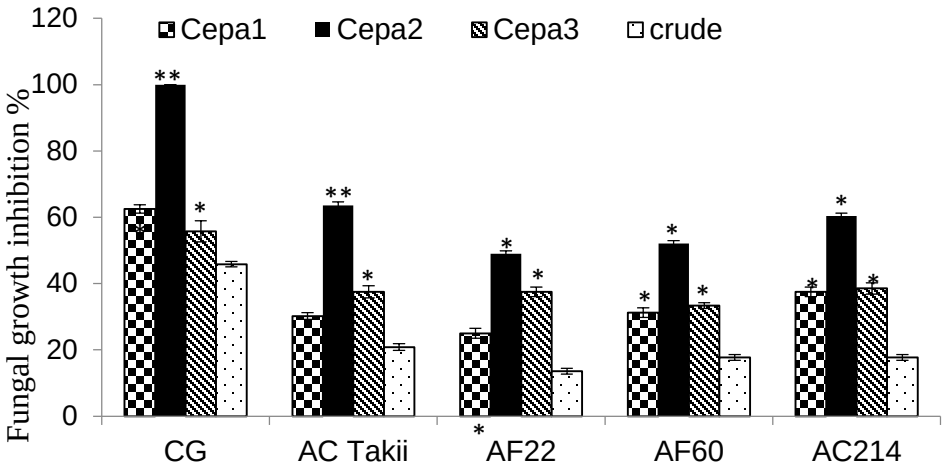


Fig. 20 Antifungal activity of the shallot crude saponin fractions (cepa1, cepa2 and cepa3) at 1000 ppm concentration against CG (*Colletotrichum gloeosporioides*), AC-TAKii (*F. oxysporum* f. sp. *cepa* Takii), AF22 (*F. oxysporum* f. sp. *cepa* 22), AF60 (*F. oxysporum* f. sp. *cepa* 60) and AC214 (*F. oxysporum* f. sp. *cepa* 214). Bars indicate the mean ± SE. The significance level was 5%, according to Tukey's test

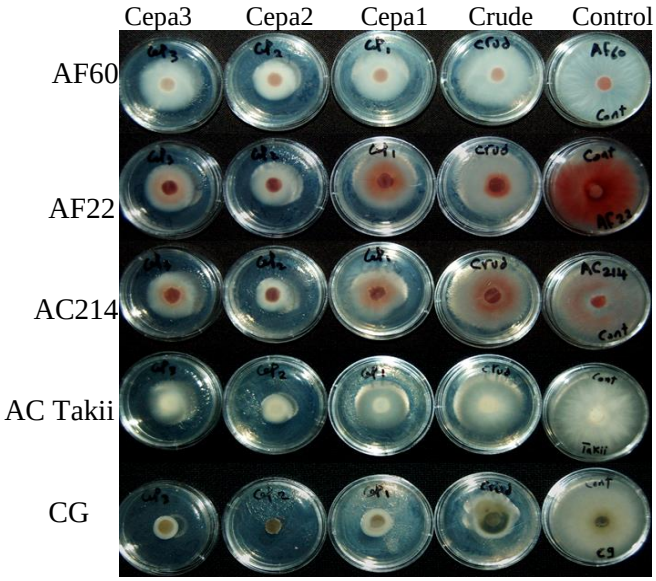


Fig. 21 *In vitro* antifungal activity of the shallot crude saponin fractions (cepa1, cepa2 and cepa3) at 1000 ppm concentration against CG (*Colletotrichum gloeosporioides*), AC-TAKii (*F. oxysporum* f. sp. *cepa* Takii), AF22 (*F. oxysporum* f. sp. *cepa* 22), AF60 (*F. oxysporum* f. sp. *cepa* 60) and AC214 (*F. oxysporum* f. sp. *cepa* 214)

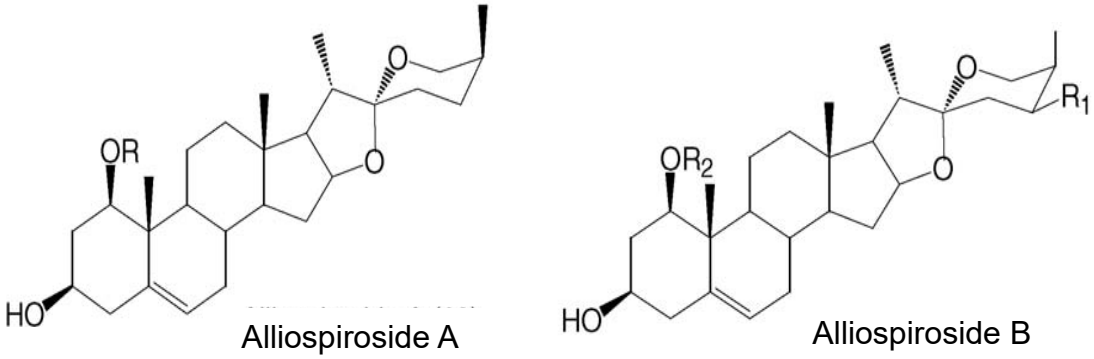


Fig. 22 Chemical structure of Alliospiroside A and Alliospiroside B

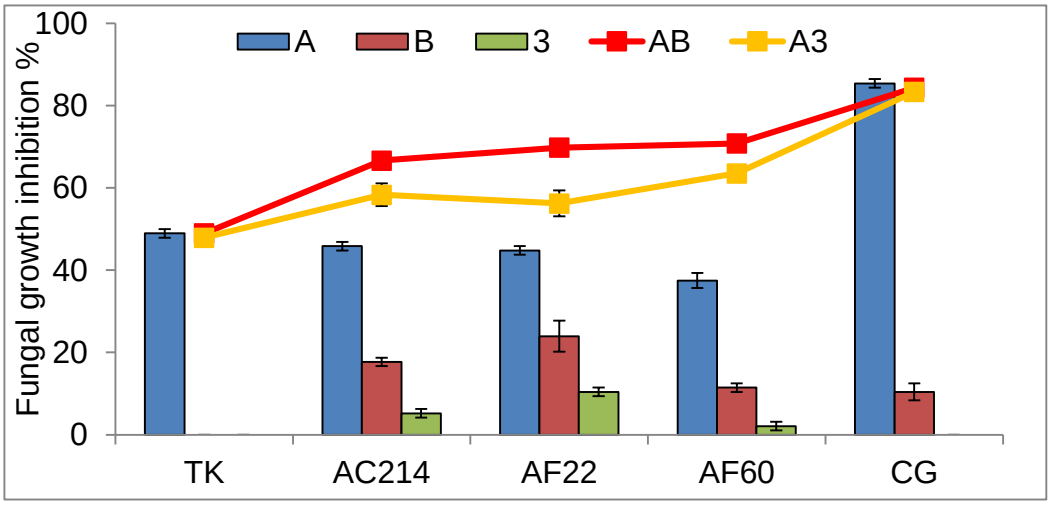


Fig. 23 *In vitro* antifungal activity of Alliospiroside A, Alliospiroside B, and compound 3 alone (150 ppm) and in combination (300 ppm, 1:1, w/w) against *F. oxysporum* f. sp. *cepa* TK, *F. oxysporum* f. sp. *cepa* AC214, *F. oxysporum* f. sp. *cepa* AF22, *F. oxysporum* f. sp. *cepa* AF80 and *Colletotrichum gloeosporioides*. Bars indicate the mean ± SE for three independent replications

Saponin profile polymorphism in different long-day onion-shallots (BC_1, BC_1S_1, BC_1S_2)

In the present part, we extracted saponin from the root tissues of different long-day onion lines, the A1 line (AC 1210) and the A1 line (AC 1211), and a C line (AC 1310) crossed with shallot 'Chaing Mai' (AC 1208) to obtain BC_1, BC_1S_1, and BC_1S_2 progeny. The disease score of these plant materials was determined under field conditions, and the relative information of the disease tolerability index is recorded in Table 2. Our objective in this part is to evaluate the saponin profile polymorphism among these different lines, to address Alliospiroside A as a chemical marker for discriminating between resistant and susceptible lines in the examined plants, and to correlate the obtained results with the disease score index of the field experiment. The TLC profile of the crude saponin extract in the different onion genotypes was extremely variable. Alliospiroside A showed an intensive accumulation in the resistant line as compared with the susceptible one. Moreover, intensive accumulation of the frustanol saponin was observed in the resistant lines as compared with the susceptible line. The TLC profile of the BC_1, BC_1S_1, and BC_1S_2 showed improvements in the total saponin content as well as the accumulation of Alliospiroside A, which indicates the genetic influence of the saponin biosynthesis genes allocated in the shallot (Figs. 24, 25, 26, and 27). The obtained results conclude that Alliospiroide A is the major compound enrolled in the defense mechanism against the *Fusarium* pathogen. Furostanol saponin may be a stored form of saponin in the root tissue, and upon plant inoculation, this furostanol saponin will be converted into a spirostanol form through beta-glucosidase (BGLU) enzymes. The *BGLU* gene family box may be highly expressed in shallot genotypes, and such genetic charters will improve onion genotypes to accelerate the biosynthesis of the spirostanol form. The biogenesis of Alliospiroside A and its conversion from the furostanol to the spirostanol forms will be a critical point for discriminating between resistant and susceptible lines; this compound can be applied in onion

genetics as a chemical marker for genotype selection, and the genes enrolled in this compound biogenesis can be a potential molecular marker. To confirm the obtained results, molecular analysis will be needed in the future to check the gene expression level in the resistant and susceptible lines related to bioformation of this compound.

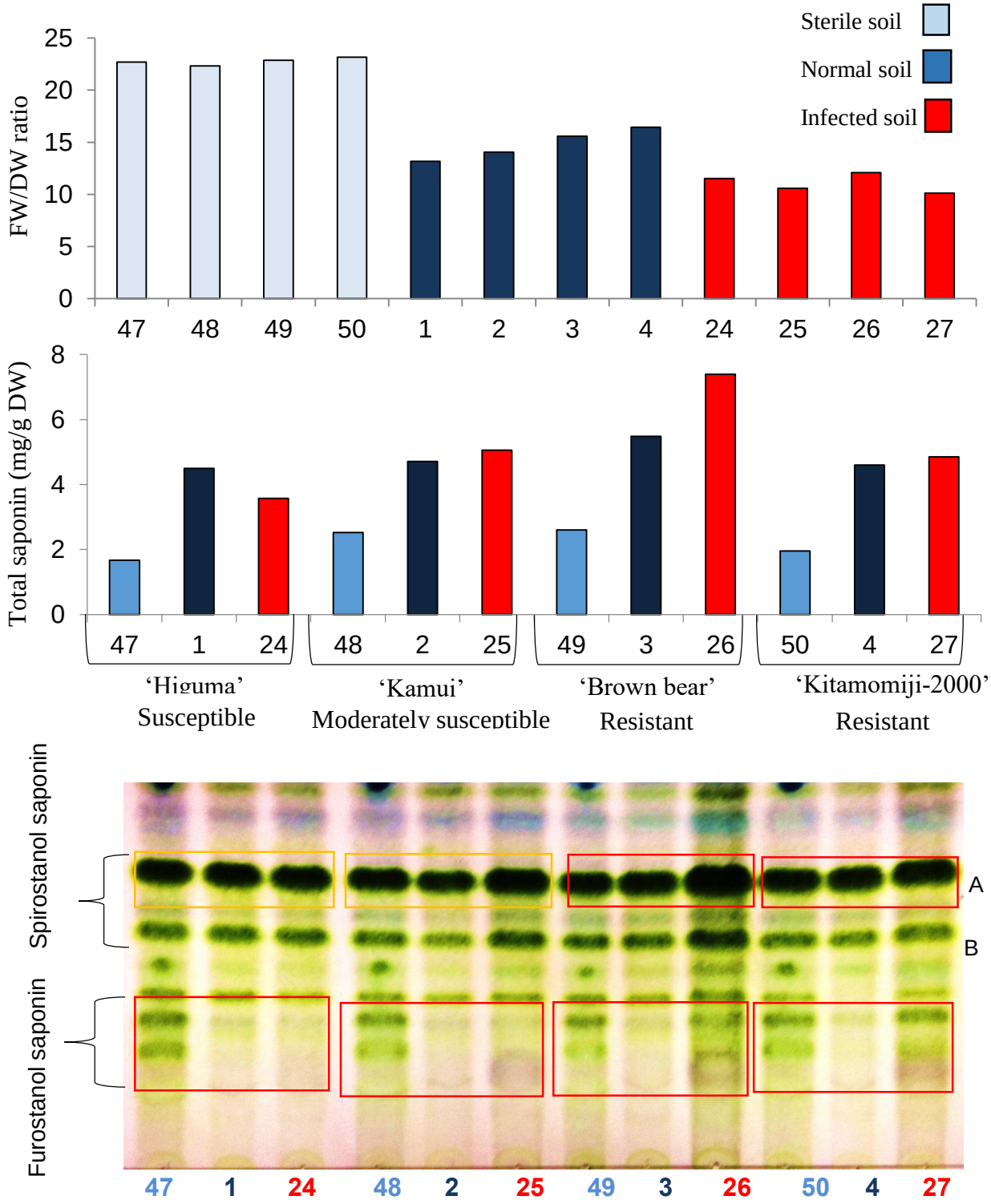


Fig. 24 Fresh weight and dry weight ratio, total saponin content and saponin TLC profile of 'Higuma' and 'Kamui' (susceptible) and 'Brown bear' and 'Kitamomiji-2000' (resistant) under sterile, normal and infected soil conditions

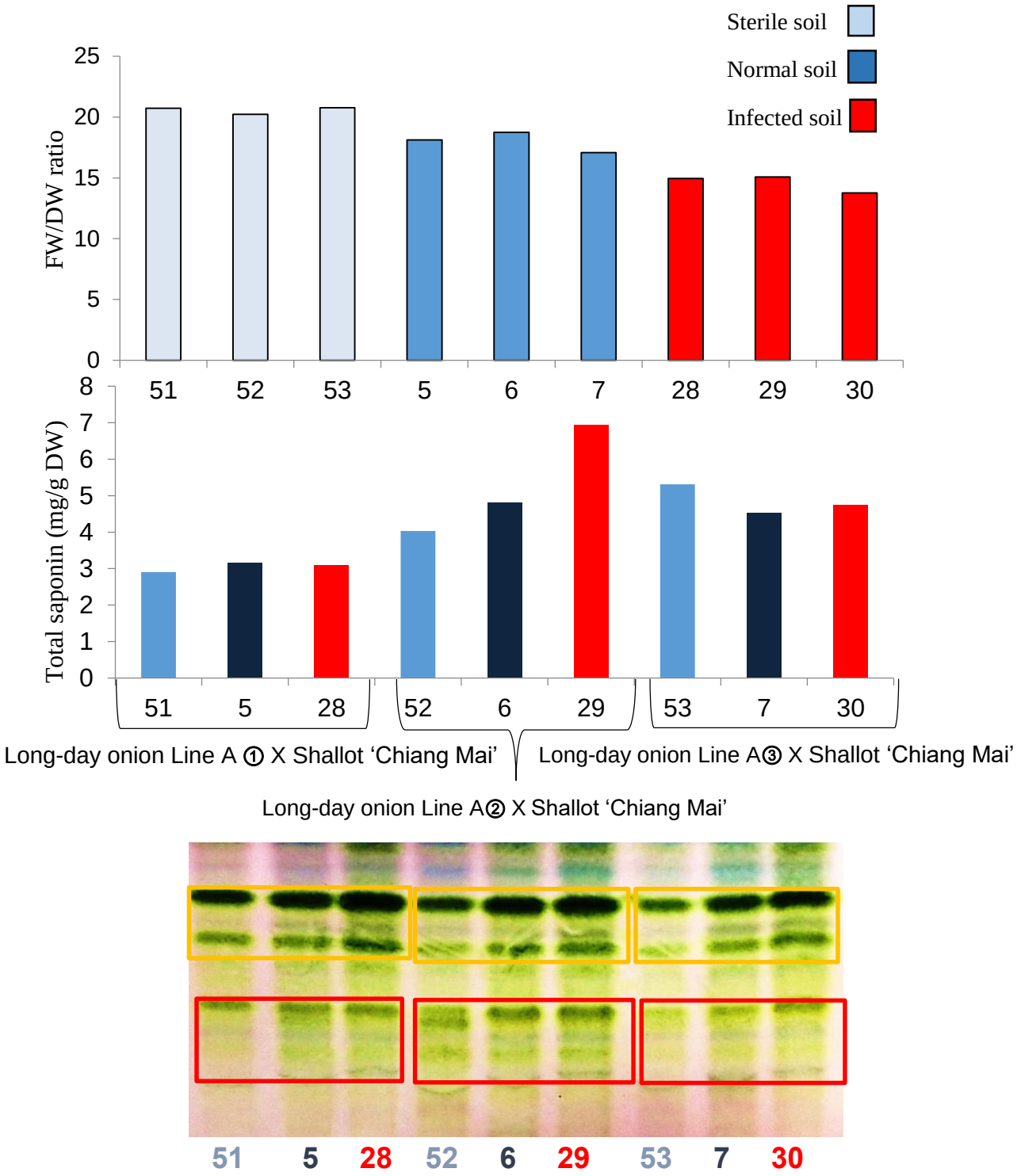


Fig. 25 Fresh weight and dry weight ratio, total saponin content and saponin TLC profiles of F_1 hybrids (long-day onion A1, A2, and A3 lines) crossed with the shallot 'Chaing Mai' under sterile, normal and infected soil conditions

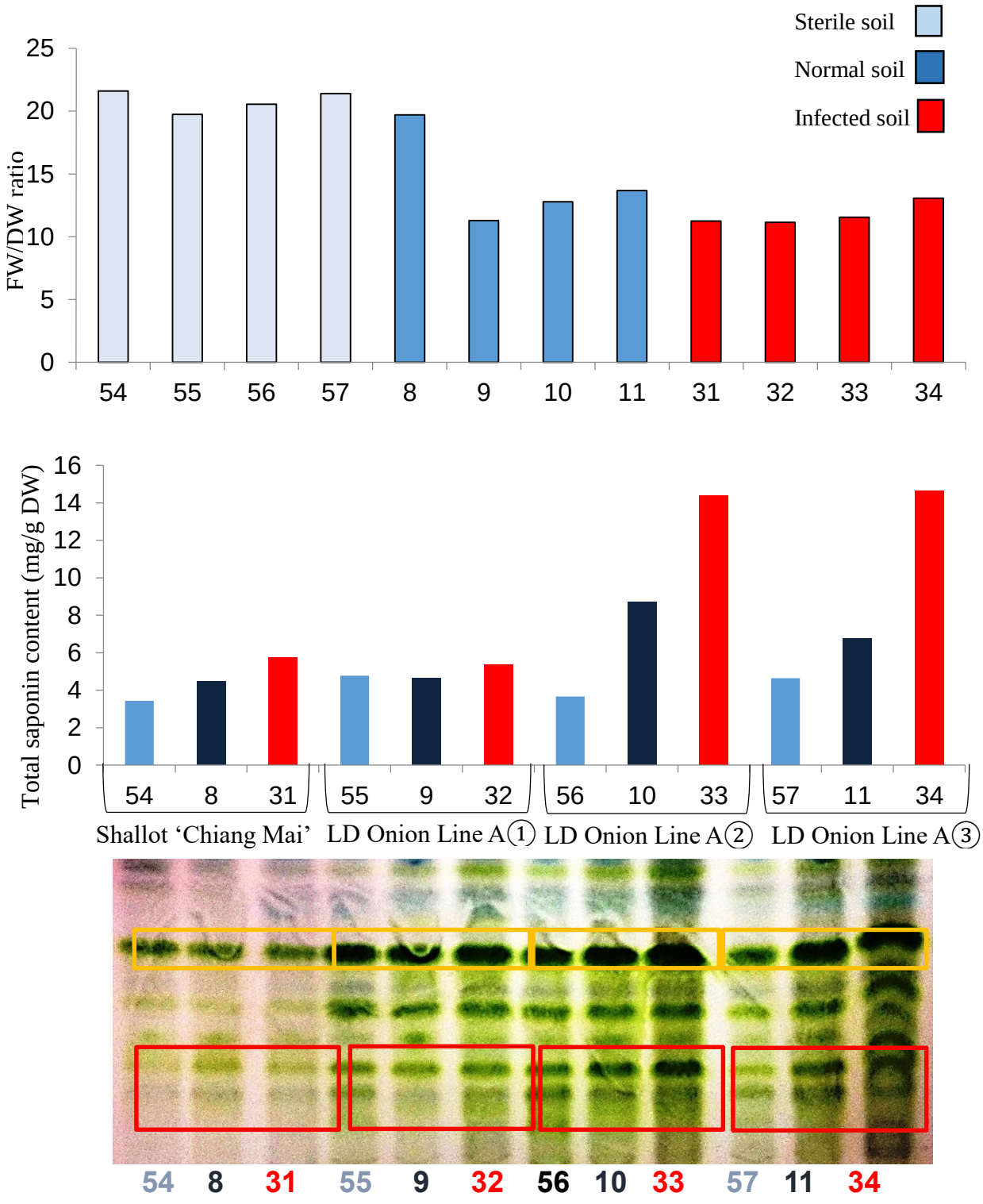


Fig. 26 Fresh weight and dry weight ratio, total saponin content, and saponin TLC profile of the long-day onion A1, A2, and A3 lines and shallot 'Chaing Mai' under sterile, normal, and infected soil conditions

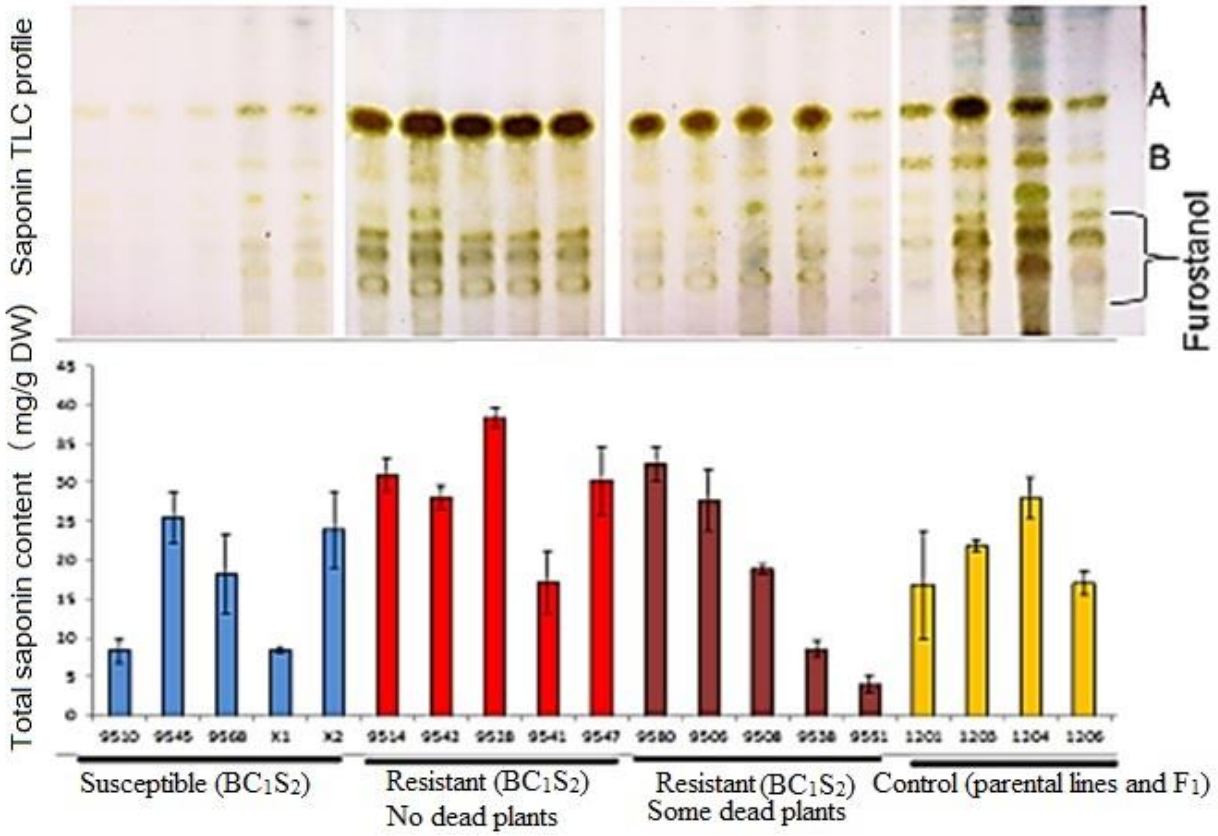


Fig. 27 Total saponin content and saponin TLC profile of the BC_1S_2 susceptible, BC_1S_2 low-resistance susceptible progeny, BC_1S_2 highly susceptible, control, and F_1 hybrid

From phenotyping to RNAseq genotyping of *A. fistulousm* with an extra chromosome from the shallot

Saponins are synthesized via the mevalonic acid (MVA) pathway (Haralampidis et al., 2002), which is ubiquitous in plants and provides the precursor 2, 3-oxidosqualene for saponin biosynthesis. The cyclization of 2,3-oxidosqalene by *oxidosqualene cyclase* (*OSC*) combined with the following modifications on the steroidal saponin skeletons, including hydroxylation and glycosidation, leads to the production of various saponins. *OSC* genes, including *dammarenediol synthase* (*DS*), *beta-amyrin* (*b-AS*), *lupeol synthase* (*LS*), and *cycloartenol synthase* (*CAS*), have been isolated in plants (Tansakul et al., 2006). However, little is known about the molecular mechanism of the biosynthetic pathway involved in steroidal saponin biosynthesis. To understand the genetic background, regulating the saponin biosynthesis in the shallot, saponin profiling from the root extract of eight *A. fistulosum* (FF) – shallot (AA) monosomic lines (MALs) was carried out. Further, total RNA was isolated from the root, bulb and leaf tissues from each monosomic line (FF+1A -8A) and parental lines (AA and FF). Large scale transcriptomic analyses using next generation sequencing (NGS) technology was applied, the obtained transcriptional data and gene annotation were uploaded in *Allium* Transcriptomic data base (*Allium* TDB). The TLC profile of the root saponin extract revealed a specific saponin spot in chromosome 2A, and this compound was isolated and identified as Alliospiroside A. In addition, furostanol saponin compounds 4 and 5 were allocated in chromosomes 1A and 2A (Fig. 28). This new finding regarding the phenotypic expression of the saponin can be important information regarding the genetic and molecular feature that controls saponin biosynthesis in the shallot, which could be regulated by chromosomes 1A and 2A.

Despite its genetic importance, the transcriptomic and genomic data of steroidal saponins are extremely limited. The limited transcriptomic data hinder the study of steroidal saponin biosynthetic mechanisms. Next generation sequencing (NGS) technology was used for the discovery and prediction of genes involved in steroidal saponin and other secondary metabolite biosynthesis. Our ultimate goal is to discover candidate genes that encode enzymes in the steroidal saponin biosynthetic pathway and to determine the chromosomal locations of several candidate genes related to saponin biosynthesis. High saponin gene expression was observed in chromosomes 1A, 2A, and AA, as compared with FF. Interestingly, *glucosyltransferase GTs* and *BGLU* genes were upregulated in chromosome 2A (Figs 29 and 30). Recently, Subramaniyam et al. (2014) showed that *GTs* can be considered a candidate involved in the biosynthesis of saponins. Furthermore, Inoue and Ebizuka (1996) and Morant et al. (2014) intensified the functional role of *BGLU* in the conversion of furostanol to a spirostanol saponin type. In addition, we were able to allocate *GTs* in chromosome 2A, which furthers our hypothesis regarding the potential role of these genes in saponin biosynthesis and subsequent improvement for disease resistance in the shallot and other related *Allium* species.

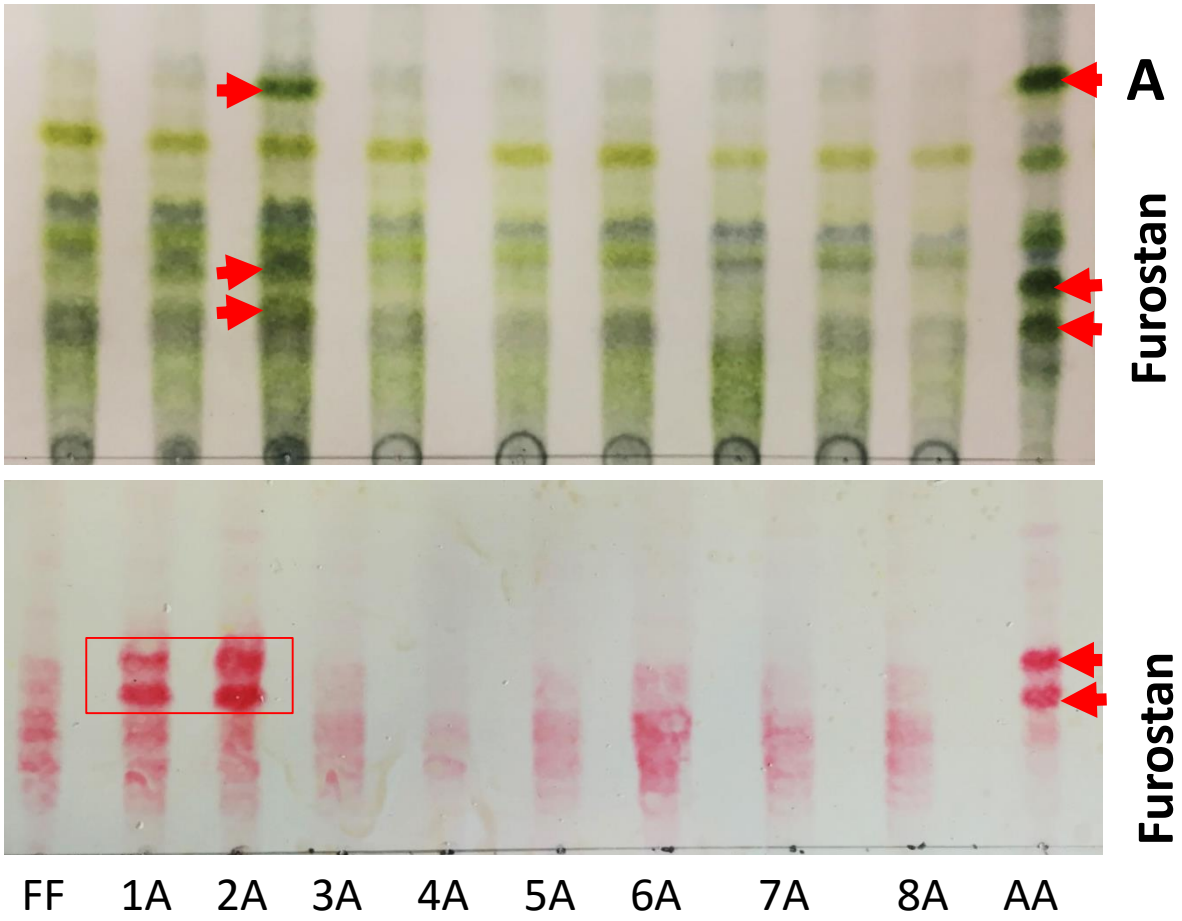


Fig. 28 TLC profiles of crude saponin extracts from a complete set of *Allium fistulosum* (FF)-shallot (AA) monosomic addition lines (1A-8A); Anisaldehyde reagent (A) and Ehrlich's reagent (B)

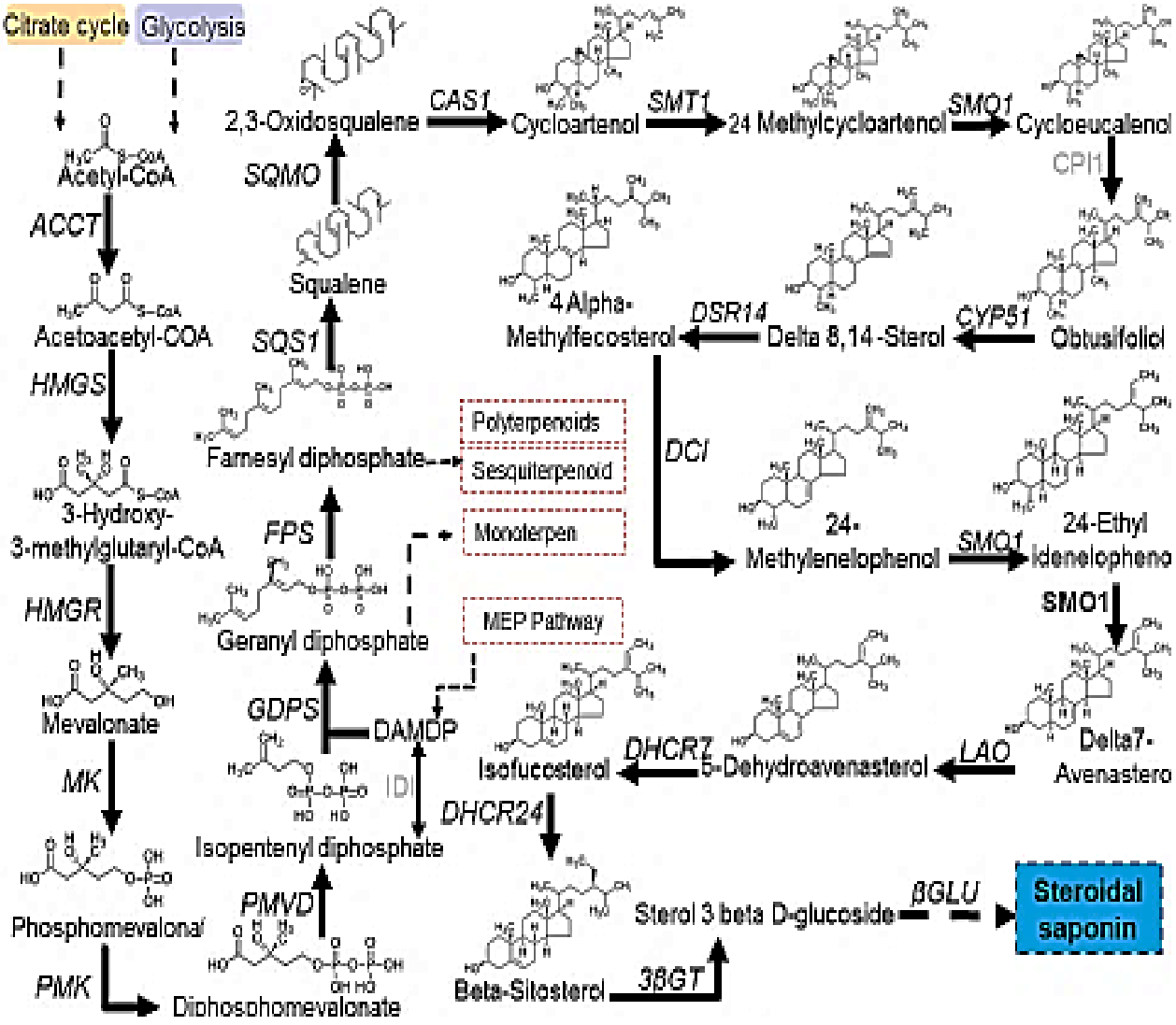


Fig. 29 Schematic representation of a steroidal saponin pathway

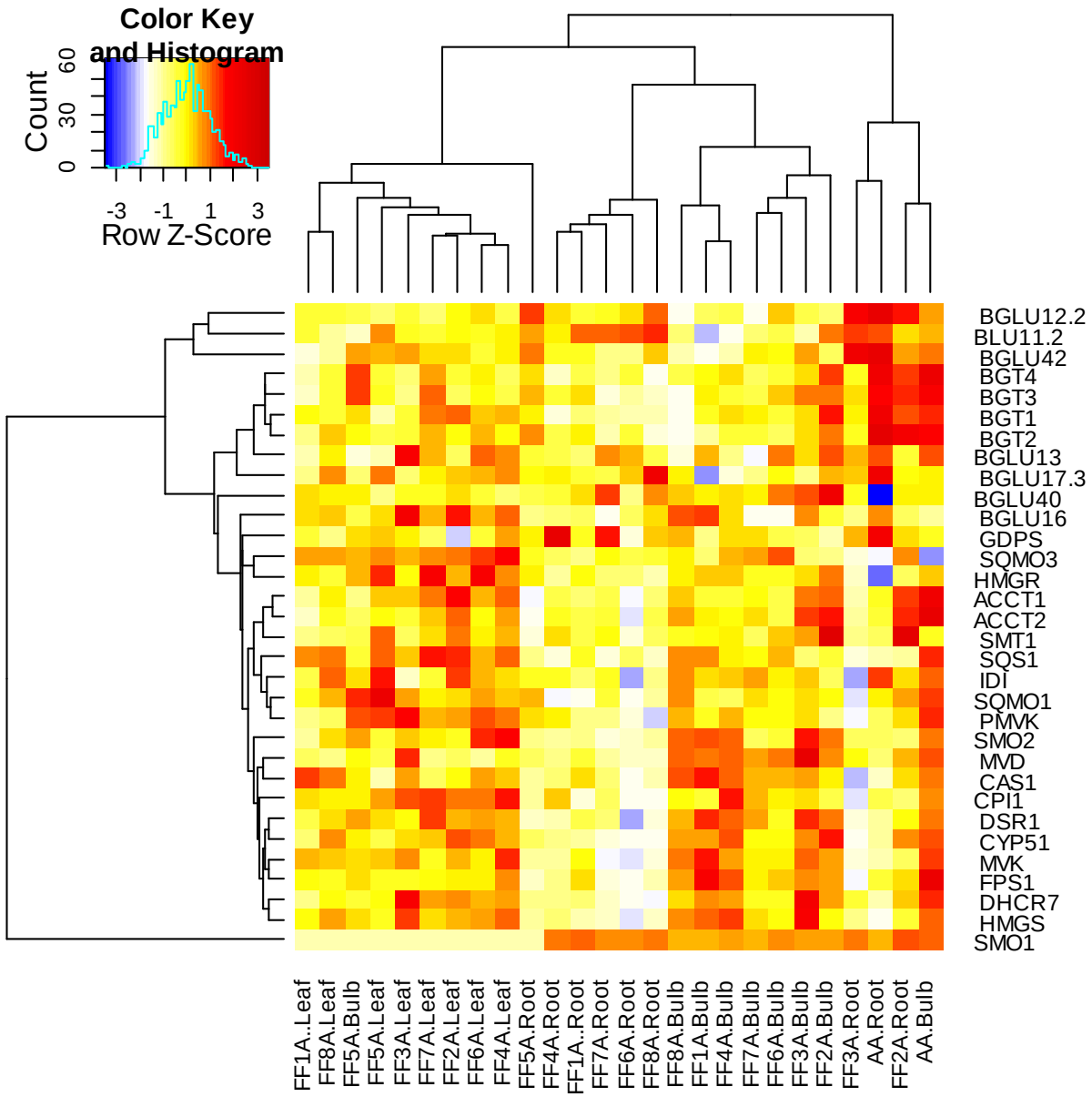


Fig. 30 Heat map and hierarchical clustering of differently expressed (log2 fold change) saponin biosynthesis genes from different tissues (root, bulb, and leaf) in a complete set of *A. fistulosum* (FF)-shallot (AA) monosomic addition lines (FF1A-FF8A)

CHAPTER V. COMPARTMENTATION AND LOCALIZATION OF BIOACTIVE METABOLITES IN DIFFERENT ORGANS OF *ALLIUM ROYLEI*

Introduction

The need for disease and pest-resistant germplasm in onion (*Allium cepa* L.) breeding has promoted studies of the biosystematic relationships between edible and wild *Allium* species of the section *Cepa* (McCollum, 1982). Domestication of wild *Allium* species started millennia ago, followed by extensive dissemination of the flavoring condiment all over the world (Hanelt, 1990). Many wild *Allium* species with the characteristic onion or garlic aroma are used as spices or vegetables by various folks (Kamenetsky and Rabinowitch, 2006). The wild *Allium* species may serve as a potential source for *Allium* crop improvement, and it may comprise genes for desired traits, as in *Allium roylei* Stearn (De Vries et al., 1992b). *Allium roylei* has proven to be a crucial bridge species, enabling introgressions of genetic resources from other related species of section Cepa into the onion, allowing disease resistance and other valuable characteristics to be introgressed into the onion (Kofoet et al., 1990). The prospect of exploiting *A. roylei* as a source of disease resistance in onion breeding is very promising, as this wild species has proven to be completely resistant to downy mildew (*Perenospora destructor*), and partially resistant to leaf blight disease caused by *Botrytis squamosa* and basal rot disease caused by *Fusarium oxysporum* f. sp. *Cepae* (De Vries et al., 1992a). Although edible *Allium* species are rich in numerous phytonutrients and bioactive metabolites (cysteine sulfoxides, flavonoids, polyphenols, saponins, *etc*.) which reportedly have many functional properties such as antimicrobial, anticancer, and

antioxidant (Prakash et al., 2007; Lanzotti et al., 2012b) little effort has been invested in evaluating the wild *Allium* species as potential sources for these bioactive metabolites with therapeutic and disease resistance properties. There is no available information concerning the distribution and localization of these bioactive metabolites in different organs of *A. roylei* and their role as a part of the defensive characteristics of the plant against *F. oxysporum* f. sp *cepae*. Therefore, understanding the distribution and functions of the defense material may elucidate new molecular and chemical markers so that better genotype selection with higher disease resistance may be introgressed into the onion-breeding program. Our recent study addresses the phytochemical investigation of the *Allium* species and their functional compounds as a chemical barrier to pathogenic attack (Mostafa et al., 2013) In continuation of our previous research, (Vu et al., 2011) a comprehensive quantitative and qualitative analysis of cysteine sulfoxides (ACSO), flavonols, polyphenols, ascorbic acid, and saponins within different organs of *A. roylei* was conducted. Localization of saponins and flavonoids in *A. roylei* organs was explored for the first time in *Allium* species together with an assay of antioxidant activity. Furthermore, the antifungal activity of the saponin and flavonoid crude extracts was tested against different fungal and bacterial phytopathogens.

Materials and Methods

Plant Materials

Allium roylei accessions '95002', '95004', and '95005' were grown in a greenhouse at Yamaguchi University, Japan. Plants were harvested in June 2011.

Chemical and reagent

Analytical grade Chemicals were purchased either from Nacalai Tesque, Inc., (Kyoto, Japan), Wako Pure Chemical Industries Ltd., (Osaka, Japan), Somatech Center House Food Corporation (Tokyo, Japan), or Merck (Darmstadt, Germany). Millipore-grade water was used for all experiments.

Extraction and analysis of S-alk(en)yl-L-cysteine sulfoxides

Allium roylei leaves, bulbs, and roots-basal stems, with a fresh weight 5 g, were microwaved for 2 min, water extracted, and homogenized. The homogenate was centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was collected and kept at -20°C. An additional 300 μl was taken in an Eppendorf tube and centrifuged at 15000 rpm for 2 min. The supernatant was diluted 10X using 0.005% Trifluoroacetic acid (TFA) buffer and filtrated using a 0.45-μm syringe-type filter (HCL-Disk3, Kanto Chemical Co., Inc., Tokyo, Japan). A 60 μl filtered sample was injected into HPLC system and quantified. The HPLC system included a pump, a degasser, a column oven, a diode array detector set to 220 nm, a data collection system (EZchrom Elite^{TM} , Hitachi High-Technologies Corporation, Tokyo, Japan), and an AQUASIL SS-1251-120 column (4.6 mm *i.d.* x 250 mm long, Senshu Scientific Co., Ltd., Tokyo, Japan). The solvent was 0.005% TFA and flowed for 15 min at a flow rate of 0.6 ml/min. A series of standards were dissolved in distilled water and analyzed. In order to obtain the mean values of respective accessions, all chemical extractions consisted of three replications. Each extraction was applied to chemical determination two times.

Extraction and analysis of flavonols

A three-gram fresh weight of leaves, bulbs, and root-basal stem tissues were cut and extracted separately using 70% ethanol (EtOH). Extracted materials were filtered by using 0.45-μm syringe-type filters. A 20 μl filtered sample was injected into the HPLC system (Hitachi L-2130) and quantified. The following HPLC conditions were applied: UV detector (360 nm), data collector (Hitachi D-2520 GPC Integrator), and Mightysil RP C18 Aqua 250-4.6 (5μm) column (Kanto Chemical. Co., Ltd., Tokyo, Japan). Samples were eluted using gradient system with a constant flow rate of 1mL/min (A, formic acid 5%; B, methanol); gradient isocratic 85-65% A for 15 min; 65-60% A for 10 min; 60-20% A for 15 min; and 20-85% A for 20 min. Quercetin, quercetin 3,4′-*O*-diglucoside, and quercetin 4′-*O*- glucoside standards were dissolved in 70% EtOH and analyzed. In order to obtain the mean values of respective accessions, all chemical extractions consisted of four replications. Each extraction was applied to chemical determinations three times.

Extraction and analysis of total phenols

Two grams of leaves, bulbs, and root-basal stem tissues were extracted using 70% EtOH. Extracted materials were adequately diluted with water, and total phenolic compound content was determined by the Folin-Ciocalteu method (Folin and Denis, 1915). One mL of the diluted extract was mixed with 1 ml of Folin-Ciocalteu's reagent. After 3 min, 1 mL of 10% sodium carbonate aqueous solution was added and mixed thoroughly, and the mixture was incubated for 60 min at room temperature. The polyphenol content was quantified spectrophotometrically at 530 nm (Hitachi, Model U-2001, Tokyo, Japan). Quantification was achieved by comparison of catechol calibration curve. In order to obtain the mean values of respective accessions, all chemical

extractions consisted of three replications and each extraction was applied to chemical determinations three times.

Extraction and analysis of total saponins

The leaves, bulbs, and root-basal stems of *A. roylei* were freeze-dried (Taitec VC-360), until a final dry weight (2 g) was obtained. The dry materials were extracted according to the procedure described by Mostafa et al. (2013). Briefly, the dry weight was exhaustively extracted at room temperature with the solvents *n*-hexane and 80% methanol (MeOH). Each solvent extraction step was conducted for one day and repeated three times with 30 min of sonication and filtration. The MeOH extract was taken to dryness in a rotary evaporator with vacuum pump v-700 (Büchi, Rotavapor® R-3) under reduced pressure at 50ºC and then partitioned between butanol (BuOH) and H_2O (1:1, v/v). The BuOH layer was filtered and then concentrated under vacuum giving a saponins crude extract. Total saponin content was determined spectrophotometrically at 473 nm (Ebrahimzadeh and Niknam, 1998). Saponin concentrations were calculated based on the average value of absorbance at each concentration of disogenin standard. In order to obtain the mean values of respective accessions, all chemical extractions consisted of three replications. Each extraction was applied to chemical determinations three times.

Extraction and analysis of ascorbic acid

Total ascorbic acid content in *A. roylei* leaves, bulbs, and root-basal stems were determined as described by Roe and Oesterling (1944) with little modification. Fresh plant material (2.5 g) was extracted with 5 ml of 10% metaphosphoric acid and 5mL of water in an icebox using a mortar and pestle, and the extract was filtered and filled with water up to 12.5 ml. One ml of filtrate was transferred into test tubes, and 2 drops of indophenol were added. One ml of 2% metaphosphoric

acid was added followed by one ml of 2% thiourea, and 0.5 ml of 2, 4-dinitrophenylhydrazine was added. All the tubes were incubated in a water path at 50ºC for 70 min. The tubes were kept in ice for cooling, and 2.5 ml 85% H_2SO_4 was added and kept at room temperature for 30 min. Ascorbic acid content was detected spectrophotometrically at 530 nm. Quantification was achieved by comparison with the ascorbic acid calibration curve. In order to obtain the mean values of respective accessions, all chemical extractions consisted of three replications. Each extraction was applied to chemical determinations three times.

Histochemcial analysis

To explore histochemical analysis of saponins, sections of fresh root were cut using a Leica CM1850 cryostat microtome at -19ºC and stained with freshly prepared 5% vanillin-glacial acetic acid perchloric acid solution (Teng et al., 2009). Flavonoid localization was carried out using outer skin layers of fresh bulbs and stained with a 1% aluminum chloride EtOH solution. The sections were observed and photographed under a light microscope (Nikon-Eclipse E100).

Antioxidant activity

The radical scavenging activity of the extracts against DPPH radicals was measured using the method of Brand-Williams et al. (1995) with a slight modification: Fresh plant materials were extracted with 70% ethanol, homogenized and filtered. A series of ethanol extract dilutions were added to freshly prepared DPPH solution (200mM MES buffer pH 6; 30% EtOH; 400µM DPPH (1:1:1, v/v/v), and the mixture was shaken gently and left to stand at room temperature in the dark for 20 min. Thereafter, the absorbance was read at 520 nm. The effective concentration having 50% radical inhibition activity (IC_{50}) expressed as mg extract/ml, was determined from the graph of the free radical scavenging activity. In order to obtain the mean values of respective accessions,

all chemical extractions consisted of four replications. Each extraction was applied to chemical determinations three times. Quercetin aglycon (1.7×10^{-2} mg/ml) and quercetin 4′-*O*-glucoside (0.5 mg/ml) was tested for their radical scavenging activity as described above.

Antimicrobial activity

Antifungal activity of the saponin crude extract isolated from root-basal stems and the flavonoid crude extracts isolated from bulb outer skin was tested on five soilborne pathogens (*Fusarium oxysporum* f. sp. *cepae* 17, *F. oxysporum* f. sp. *cepae* TK, *F. oxysporum* f. sp. *cepae* TKN, *F. oxysporum* f.sp. *cepae* 12, and *F. oxysporum* f. sp. *cepae* 13) as a specific pathogen for *Allium* species and one airborne pathogen (*Colletotrichum gloeosporioides*) (Table 3). Antifungal activity was assessed by *in vitro* agar diffusion test. Briefly, 3.2 cm Plates of Potato Dextrose Agar (PDA) mixed with saponin and flavonoid crude extracts at three different concentrations (100, 500, and 1000 ppm) were inoculated with a 5 mm plug containing the fungi. Plates were incubated at 25ºC, and the fungi's radical growth was measured after seven days. Antibacterial properties of the saponin and flavonoid crude extracts were assessed for five bacterial strains (*Agrobacterium tumefaciens, Agrobacterium rhizogenes, Burkholderia glumae, Clavibacter michiganensis* and *Ralstonia solani*) (Table 3). The antibacterial activity was assessed by disc diffusion test on Nutrient Agar (NA) medium. One ml of the tested organism suspension was spread on the NA medium plate using a glass rod. The petri dishes were left at room temperature for 1 h. Using a cork borer, a 10-mm disc of the NA medium was removed, and three different concentrations (100, 500, and 1000 ppm) of the saponin and flavonoid extracts were loaded on the inoculated plates. After 30 min, the plates were incubated at 37ºC for 48 h. The definite zone of inhibition was measured and expressed in millimeters. All microbes were obtained from the Department of

Biological and Environmental Science, Molecular Plant Pathology, Faculty of Agriculture, Yamaguchi University, Japan.

Table 3. Fungal and bacterial isolates used in this study

Pathogen	Isolate (host plant)	Source
F. oxysporum f.sp. *cepae* 17	FOC17 (Bunching onion)	Yamaguchi University
F. oxysporum f.sp. *cepae* TK	FOCTK (Bulb onion)	Yamaguchi University
F. oxysporum f.sp. *cepae* TKN	FOCTKN (Bulb onion)	Yamaguchi University
F. oxysporum f.sp. *cepae* 12	FOC12 (Bunching onion)	Yamaguchi University
F. oxysporum f.sp. *cepae* 13	FOC13 (Bunching onion)	Yamaguchi University
Agrobacterium tumefaciens	MAFF301001 (*Prunus* sp.)	National Institute of Agricultural Science (NIAS)
Agrobacterium rhizogenes	MAFF210265 (Melon)	National Institute of Agricultural Science (NIAS)
Burkholderia glumae	YAR-1(Rice)	Yamaguchi University
Clavibacter michiganensis	T-1(Tomato)	Yamaguchi University
Ralstonia solani	YARS-1 (Tomato)	Yamaguchi University

Statistics

Values are expressed as the mean ± standard error (SE). The analysis of variance carried out using SPSS, Inc., 11.5. The significant effects were determined by the magnitude of the F-value ($p < 0.05$). The different metabolite means were separated by the Tukey's Honestly Significant Difference (HSD) test, and the antimicrobial activity was analyzed by the Dunnett's comparison test.

Results and Discussion

Cysteine sulfoxides

Determination of total cysteine sulfoxide (ACSO) content was carried out by means of HPLC. Methiin (MeCSO) was first separated at a retention time of 6.4 min, followed by alliin (AlCSO) with a retention time of 7.4 min and isoaliin (PeCSO) with a retention time of 9.4 min (Fig. 31A). A significant difference ($p > 0.05$) was detected in the ACSO content (mg/g FW) of *A. roylei* bulbs ranging from 1.59–2.03, of root-basal stems ranging from 1.48–1.93 and of leaves ranging from 0.37–0.79 (Fig. 31B). Since there is no available data regarding ACSO content in *A. roylei*, we compared our results with other *Allium* species. Our findings revealed substantially higher ACSO content (mg/g FW) within different organs of *A. roylei* than that reported in common onion bulbs, roots, and leaves (0.39, 0.39, and 0.40, respectively (Hovius and Goldman, 2005). Our findings were fairly consistent with the ACSO levels in shallot (*A. cepa* L. Aggregatum group) bulbs as reported previously (Yoo and Pike, 1998; Fritsch and Keusgen, 2006). However, previous reports didn't consider all three organs considered in this study. MeCSO, AlCSO, and PeCSO (mg/g FW) were detected in the three organs of *A. roylei* investigated. MeCSO content was the highest, followed by PeCSO and AlCSO content, but their distribution within the different organs showed significant differences ($p > 0.05$). MeCSO exhibited the highest concentrations in the bulbs, root-basal stem organs averaged 1.02, and the leaves had the lowest average at 0.11. However, PeCSO, predominant in the leaves, averaged 0.40. AlCSO was detected in trace amounts in the all plant organs, ranging between 0.04 and 0.11. The ACSO distribution pattern in *A. roylei* organs reveals a domination of MeCSO with detectable amounts of PeCSO in the leaves. This result is different

from the ACSO pattern of the common onion, where PeCSO is the characteristic compound in the different organs (Lawson et al., 1991a). MeCSO is ubiquitous in the genus *Allium*, and the ratio of MeCSO in combination with other CSOs is variable within the different species and is mainly correlated with their usage as vegetables or spices (Krest et al., 2000). In the present study, *A. roylei* expressed high MeCSO, which is associated with a hot pungent taste and strong sulfur smell; this could yield an important insight to onion breeders for taste and aroma development. In addition to the critical role of ACSO in determining the characteristic smell and taste of *Allium* species, these substances are physiologically active and used as antibiotic and antitumor agents (Kusterer et al., 2010). *Allium roylei* leaves and inflorescences were reported to be used as a condiment, whereas bulbs are used to relieve headaches by local populations in India (Kohli and Gohil, 2009).

Flavonol

Significant differences ($p > 0.05$) have been found in the total flavonol content within the different organs of *A. roylei* accessions. Higher levels of total flavonol were detected in the outer layers (second, first, and dry skin) of the bulbs (133.92 mg/100g FW) than in the leaves (30.36 mg/100g FW); however, there was no trace of any flavonol in the root-basal stems (Fig. 32B). The observed results can be explained by the high activity of phenylalanine ammonia-lyase, responsible for flavonol synthesis, which is allocated to the outer layers of the bulb (Hirota et al., 1999). Quantitatively speaking, previous literature has reported that the total amount of flavonol in onion bulbs is strongly associated with their color; the richest in these metabolites is the red onion, and the lowest is the white onion (Rodrigues et al., 2011). The value of total flavonol in the *A. roylei* bulb was higher than that reported in different onion cultivars, which ranged between 5 and 18.6 mg/100g FW (Price and Rhodes, 1997; Lee et al., 2008), 12.21 and 52.43 mg/100g FW (Sellappan and Akoh, 2002) 116.7, 102.3 and 67.3 mg/100g FW in the French shallot, Italian shallot, and red

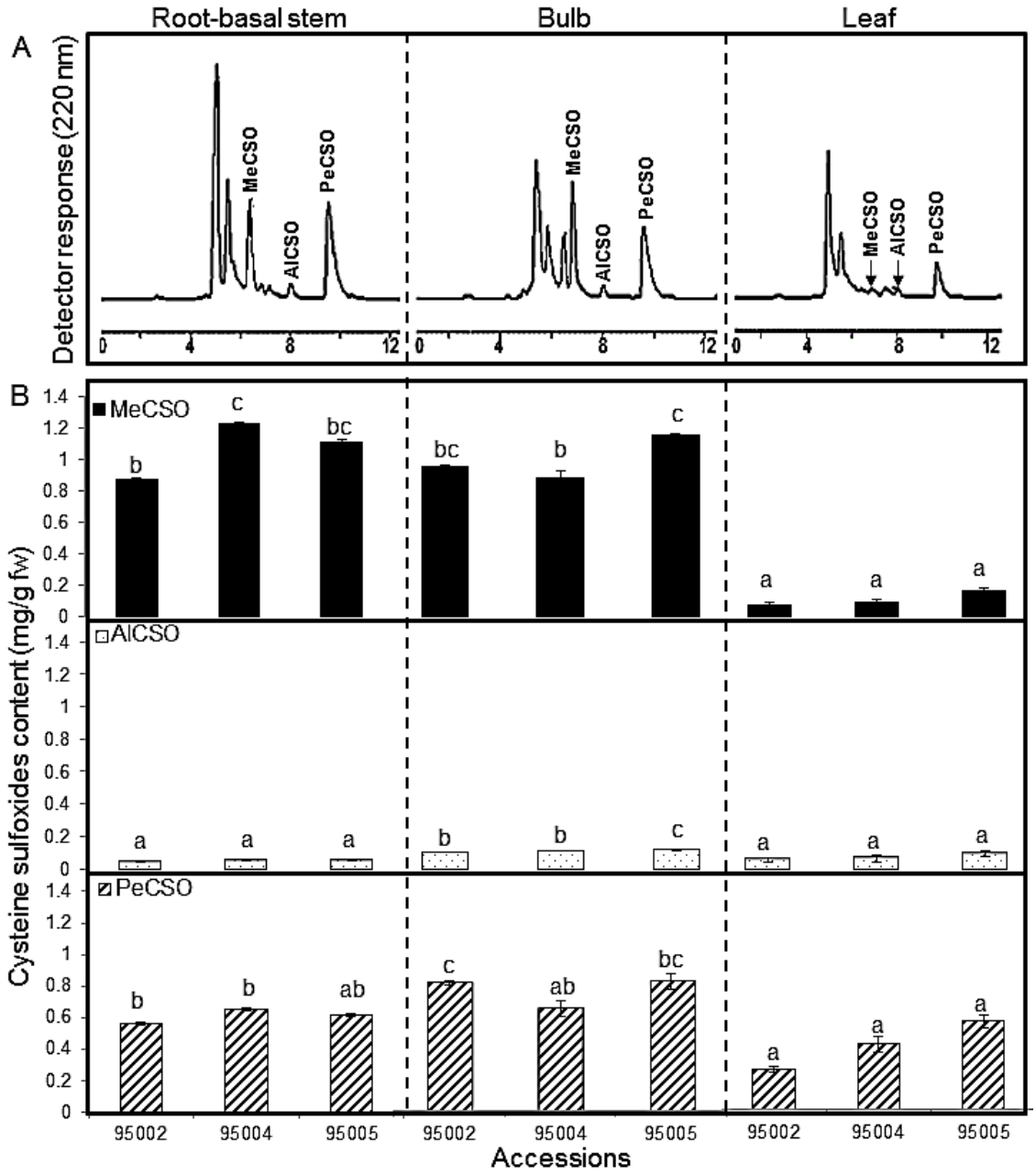


Fig. 32 Determination of total cysteine sulfoxides: methiin (MeCSO), aliin (AlCSO), and isoaliin (PeCSO) in *A. roylei* root-basal stem, bulb, and leaf Accessions '95002', '95004', and '95005'. A: Representative HPLC Chromatogram and B: cysteine sulfoxides content. Bar indicates ± SE (n = 3)

onion, respectively, (Bonaccorsi et al., 2008) and between16 and 149.75 mg/100g DW in *A. odorum* and *A. fistulosum* leaves, respectively (Miean and Mohamed, 2001). In general, flavonol levels reported in this study were higher than the values reported earlier. The procedural differences in the extractions and analyses as well as the characteristics of the species examined may explain these differences. We found two quercetin derivatives: quercetin 3,4′-*O*-diglucoside, quercetin 4′-*O*- glucoside, and quercetin aglycone (Fig. 32A). The quercetin glucoside and quercetin aglycone content varied significantly between organs, ranging from threefold to fourfold differences. Statistically significant higher levels of quercetin 3,4′-*O*-diglucoside, quercetin 4′-*O*-glucoside, and quercetin aglycone in *A. roylei* accessions were detected in the bulbs (ranges: 29.02–36.9, 48.12–58.85, and 44.96–49.31 mg/100g FW, respectively). However, these values were lower in the leaves (ranges: 4.54– 8.31, 12.52–14.1, and 9.1–11.23 mg/100g FW, respectively).The dominant flavonol in the bulb and leaf was ranked as follows: quercetin 4′-*O*-glucoside representing 41.12–42.66% > qurecetin 34.7–33.62% > quercetin 3,4′-*O*-diglucoside 24.16–22.16%. Our querectin glucoside and quercetin aglycone values were twofold to threefold higher than those reported in yellow and red onion cultivars (Perez-Gregorio et al., 2010). In our opinion, this high level of flavonol, especially quercetin aglycone, can be considered a characteristic feature of *A. roylei* which had a red- brownish bulb color that can be correlated with other red and yellow onions that show the same high levels of quercetin, ranging from 5.4–28.6 and from 11.7–20.2 mg/100g FW, respectively (Patil et al., 1995). Qualitatively speaking, the accumulation of flavonol in the bulb outer layer and the leaf rather than in the root can be linked to the role of these metabolites in the defense mechanism, in addition to their role in protecting plant cells against UVB radiation (Lake et al., 2009).

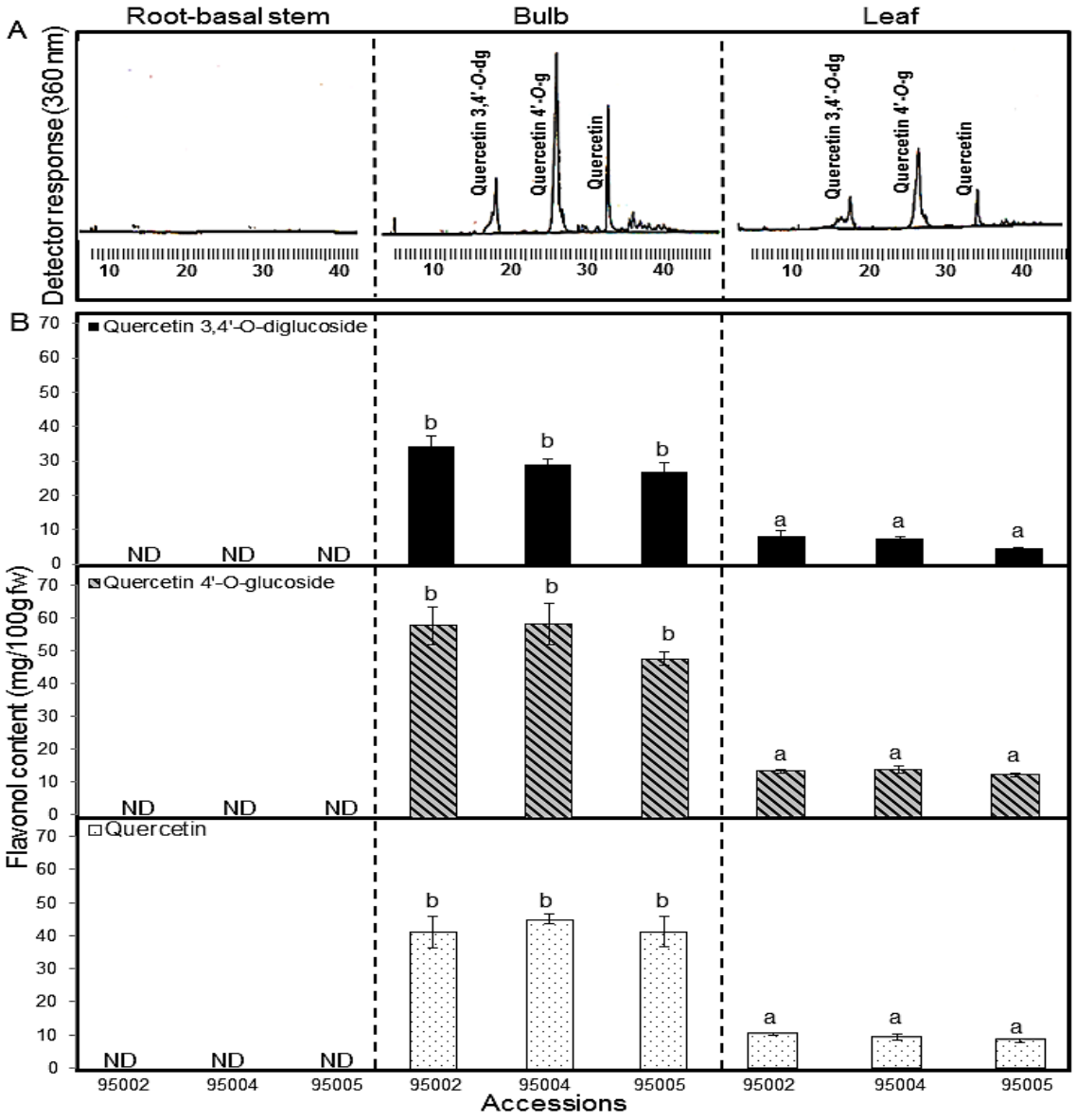


Fig. 32 Determination of total flavonol: Quercetin 3, 4′-*O*-diglucoside, Quercetin 4′-*O*- glucoside, and Quercetin in *A. rolyei* root-basal stem, bulb, and leaf Accessions '95002', '95004', and '95005'. A: Representative HPLC Chromatogram and B: Flavonol Content. Bar indicates ± SE (*n* = 4)

Polyphenol

The total phenol content determined by the Folin-Ciocalteu method expressed as catechol equivalents is presented in Fig. 33. The highest phenol content (mg/100g FW) in *A. roylei* accessions was recorded in the leaves, ranging between 97.99 and 119.72; then in the bulbs, ranging between 80.57 and 97.82; and the lowest in the root-basal stem, ranging from 34.82–44.16. The results obtained reveal a significant ($p > 0.05$) difference of total phenol content within different organs of *A. roylei;* this could be associated with the physiological and morphological importance of these metabolites in the plant. Phenolic compounds may act as phytoalexines (Popa et al., 2008) antifeedants, attractants for pollinators, or as plant pigmentation and protection against UV light (Nencini et al., 2011; Ignat et al., 2011), the high accumulation of phenol content in the bulbs and leaves as compared with the root-basal stem strongly supports this assumption. This organ-dependent repartition of the total phenol content has recently been reported for *A. roseum* with the highest content in the flowers, bulbs, and leaves (135, 44.3, and 55.9 mg GAE/ 100g FW, respectively (Dziri et al., 2012). Our total phenol levels are slightly higher than the values reported in different commercial onion cultivars, which ranged between 25.2 and 75.9 mg/100g FW (Lombard, 2000) and between 34.2 and 62.4 mg/100g FW (Galdon et al., 2009). However, it was considerably lower than the values reported in yellow onion cultivars, which ranged between 73.3 and 180.8 mgGAE/100g FW (Sellappan and Akoh, 2002). Total phenolic levels in different species vary considerably and mainly depend on the plant species/cultivars, the extraction procedure, or the growing region (Bilyk et al., 1984).

Ascorbic acid

In the present study, *A. roylei* accessions have particularly abundant ascorbic acid content within different organs, ranging from 14.59–112.22 mg/100g FW. The highest accumulation of ascorbic acid (mg/100g FW) was detected in the leaves, ranging from 85.32–112.22, then in the bulbs, ranging from 45.22–69.74; considerably less content was found in the root-basal stem, ranging from 14.95–22.09 (Fig. 33). The high level of ascorbic acid in the leaves suggests that ascorbic acid plays an essential role in the plant's antioxidant response toward radiation exposure. Previous studies have pointed out that ascorbic acid is capable of reacting with radicals generated during radiation exposure (Jimenez et al., 2011). The ascorbic acid levels in *A. roylei* were higher than those recorded in the white shaft and green leaves of leek cultivars (*A. ampeloprasum* var. *porrum*), which varied from 0.89–3.55 mg/g DW (Bernaert et al., 2012) and in *A. schoenoprasum* leaves, 24.59 mg/100g FW (Andarwulan et al., 2012). The high ascorbic acid content in *A. roylei* has important relevance, from a nutritional point of view, that makes this plant promising for nutritional enhancement of other edible *Alliums* through breeding programs.

Saponins

Despite much research that has addressed the role of saponins as a remarkable antifungal metabolites against different pathogens (Barile et al., 2007), few investigations have considered characterizing the distribution of total saponins within the different organs in *Allium* species. This information is very important, especially in the vegetable crops research, for obtaining the most valuable plant material with optimum nutritional components for proper harvesting or breeding strategies (Dong et al., 2003; Lim et al., 2005). Our results have revealed the highest accumulation of total saponin content (mg/g DW) in the root-basal stem, ranging between 12.26 and 15.42, followed by that in the leaf, ranging from 5.10–8.97, and the lowest detected in the bulb, ranging

from 4.6–5.33 (Fig. 33). These findings agree with our previous report (Mostafa et al., 2013), where saponins were highly detected in the roots of *A. nigrum* as compared with other organs. The same tendency was reported in *Medicago truncatula*, with high accumulations of saponins, 5.924 mg/g DW in the root and 1.064 mg/g DW in the leaf (Huhman et al., 2005). From a biological point of view, the massive accumulation of saponins in the root tissue as compared with other tissues suggests that saponins are responsible for protecting plants against many soilborne pathogens underground where the root-basal stem is mainly found. Further, the quantitative variance of saponins and their translocation from the root into the bulb, leaf, or flowers could be associated with the developmental growth stage and external factors, including interaction with insects and plant pathogens (Szakiel et al., 2011). The future prospects of saponin compounds as chemical markers for resistance against fungal pathogens would be an interesting point of research for onion breeders.

Histochemcial observations of roots and bulb skin

In the root section, cells of the epidermis, endodermis and primary xylem did not show any color reaction, whereas a light to dark pinkish-brown color appeared in the cortex and phloem (Fig. 34A). The outer skin section of the *A. roylei* bulb showed a massive accumulation of flavonol in the epidermal cells, particularly quercetin, which reacts with the reagent, giving a fluorescent greenish-yellow color (Fig. 34B).

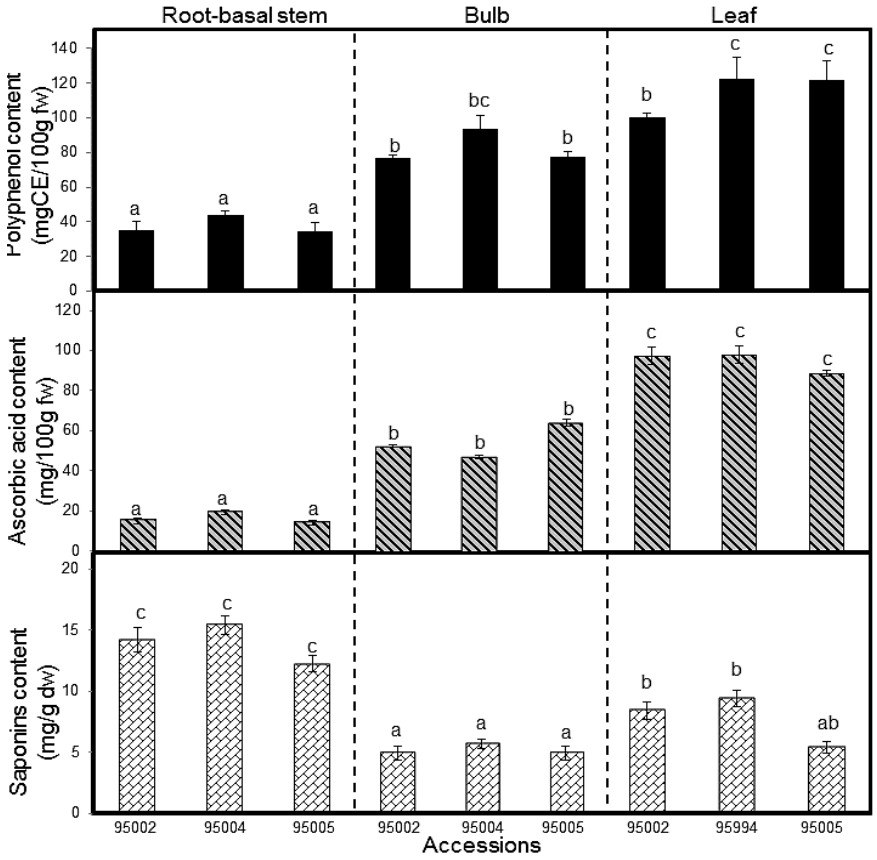


Fig. 33 Determination of total phenols, ascorbic acid, and saponins content in *A. roylei* root-basal stem, bulb, and leaf Accessions '95002', '95004', and '95005'. Bar indicates ± SE (*n* = 3)

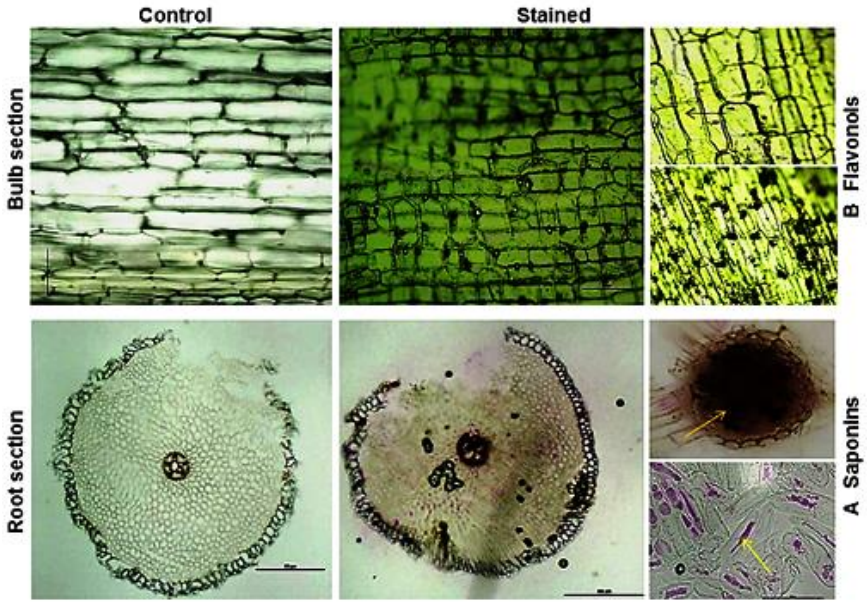


Fig. 34 A: Localization of saponin in the root section and B: Flavonoid accumulation in the bulb section of *A. roylei*

Antioxidant activity

The three ethanol extracts of the root-basal stem, the bulb, and the leaf were assayed for antioxidant activity against the DPPH radical (Table 4, 5). Free radical scavenging activity was expressed in terms of IC_{50} (mg/ml). There was no significant variation of IC_{50} among the three ethanol extracts, ranging from 0.649–0.757 mg/ml. The bulb extract was the most powerful scavenger for reducing the DPPH radical with IC_{50} (0.649–0.662) of the root-basal stem (0.710–0.715) and of the leaf (0.747–0.757 mg/ml). This result contradicts with *A. roseum* and *A. neapolitanum* flowers and leaves as being the most powerful antioxidant in the DPPH test in respect to bulbs (Nencini et al., 2011; Dziri et al., 2012). However, their DPPH activity was not correlated with the total phenol content in these organs and it was mainly attributed to flavonoid content. In our study, *A. roylei* showed a considerable amount of flavonol in the bulb as compared to the leaf. The DPPH scavenging activity using flavonol standards revealed a remarkable radical scavenging power for quercetin aglycon (Q) with IC_{50} (8.2×10^{-3} mg/ml), which is approximately 67 times greater than quercetin 4′-*O*-glucoside (Q4′G) IC_{50} (5.5×10^{-1} mg/ml). However, quercetin 3,4′-*O*-diglucoside did not showed any scavenging activity. These results indicated that the flavonol antioxidant activity is mainly due to Q contribution which is considerably high in *A. roylei* bulb organ. In addition, we calculated the contribution percentages (CP) of Q and Q4′G in the DPPH scavenging IC_{50} (Q-CP= Estimated Q content in the plant extraction IC_{50} (mg/ml) / Q standard IC_{50} (mg/ml) × 100; Q4′G-CP = Estimated Q4′G content in the plant extraction IC_{50} (mg/ml) / Q4′G standard IC_{50} (mg/ml) × 100). The 69.4 % of the CP was observed in Q and 1.2 % in Q4′G in the bulb organs. While, these CPs were very low in the leaf with 18.5 for Q and 0.3% for Q4′G. Generally speaking, our data revealed a high reducing power of *A. roylei* extracts as compared with previous IC_{50} levels reported in different onion cultivars, ranging from 1.44–4.20

mg/mL in the red onion and from 1.84–4.67 mg/ml in the violet onion (Prakash et al., 2007). However, it was lower than values reported previously in methanol extracts of five *Allium* species (*A. nevsehirense*, *A. sivasicum*, *A. dictyoprosum*, *A. scrodoprosum*, and *A. atroviolaceum*) which showed IC_{50} ranging between 0.079 and 0.104 mg/ml (Szakiel et al., 2011). The most probable reason for this variation in antioxidant activity in *A. roylei* as compared with other species might be due to variation in the quantities of flavonols especially quercetin, phenolic acid, and ascorbic acid (Tepe et al., 2005; Diplock et al., 1998).This hypothesis was supported with our HPLC and spectral analyses of these metabolites in each organ which revealed a high quercetin, phenolic acid, and ascorbic acid content in the bulb and leaf organs. On the other hand, these metabolites were not detected or were detected only in small amounts in the root-basal stem organ. In our opinion, the powerful antioxidant activity of the root-basal stem extract could be encountered with the high ACSO and saponin content. Similar reports have attributed the antioxidant activity of some *Allium* species to organosulfur compounds and their precursors (Kim et al., 1997; Griffiths et al., 2002). Crude saponin extract from the root-basal stem organ revealed a strong antioxidant activity in the DPPH test (data not shown). The role of saponin compounds as an antioxidant agent has not yet been reported and is still unclear; future studies are needed to confirm this new finding.

Antimicrobial activity

The crude saponin extract from the root-basal stem and the crude flavonoid extract from the bulb outer skin were tested for their antimicrobial activity against a number of fungal and bacterial pathogens. All extracts showed significant antifungal activity depending on their concentration and the tested fungal pathogen (Fig. 35). The obtained results reveal a potent antifungal activity of the crude saponin extract against *C. gloeosporioides* and *F. oxysporum* f. sp. *cepae* isolates at 1000 ppm. Maximum inhibition of fungal growth (47.76%) was recorded with *C. gloeosporioides*

Table 4. DPPH scavenging activities IC_{50} (mg/ml) of different *A. roylei* organs extracts

Plant sample	Inhibition of DPPH radical IC_{50} (mg/ml)		
	95002	94004	95005
Leaf	0.748 ± 0.008	0.747 ± 0.008	0.757 ± 0.004
Bulb	0.662 ± 0.016	0.648 ± 0.026	0.654 ± 0.028
Root-basal stem	0.715 ± 0.008	0.710 ± 0.006	0.711 ± 0.010

DPPH scavenging activity of four replications followed by Means (n = 4, ± SE)

Table 5. Analysis of variance for DPPH in different organs and accessions *of A. roylei*

Source	Degree of freedom	Sum of squares	Mean of squares	*F* value
Total	36			
Accessions	2	0.000	0.000	0.154
Organs	2	0.056	0.028	29.311***
Error	27	0.026	0.001	

***significant at 5% level

However, this activity was variable among *F. oxysporum* f. sp. *cepae* isolates. *F. oxysporum* f. sp. *cepae* 12 and 13 were the isolates most sensitive to the crude saponin with 41.33% growth inhibition. *F. oxysporum* f. sp. *cepae* TK and TKN were more resistant, showing 10.43 and 9.11% growth inhibition, respectively. Our results are in agreement with previous studies, which pointed out different responses of *F. oxysporum* strains against saponin compounds. Lanzotti et al. (2012a) reported that *F. oxysporum* f. sp. *lycopersici* was not affected by saponin compounds isolated from *A. cepa*. However, moderate to high responses of *F. oxysporum* and *F. oxysporum* f. sp. *lycopersici* toward saponin compounds isolated from *A. minutiflorum* were reported (Barile et al., 2007). The variability of *A. roylei* crude saponin activity against *F. oxysporum* f. sp. *cepae* could be explained by the ability of some fungal pathogens to produce enzymes that degrade saponin into nontoxic molecules and the nature of the aglycone structure (Lanzotti et al., 2012a; Morrissey et al., 1999). All the crude saponin activity showed a statistically significant difference as compared with the disogenin standard within the same tested fungal pathogens. The crude saponin extract did not show any antibacterial activity toward the examined bacteria (Table 6). Previous studies have reported that bacteria in general are less sensitive to saponin as compared with fungi (Avato et al., 2006). The crude flavonoid extract was not effective against most of the tested fungal pathogens at 1000 ppm (Fig. 35). *Fusarium. oxysporum* f. sp. *cepae* isolates showed high (4.37–9.11%) to complete resistance to flavonoid doses. Furthermore, the highest inhibition (11.65%) was recorded with *C. gloeosporioides*. This result indicates that flavonoids' role in defending the plant against *F. oxysporum* f. sp. *cepae* is limited as compared with that of saponins, which seem to be the major metabolites involved in plant protection. Similarly, fair to low antifungal activity of flavonoid compounds was recorded against *Aspergillus niger* and *Candida albicans* (Rauha et al., 2000). However, our results contradicts those of Skerget et al. (2009) who showed high antifungal

activity of the onion skin extract against *A. niger* and *T. viride* (35.6 and 63.0%, respectively). The crude flavonoid extracts exhibited different antibacterial activity among test bacterial pathogens, ranging between 5.6 and 22 mm (Table 6). High affinity of the flavonoids' antibacterial activity was recorded with *Ralstonia solani*, *Agrobacterium tumefaciens*, and *Burkholderia glumae* (22, 16 and 5.6 mm, respectively). However, *Agrobacterium rhizogenes* and *Clavibacter michiganensis* were highly resistant. The same tendency of considerable antibacterial activity was recorded in the flavonoid extract from the yellow onion (GO cultivar) against gram-positive bacteria as compared with the flavonoid extract from the white onion (FE cultivar) (Santas et al., 2010). The observed results demonstrate that the antibacterial activity of the crude flavonoid extract was generally higher than their antifungal activity. *A. roylei* ACSO can be implemented in the taste and aroma development with other edible *Allium* species. The prospects of *A. roylei* saponin compounds as chemical markers for resistance against fungal pathogens would be an interesting point of research for onion breeding.

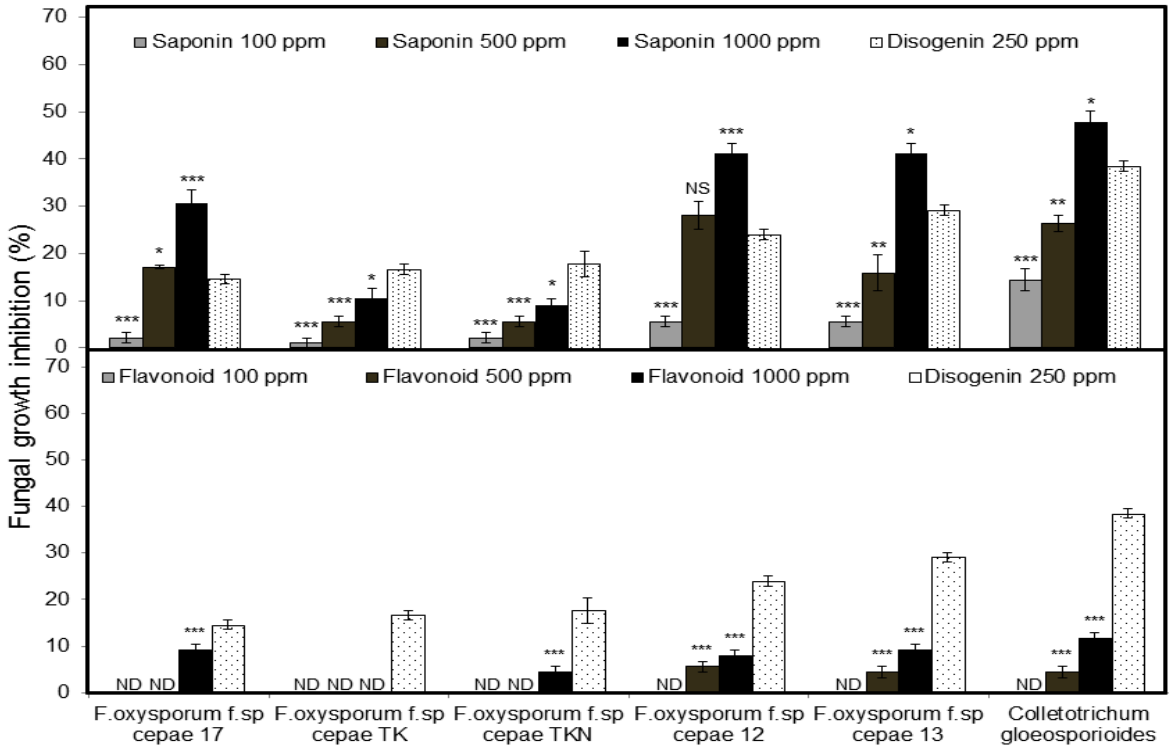


Fig. 35 Fungal growth inhibition (%) of saponin and flavonoid crude extracts at three different concentration (100, 500 and 1000 ppm). Data are expressed as mean ± SE (n = 3). Asterisks denote significant differences between treatments and disogenin standard determined by Dunnett's test (NS = not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$). ND = not detected

Table 6. Antibacterial activity of saponin and flavonoid crude extracts

Bacterial pathogen	Bacterial growth inhibition (mm)						
	Crude saponin (ppm)			Crude flavonoids (ppm)			Ampicillin (ppm)
	100	500	1000	100	500	1000	250
Agrobacterium tumefaciens	ND	ND	ND	5.6±0.66***	13±0.33**	16±0.30 NS	19.6±0.88
Agrobacterium rhizogenes	ND	ND	ND	ND	ND	5.6±0.57***	18±1.15
Burkholderia glumae	ND	ND	ND	ND	5.6±0.33***	5.6±0.33***	14.3±1.45
Clavibacter michiganensis	ND	ND	ND	ND	ND	ND	14.6±1.45
Ralstonia solani	ND	ND	ND	9±0.33***	15±0.88***	22±0.33NS	25.6±2.02

Data are expressed as mean ± SE (n = 3). Asterisks denote significant differences between treatments and Ampicillin standard determined by Dunnetts test (NS = not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$). ND = not detected

Quantitative and qualitative Saponin profiling in F_1, allotriploid and amphidiploid lines of shallot and *A. roylei*

To investigate the significant impact of *A. roylei* saponin on other edible *Allium* species, a crossbreeding between *A. roylei* and *A. cepa* Aggregatum group L. was carried out and the F_1 hybrid was subjected to chromosomal doubling and then backcross with shallot to obtain the amphidiploid and allotriploid lines (Fig. 36). Saponin profiling from each line was obtained and the antifungal activity of the crude saponin extract from each line was examined against different *Fusarium* pathogens. Differences were observed between parental lines *A. cepa* L. Aggregatum group (AA) and *A. roylei* (RR), and introgressed lines including allotriploid (AAR) and the amphidiploid (AARR) in the total saponin content (Fig. 37). Significant higher saponin content was observed in the amphidiploid and allotriploid plant in comparison with the *A. cepa* L. Aggregatum group. The thin-layer chromatography also showed qualitative differences in saponins between these genotypes. The present study revealed that, the triploids and amphidiploid lines had significantly higher saponin content and more antifungal activities against examined *Fusarium* pathogens in comparison with shallot (Fig. 38). The additional saponin content of the allotriploids and amphidiploid lines would be derived from *A. roylei* saponin biosynthesis controlled by the introgressed genes located on the extra chromosomes of *A. roylei*. The observations of chemical modifications in the *A. cepa* L. Aggregatum group–*A. roylei* addition lines would bring helpful information regarding chromosome manipulation to improve the consumer quality as well as the disease resistance of *A. cepa* L. Aggregatum group. The introgression of desirable traits, such as *Fusarium* or downy mildew resistance, from *A. roylei* to *A. cepa* L. Aggregatum group is promising for the future, as a BC_3 generation could be produced via initial trials of selfing and backcrossing of the *A. cepa* L. Aggregatum group –*A. roylei*. The

same observation of such kind of saponin modification was observed in our previous study where a transformation of a specific saponin compound from shallot to *A. fistulosum* was resulted in an improvement in the total saponin content and plant resistance against *Fusarium* pathogen (Hoa et al., 2012). The same tendency of saponin modification was also observed in our recent study of the saponin profile of allotriploid (FFR) and amphidiploid (FFRR) lines of *A. fistulosum-A. roylei* (Ariyanti et al., 2015).

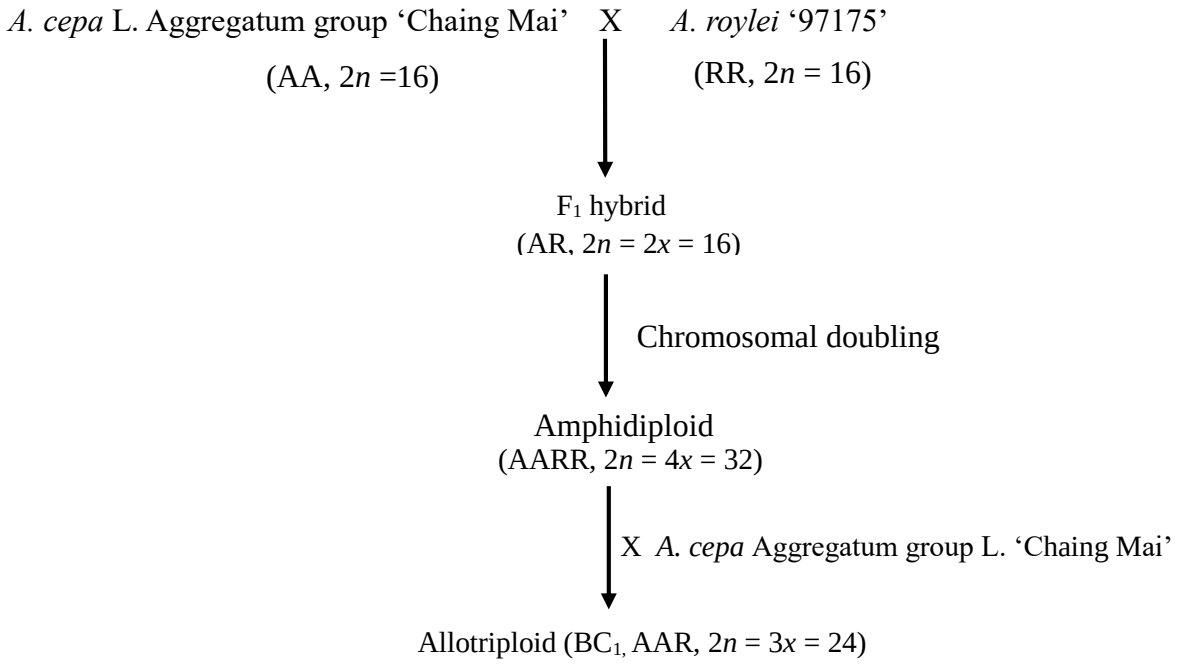


Fig. 36 Method for producing alien addition lines of *Allium cepa* L. Aggregatum group with extra chromosomes of *A. roylei*

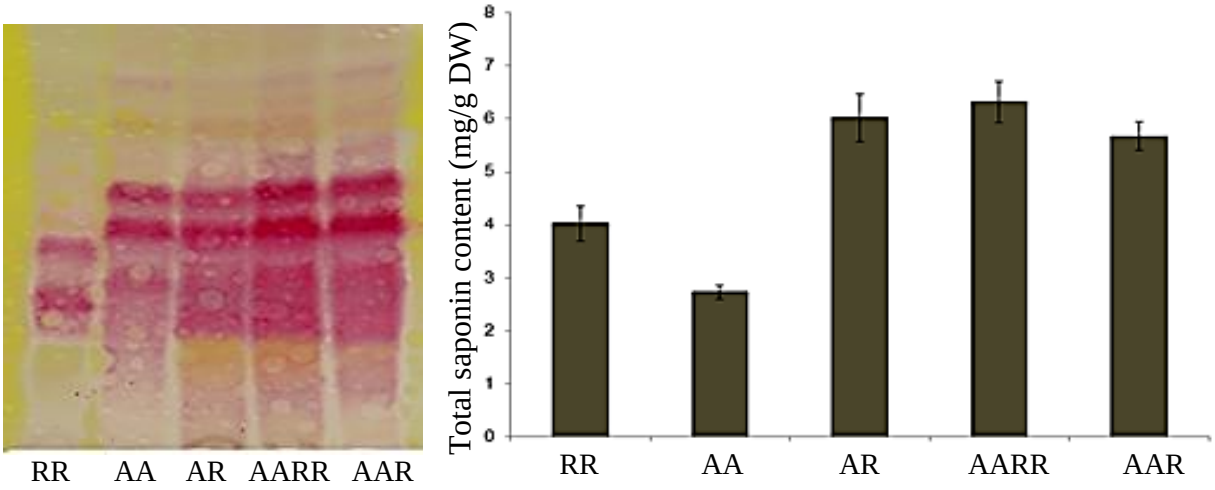


Fig. 37 (A) Comparative saponin TLC profile from *A .roylei* (RR), *A. cepa* Aggregatum group (AA), F_1 (AR), amphidiploid (AARR) and triploid (AAR) crude saponin extract. (B) Total saponin content in each genotype. Bars indicates mean ± SE (*n*=3)

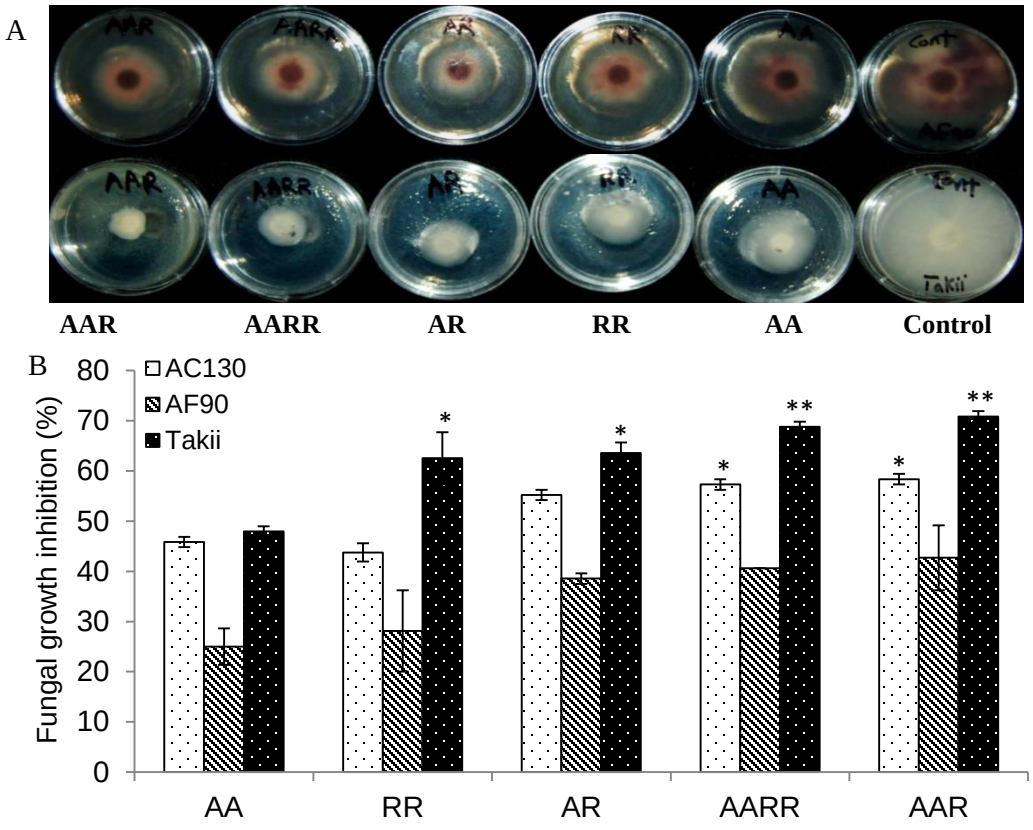


Fig. 38 *In vitro* antifungal activity of the crude saponin against *F. oxysporum* f. sp. *cepa* strain AC130, AF90 and Takii. (A) Agar diffusion antifungal test and (B) fungal growth inhibition %. Bars indicate means ± SE (*n*=3). Significant level at 5% level according to Tukey's HSD test

CHAPTER VI. INTEGARTING TRANSCRIPTOME AND TARGET METABOLOME VARIABILITY IN DOUBLED HAPLOIDS OF *ALLIUM CEPA* FOR ABIOTIC STRESS PROSPECTING

Introduction

Onions (2*n* =16) are the third most important crop worldwide; most commercially grown cultivars are F_1 hybrids (Alan et al., 2003). Onions are generally considered to be drought sensitive; this limits its production to areas with adequate irrigation facilities, which influences the production price (Brouwer et al., 1989). Abiotic stresses have a significant economic impact; it has been suggested that they reduce the average yield by > 50% for most major crop plants (Atkinson and Urwin, 2012). This is expected to increase drastically with global climate change, affecting global agricultural systems and resulting in a mandate for stress-tolerant crop varieties (Takeda and Matsuoka, 2008; Newton et al., 2011). In Southeast Asian countries, shallots (2*n*=16) which are closely related to onions, are an economically important crop because of their pungency and adaptability to environmental stresses (Sulistyaningsih et al., 1997); however, the molecular and metabolic architecture underlying this tolerability is still unclear. Inheritance of genetic materials from shallot to the Japanese bunching onion (*A. fistulosum*) enhanced *A. fistulosum's* flavonoid profile, one major metabolites that interacts with abiotic stress (Masuzaki et al., 2006), amino acid content and cysteine sulfoxide production during the summer season (Masamura et al., 2011), sucrose and fructan content in the winter season (Yaguchi et al., 2008), and shallot specific saponin compounds for *Fusarium* basal rot resistance (Vu et al., 2012). These reports gave insight into the

potentiality of shallot as a prospective genetic resource for the future breeding of onions toward biotic and abiotic stress tolerance.

The shallot is a heterozygous species; therefore, hybrids of the shallot and the onion will show variations in many characters. This will hamper the progress of the breeding program. In addition, development of an inbred onion line may require 5-10 years, depending on the complexity of the traits under selection. Double haploid (DH) techniques that use the chromosomal doubling of haploid plant can shorten the time needed, offer homozygous pure lines, and provide precious materials for genomic analysis (Sulistyaningsih et al., 1997; Alan et al., 2003). With the development of genomic initiatives to outline genome and gene expression in the context of plant-environment interaction, metabolic profiling is required for a better understanding of plant response mechanisms against various environmental stresses (Peremarti et al., 2014). MS-based metabolomics allow concomitant detection of several hundred metabolites as a snapshot of metabolomics phenomena (Sawada et al., 2009). Integrated metabolomics, genomics, and transcriptomics, referred to as *omics technologies*, play an important role in phytochemical genomic and crop breeding in sequenced plants (Saito and Matsuda, 2010).

In this context, knowledge of the environmental stress adaptability of DHA, a cultivar of tropical origin, as compared to DHC is gaining importance. The aim of the present study was to discriminate between DHC, DHA, and F_1 genotypes using target metabolite analyses through chemical assignment for major metabolic classes, including metabolism of amino acids, carbohydrates, phospholipids, flavonoids, and alkaloids by LC-MS/MS integrated with the transcriptional profiling of their relevant genes under normal conditions. The obtained data will be useful for elucidating gene function for understanding biological variations in DHC, DHA, and F_1

genotypes and for demonstrating a technical ability to reveal the expected coherence between metabolic traits and abiotic stress parameters in the context of *Allium* genotypes.

Materials and Methods

DHC, DHA, and F_1 production

Shallot strain 'Chiang Mai' from Thailand and the long-day onion stain 'Sapporo-ki' from Japan were used to establish the DHC and DHA according to Sulistyaningsih et al. (2006), with some modifications (Fig. 39 a and b). DHC grown in pots were used as donors and DHA was used as recipients for crossing to obtain F_1 hybrids.

Total RNA extraction and transcriptional analysis

The total RNA of the DHC, DHA, and F_1 was isolated from the fresh leaf tissue using RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Using the total RNA of each plant, cDNA was synthesized using a First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) with AMV reverse transcriptase. Next-generation sequencing for the isolated RNA was carried out at the Bejin Genomic Institution (BGI) using the Illumina GA/HiSeq System (HiSeq 2000 series) combined with HiSeq control software for program control, real-time analyzer software to do on-instrument base-calling, and CASAVA for secondary analysis. The gene expression dataset was uploaded in the *Allium* Transcript Database (*Allium* TDB, http://allium.kazusa.or.jp/).

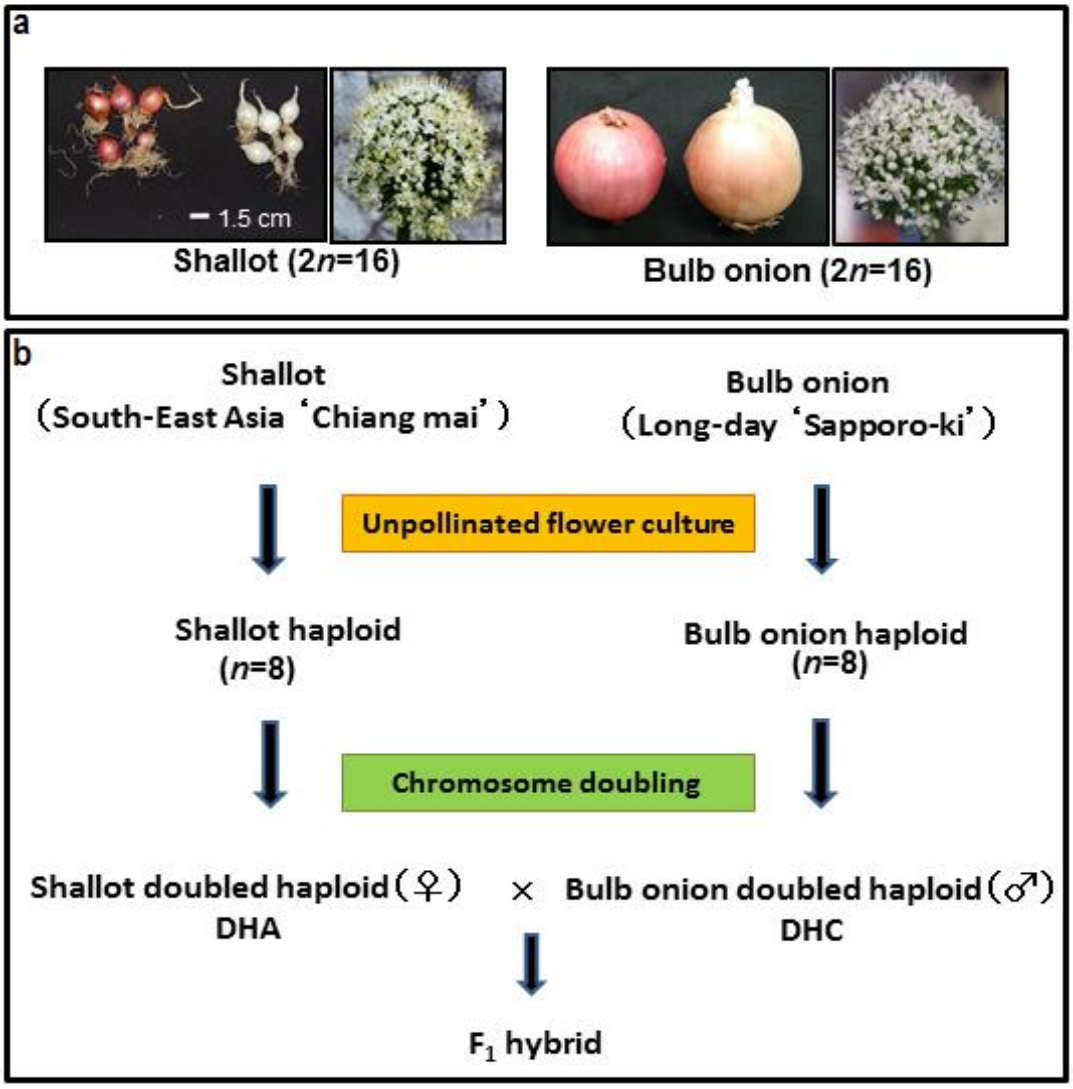


Fig. 39 Plant materials production: long-day bulb onion 'Sapporo-ki' and shallot 'Chaing-Mai' (a) and Scheme diagram for DHC, DHA and F_1 production (b).

Extraction of Metabolites

The leaf tissue samples of each genotypes were pooled, freeze-dried, powdered, and divided into three group representing three analytical replication. Freeze-dried samples (4 mg DW) in 2 ml tubes were extracted with 80% MeOH that contained 2.5 μM lidocaine per mg of dry weight using a mixer mill (Shake Master Neo, BMS, Tokyo, Japan) with 5 mm zirconia beads for 1000 rpm for 5 min at 4°C. After centrifugation for 10 min, the liquid samples were prepared using a liquid handling system (MICROLAB STAR PLUS, Hamilton Co., Reno, NV, USA); 50 ul of the supernatant was transferred to a 96-well formatted collection plate and they were dried, resulting in 250 μl of LC-MS grade pure water.

LC−MS analysis

A liquid sample (1 μL) was analyzed using the UPLC-TQS (Waters Corp., Watertwon, MA, USA). Analytical conditions were as follows. LC: column, ACQUITY UPLC HSS T3 (1.8 μm, 1.0 mm ×100 mm, Waters Corp.); solvent system, solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid); gradient program, 99.9% A/0.1% B at 0 min, 99.9% A/0.1% B at 0.25 min, 91.0% A/0.9% B at 0.40 min, 83.0% A/17.0% B at 0.80 min, and 0.1% A/99.9% B at 1.90 min, 0.1% A/99.9% B at 2.10 min; 99.9% A/0.1% B at 2.11 min flow rate, 0.240 ml/min.

Results

Metabolic variations in DHC, DHA, and F_1

A total of 113 distinct metabolites were positively annotated using reference data of the retention time and the mass spectrum with authentic compounds. To validate the biological data based on a statistically significant difference, PCA loading plots were performed on the metabolite dataset (113 metabolites, 3 genotypes, 3 leaf tissues) to build models that can visualize the relationship between the metabolite signature and the genotypes (Fig. 40). Clear discrimination of the three genotypes showed 85.8% of the variance in PC1 and 7.2% in PC2 with an accumulative proportion of variance of 93%. The entire dataset was further analyzed using a Volcano plot (Figs. 41, 42, and 43) to give a scatter overview of the metabolites exhibit a significant genotype variance (DHA vs. DHC, F_1 vs. DHA, and F_1 vs. DHC). Of the complete set of 113 metabolites, 49 metabolites were found to be statistically different within the genotypes (Log10 $P < 0.05$ and log2 fold changes > 1.2). The genotype-metabolite fingerprints are demonstrated in Table 7. To determine the metabolite flux, a Venn diagram (Fig. 44) of the compounds whose accumulations were significantly ($P > 0.05$) altered by genotype carried out. Of the 49 metabolites, 10 metabolites were characteristic for DHC, 11 for DHA, and 14 for F_1, and 14 were mutual among the three genotypes. It was interesting to observe that of the 14 mutual metabolites, 10 were shared between DHA and F_1, three between DHC and F_1, and one between DHC and DHA. This observation indicates that the metabolic flux in the F_1 genotype was mainly directed from the DHA metabolic pool rather than that of DHC, which may offer a prediction as to the capability of F_1 as stress-tolerant candidate, emphasizing the potential of DHA as an important bioresource for plant breeding. The

identified metabolites represent a small number of distinct metabolic classes, the most predominant involving amino acid, organic acid, and carbohydrate metabolism (including monosaccharaides, oligosaccharides, and sugar alcohol), shikimate pathway metabolism (including phenylpropanoids and alkaloids), and phospholipid metabolism. In the present study, DHA and F_1 showed a distinctive accumulation of free aromatic and non-aromatic amino acids and their derivatives in compare with DHC genotype. Organic acids and their derivatives were detected in DHC, including Aspartic acid and beta-homoglutamine, however, malic acid was specific to DHA and F_1. The most important metabolic variation was observed in the carbohydrate metabolism. An increase in carbohydrates was detected in the DHA and F_1, which seem to be a characteristic feature for those genotypes as compared to the DHC. Furthermore, an important signaling molecules and osmolytes that influenced the plant's stress response were exclusively detected in DHA and F_1 such as alkaloids (trigenolline and indole-3-carboxaldehyde), flavonoids (luteoline, kampferol, quercetin, and cyanidine glucoside), phospholipid (phosphocholine, glycerol-3-phosphate, glycerol-3-phosphocholine, and Glyceraldehyde-3-phosphate), quaternary ammonium compounds (carnitine and dimethylglycine), and vitamins including vitamin B6 (pyridoxine) and Vitamin B5 (pantothenic acid). DHC accumulated an important class of quaternary ammonium compounds such as betain and dimethylglycine, in addition to nucleoside molecules, such as inosine and cytidine.

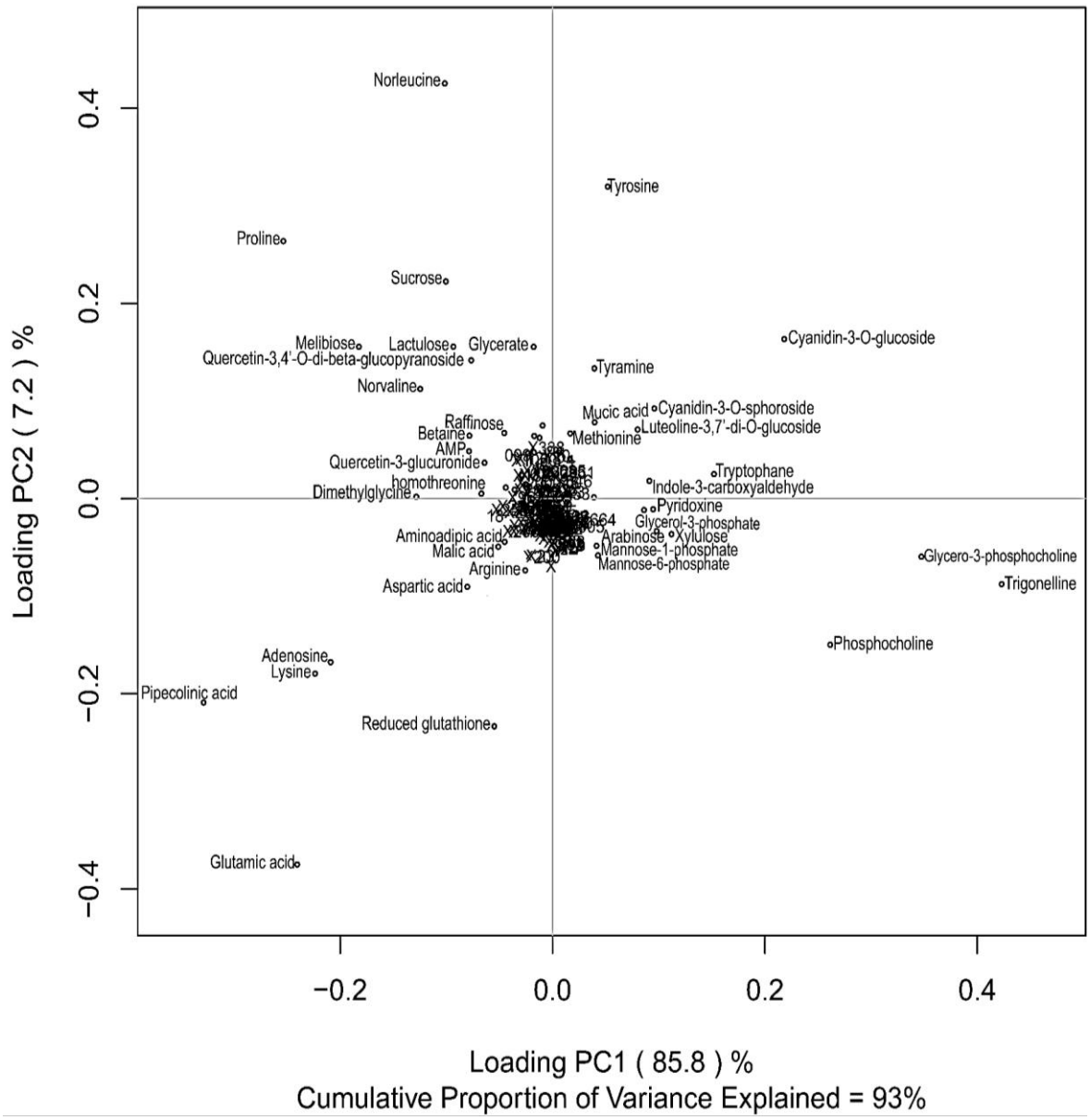


Fig. 40 PCA loading plots of LC-MS/MS data representing the metabolites discrimination among the three genotypes (DHC, DHA and F_1) with percentage of variance captured by each PC.

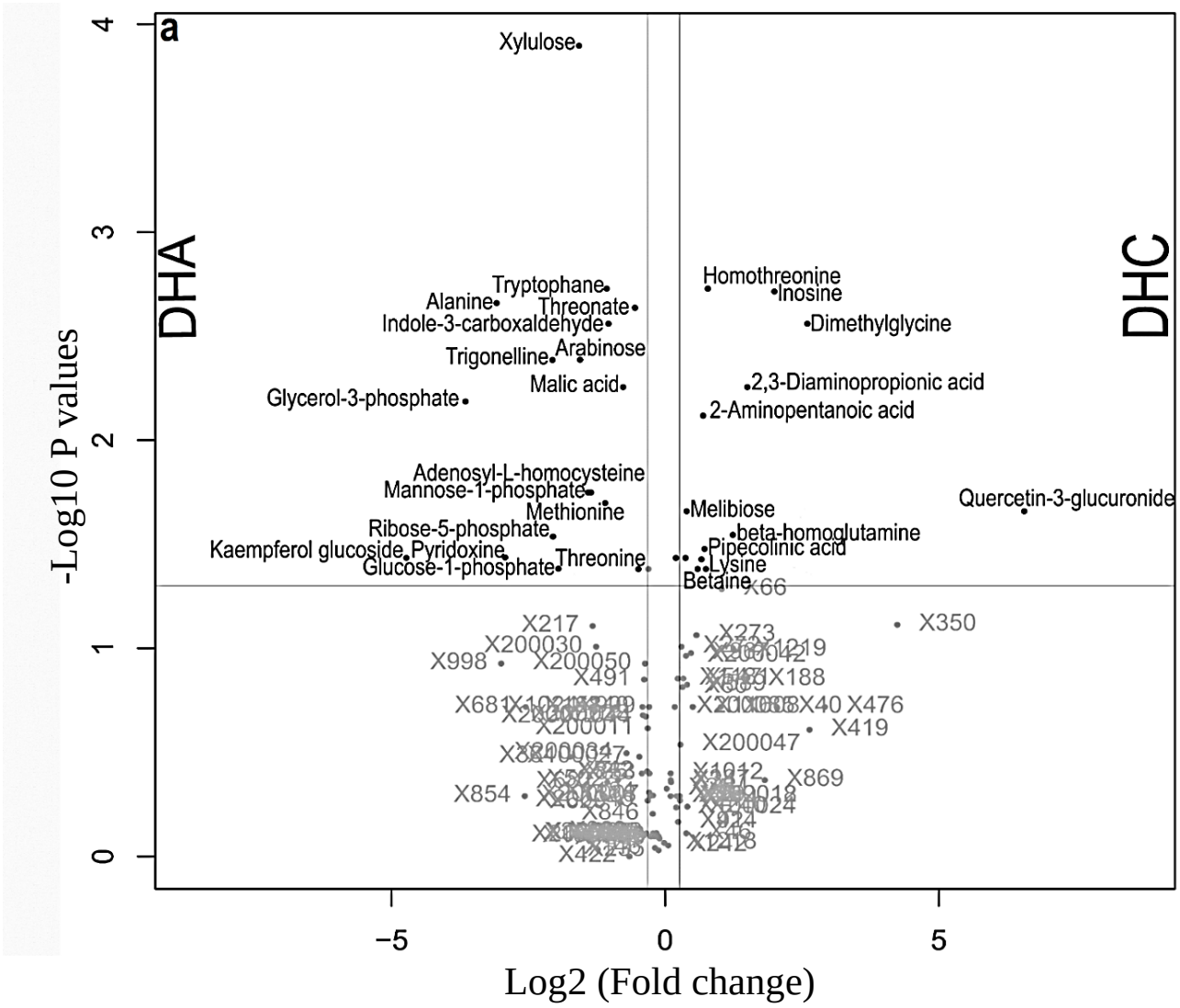


Fig. 41 Volcano plots of genotype-metabolic comparison: DHA versus DHC. Significant difference with $P < 0.05$ and >1.2-fold intensity ratio are shown as black spots. Gray spots mean no significant differences.

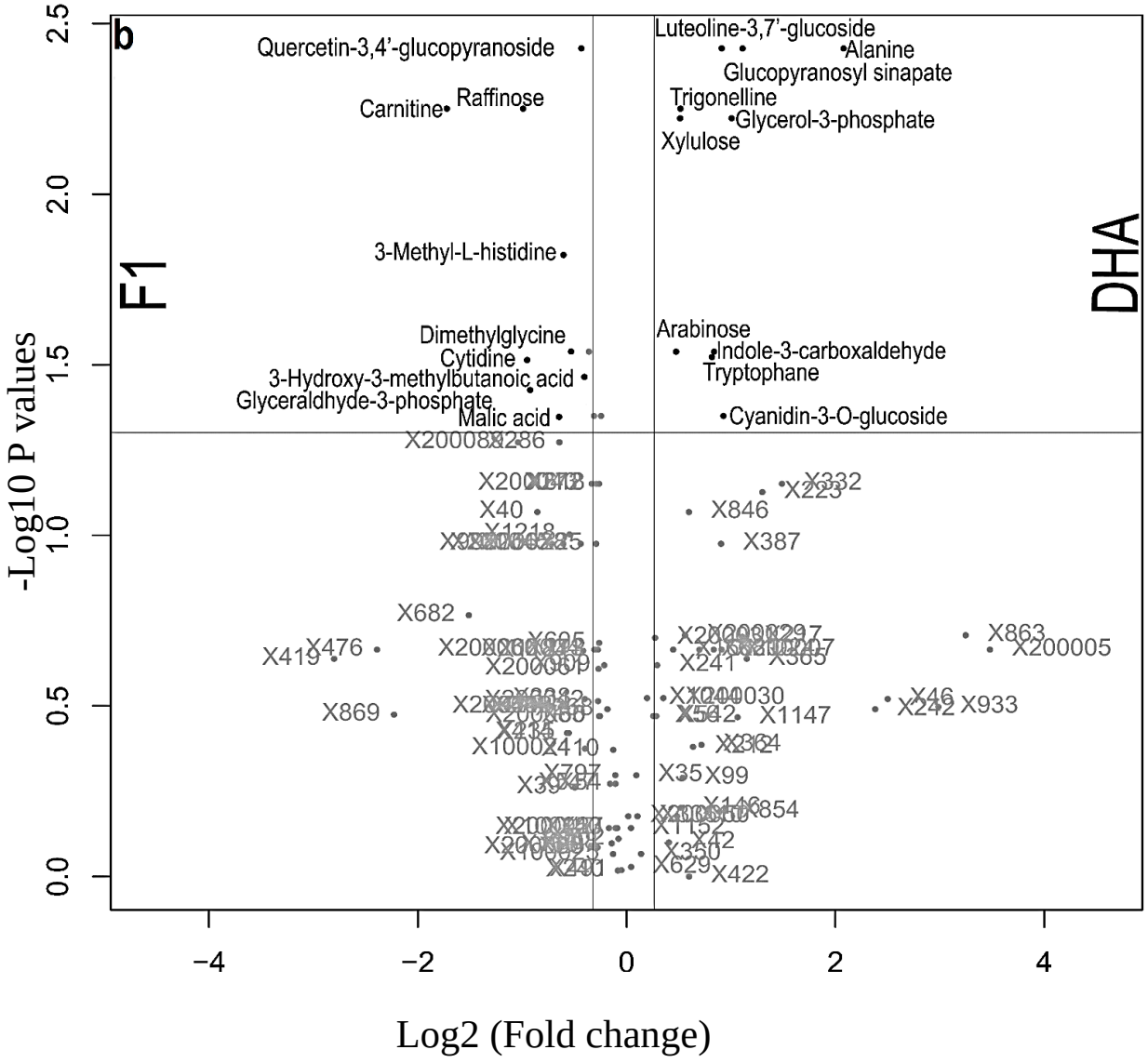


Fig. 42 Volcano plots of genotype-metabolic comparison: F_1 versus DHA. Significant difference with $P < 0.05$ and >1.2-fold intensity ratio are shown as black spots. Gray spots mean no significant differences.

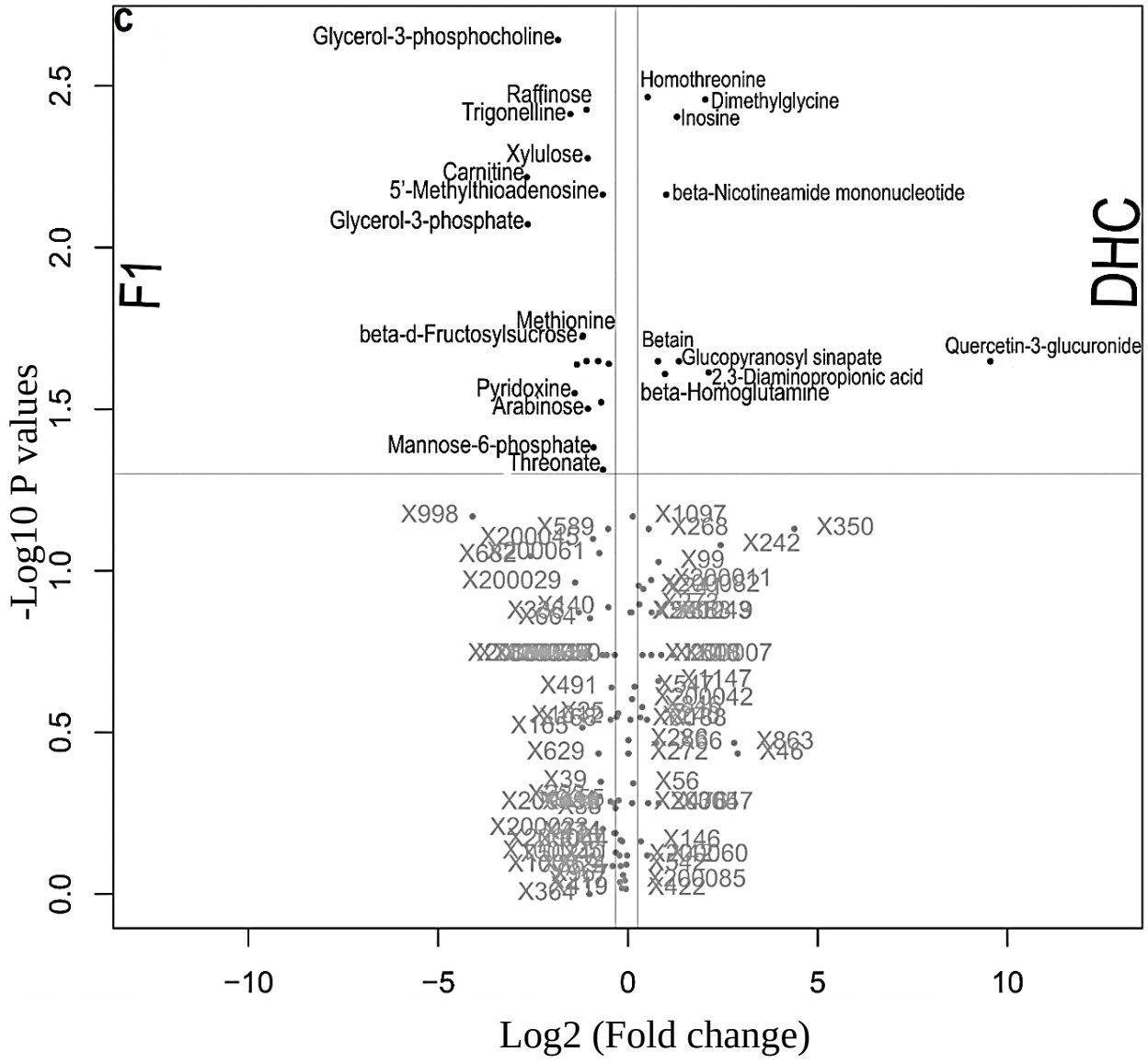


Fig. 43 Volcano plots of genotype-metabolic comparison: F_1 versus DHC. Significant difference with $P < 0.05$ and >1.2-fold intensity ratio are shown as black spots. Gray spots mean no significant differences.

Table 7 Represents list of metabolites statistically (Log10 $P < 0.05$ and log2 fold changes > 1.2) different within the genotypes

DHC	DHA	F1
Amino acids, organic acids and its derivatives	**Amino acids, organic acids and its derivatives**	**Amino acids, organic acids and its derivatives**
Aspartic acid	Methionine	Methionine
Proline	Tryptophane	Methyl-L-histidine
Lysine	Threonine	Lysine
Beta-homoglutamine	Malate	Malate
Aminopentanoic acid	Alanine	3-hydroxy-3-methylbutanoic
2,3-Diaminopropionic acid	Threonate	Threonate
Pipecolinic acid	S-Adenosyl-L-homocysteine	Pipecolinic acid
Beta-homothreonine	-	Homocysteine
Carbohydrates	**Carbohydrates**	**Carbohydrates**
Melibiose	Arabinose	Arabinose
-	Glucose-1-phosphate	Raffinose
-	Mannose-1-phosphate	Mannose-1-phosphate
-	Ribose 5-phosphate	Mannose 6-phosphate
-	Xylulose	Xylulose
-	-	1F-beta-D-Fructosylsucrose
Alkaloids, phospholipids and its derivatives	**Alkaloids, phospholipids and its derivatives**	**Alkaloids, phospholipids and its derivatives**
Beta-Nicotinamide mononucleotide	Glycerol-3-phosphate	Glycerol-3-phosphate
-	Indole-3-carboxaldehyde	Glyceric acid
-	Trigonelline	Trigonelline
-	-	Glycero-3-phosphocholine
-	-	Glyceraldehyde 3-phosphate
-	-	Phosphocholine
Phenolic and Flavonoids	**Phenolic and Flavonoids**	**Phenolic and Flavonoids**
Quercetin-3-Glucuronide	Cyanidin 3-O-glucoside	Quercetin-3,4'-O-di-beta-glucopyranoside
1-O-b-D-glucopyranosyl sinapate	1-O-b-D-glucopyranosyl sinapate	-
-	luteolin-3',7-di-O-glucoside	-
-	Rhamnoside_Kaempferol-3-O-b-glucopyranosyl-7-O-a-	-
Quaternary ammonium	**Quaternary ammonium**	**Quaternary ammonium**
Dimethylglycine	-	Dimethylglycine
Betaine	-	Carnitine
-	-	Cytidine
Vitamins and Nucleosides	**Vitamins and Nucleosides**	**Vitamins and Nucleosides**
Inosine	Pyridoxine	Pyridoxine
-	5'-Deoxy-5'-Methylthioadenosine	5'-Deoxy-5'-Methylthioadenosine
-	-	Pantothenic acid

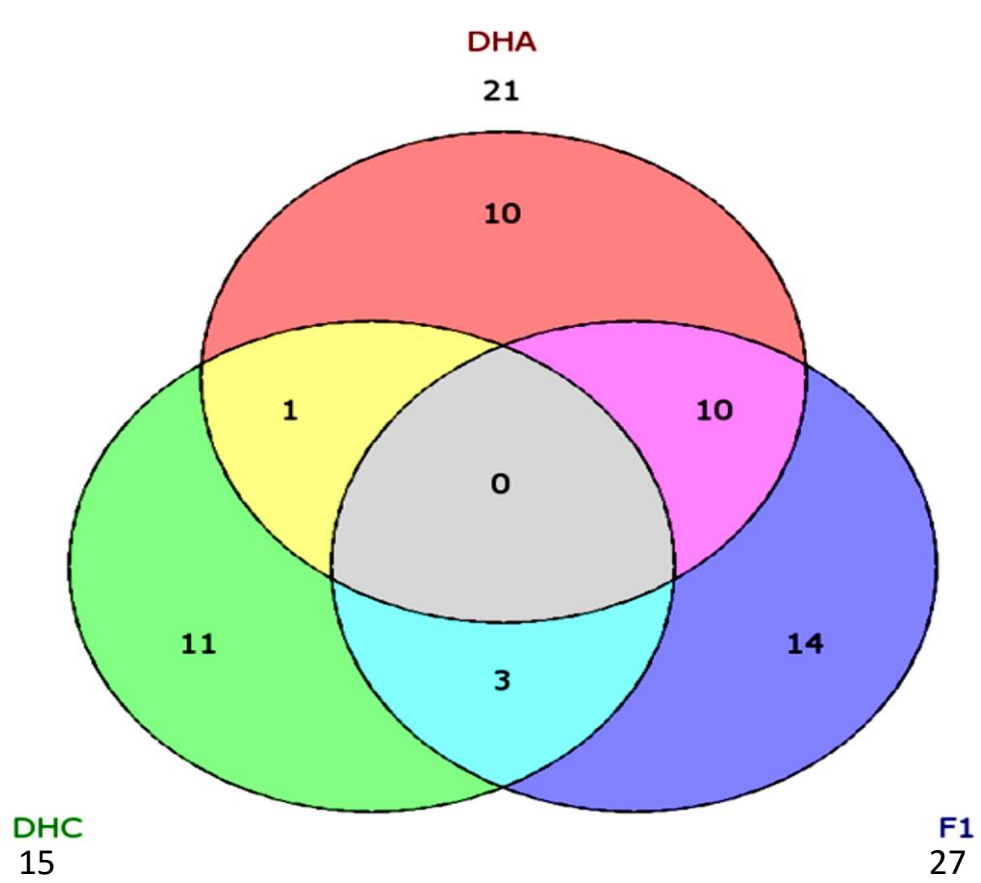


Fig. 44 Venn diagram of the compounds whose accumulations were significantly ($P > 0.05$) altered by genotype.

Transcriptomic variations in DHC, DHA and F_1

Transcriptional profiling revealed differences in gene expression between the three genotypes under normal conditions. The gene expression dataset was uploaded in the *Allium* Transcript Database (*Allium* TDB, http://allium.kazusa.or.jp/). We initially compared the gene expression of DHA and F_1 genotypes with the DHC as a control to identify genotype-specific stress-related genes. A total of 77 gene expression (log2 RPKM values) representing the following functional categories of primary and secondary metabolism was illustrated with heatmap2 using open source statistical package R, version 3.0.2 (http://www.r-project.org/). The amino acid biosynthesis genes showed up-regulation in the DHA and F_1 as compared to the DHC (Fig. 45a). This includes the genes that encode *homoserine dehydrogenase* (*HSD*), *homoserine kinase* (*HSK*), *threonine synthase* (*TS*), *cystathionine-γ-synthase* (*CGS*), *methionine synthase* (*MS*), *threonine deaminase* (*TDA*), *S-adenosylmethionine synthase* (*SAMS*), S-adnosylmethionine decarboxylase (*SAMDC*), *threonine aldolase* (*THA*), *1-aminocyclopropane-1-carboxylate synthase* (*ACCS*) and *1-aminocyclopropane-1-carboxylase oxidase* (*ACCO*) with 2.54-, 2.45-, 2.13-, 2.82-, 4.26-, 3.19-, 2.95-, 1.70-, 3.23-, 4.21-, and 2.22-fold increases, respectively in DHA and 1.43-, 1.09-, 1.88-, 2.50-, 1.62-, 5.24-, 2.37-, 3.14-, 6.22-, 3.15-, and 1.59-fold increases in F_1, respectively, as compared to DHC.

Similarly, transcriptomic profiling revealed differences between the genotypes in the regulation of carbohydrate metabolism (Fig. 45b). The synthesis of osmoprotectants such as ROFs was specifically up-regulated in the DHA and F_1, including the genes encoding *galactokinase* (*GALK*), *α-galactosidase* (*GAL*), *galactinol synthase* (*GolS*), and *raffinose synthase* (*RS*) with 2.16-, 2.04-, 4.70-, and 3.04-fold increases in DHA and 1.24-, 1.16-, 17.70-, and 10.98-fold increases in F_1, respectively. The DHA and F_1 genotypes appeared to promote sucrose and alcohol

sugar synthesis, including those genes encoding *sucrose synthase* (*SucS*), *Hexokinase* (*HK*) and *Mannose-6-phosphate isomerase* with 4.92-, 2.14-, and 2.17-fold increases in DHA and 1.63-, 1.75-, and 7.25-fold increases in F_1, respectively. In addition, genes involved in diverting the carbon flux to monosaccharide and polysaccharide syntheses that encode *cellulose synthase* (*CS*), *xylose isomerase* (XylA), *deoxyxylulose-5-phosphate synthase* (*DXS*), UDP-glucose-4 epimerase (*GluE4*),*UDP-glucose 6-dehydrogenase* (*GlucDH*), and *UDP-arabinose 4-epimerase* (*AraE4*) were up-regulated in DHA (5.62-, 2.44-, 1.98-, 1.83-, 3.79-, and 4.0-fold changes, respectively) and F_1 (2.83-, 2.33-, 1.99-, 7.53-, 3.78- and 3.16-fold changes, respectively).

Shikimate, flavonoid and phospholipid metabolism were similarly up regulated in DHA and F1 (Fig. 45c). This includes genes that encode phosphoenolpyruvate *carboxykinase* (*PCK*), *shikimate kinase* (*SK*), *3-dehydroquinate synthase* (*3DHQS*), *3-phosphoshikimate 1-carboxyvinyltransferase* (*AroA*), and *chorismate synthase* (*AroC*) were highly expressed in DHA with 4.71-, 2.25-, 2.88-, 3.61-, and 2.88-fold changes and 2.61-, 1.89-, 1.77-, 2.10- and 3.07-fold changes in F_1, respectively. These genes direct the carbon flow to chorismate synthesis, which is considered a branching point to the phenylpropanoid and tryptophan synthesis pathways. *Phenyl-amonia lyase* (*PAL*) and *4-coumarate CoA ligase* (*4CL*), representing the upstream of the phenylpropanoid and flavonoid pathways were up-regulated in DHA (1.70- and 6.03-fold increases, respectively) and F_1 (1.61- and 5.10-fold increases, respectively). Flavonoid biosynthesis genes that encode *flavanone 3-hydroxylase* (*F3H*), *dihydroflavonol 4-reductase* (*DFR*) and *leuco anthocyanidin dioxygenase* (*LACDO*) were also highly expressed in DHA with 1.81-, 13.33-, and 31.30-fold increases, respectively, and in F_1 with 5.99-, 11.22-, and 38.21-fold increases, respectively. Moreover, genes involved in the tryptophan and trigonelline synthesis that encodes *anthranilate synthase* (*ASA*), *anthranilate phosphoribosyltransferase* (*APRT*) and

nicotinamidase (*PNC*) were up-regulated in DHA (3.17-, 1.85-, and 2.40-fold changes, respectively) and F_1 (2.0-, 1.99-, and 2.18-fold changes, respectively). The phospholipid metabolism that includes genes that encode *phosphorylcholine cytidylyltransferase* (*CCT*), *phospholipase-A2* (*PLA*2), *lysophospholipase* (*LysoPL*), *Glycerol phosphoryl diesterphosphodiesterase* (*GP-PDE*) and *glycerol-3-phosphate dehydrogenase* (*GP-DH*) were up-regulated in DHA with 5.05-, 3.44-, 1.61-, 2.71-, and 2.15-fold increases, respectively, and in F_1 with 3.82-, 2.05-, 1.55-, 2.16-, and 2.58-fold increases respectively. These genes are responsible for directing the lipid pathways into glycerol-3-phosphocholine and glycerol-3-phosphate synthesis.

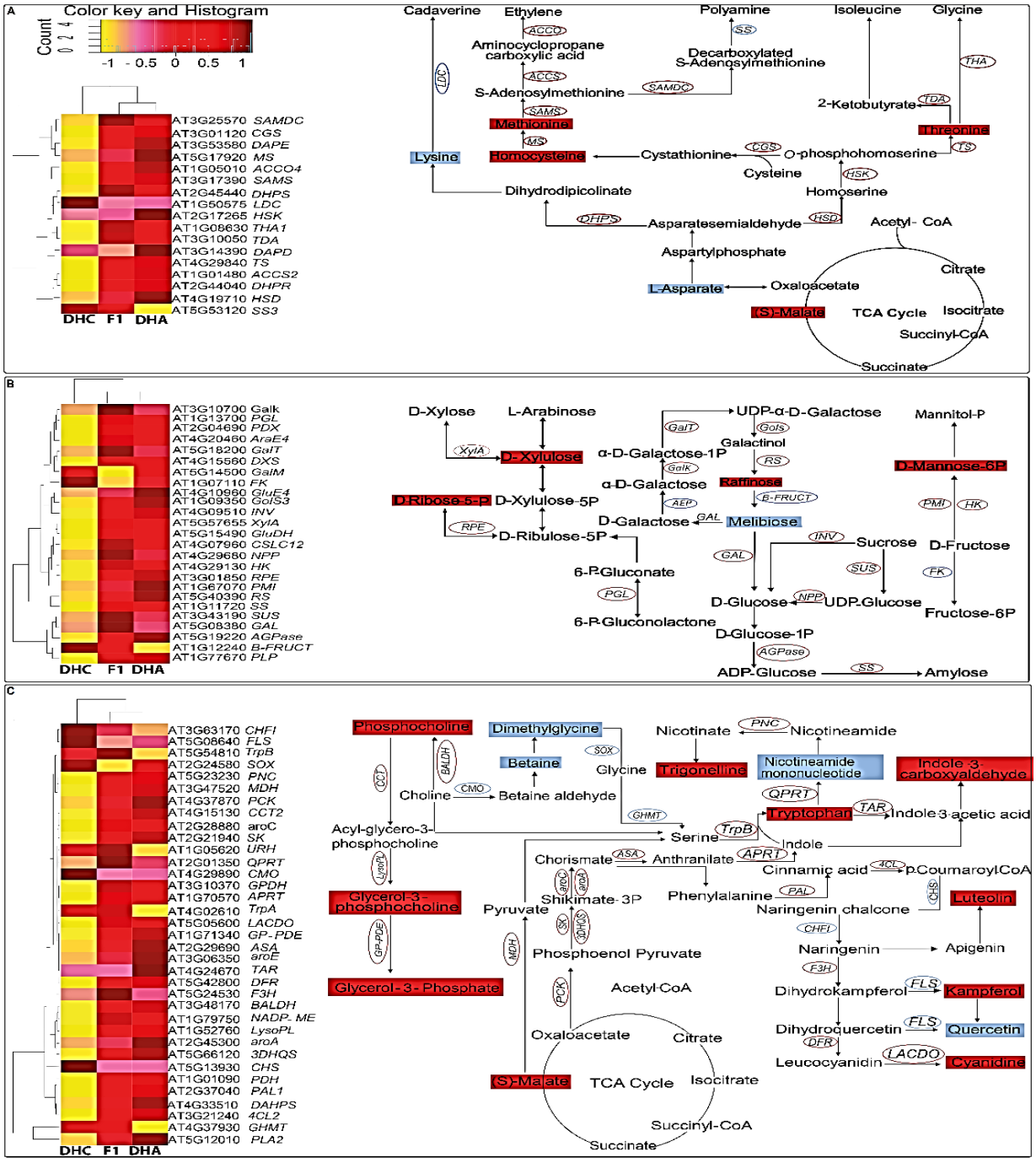


Fig. 45 Model summarizing metabolites change and gene expression analysis associated with amino acid biosynthesis pathway (a), carbohydrate biosynthesis pathway (b) and shikimate, flavonoid, tryptophan, and phospholipid biosynthesis pathway (c) in the DHC, DHA and F_1. Metabolites and genes with significant accumulation ($P < 0.05$) in DHA and F_1 highlighted with red color and DHC with blue

Discussion

To explore the genetic variation between DHC, DHA and F_1 genotypes in response to environmental stresses, the association between targeted metabolic profiling and the transcriptomic level was investigated. At the transcriptional level, substantial variation has been observed among genotypes; many of these transcripts seem to be tangled in different aspects of primary as well as secondary metabolism. Amino acid, carbohydrate, alkaloid, phospholipid, and flavonoid metabolisms showed a significant variations ($P > 0.05$) among genotypes, suggesting that these metabolic changes may reflect genetic adaption to environmental stimuli. Such trends in metabolic accumulation were reported in different species under different environmental stresses (Parker et al., 2009; Evers et al., 2010; Dauwe et al., 2012)

The induction of free amino acids as osmolytes in response to abiotic stress is thought to play a pivotal role in plant stress tolerance through intracellular pH regulation, detoxification of reactive oxygen species, xenobiotics, UV, and heavy metals (Nuccio et al., 1999; Zagorchev et al., 2013). Threonine and methionine, substrates for isoleucine synthesis via aspartate-derived pathway, were significantly higher in the DHA and F_1 genotypes as compared to the DHC genotype. *HSK* catalyzes the formation of *O*-phosphohomoerine from homoserine and leads to the formation of threonine or methionine through competitive affinities of the threonine synthase (*TS*) and *CGS* (Curien et al., 1996; Joshi et al., 2010). In the case of methionine catabolism, *SAMS* directs 80% of the metabolic flux of methionine into S-adenosylmethionine which is used to methylate nucleic acid, proteins and many plant metabolites (Joshi et al., 2010). Our results clearly show the overexpression of *TS*, *MS*, *CGS*, *TDA*, and *SAM* genes in DHA and F_1, which is in accordance with the same tendency of expression in stress-tolerant plants under drought and osmotic and salt stresses (Wu et al., 2004; Mitsuda and Ohme-Takagi, 2009). Recent studies have

suggested that threonine and methionine regulate isoleucine homeostasis under osmotic stress conditions; for example, increased isoleucine accumulation was observed in potatoes' *TS* expression (Zeh et al., 2001), potatoes *CGS* overexpression (Dancs et al., 2008), and *Arabidopsis CGS* expression (Hacham et al., 2008). Methionine is also a substrate for the synthesis of various important stress tolerance polyamines, such as putrescine, spermidine, and spermine (Alcázar et al., 2010), and this pathway involves S-Adenosylmethionine as a primary methyl donor. Moreover, S-adenosyl methionine is also a source for ethylene synthesis (van de Poel et al., 2013), reinforcing the pivotal role of methionine in plant stress responses. The overexpression of the threonine and methionine metabolism pathway in DHA and F_1 highlights the correlation between metabolic and genomic dataset and reflects the adaptability of DHA and F_1 to abiotic stress as compared to DHC.

In addition to non-aromatic amino acids such as methionine, aromatic amino acids such as tryptophan were characteristic in DHA and F_1. Our results show the same tendency of gene expression as in *Arabidopsis* plants under oxidative stress by increasing the tryptophan biosynthesis pathway (*TrpA* and *TrpB*), suggesting that there is a coordinating regulation of the entire tryptophan pathway and stress responsive mechanism (Zhao et al., 1998).

Abiotic stress tolerance in plants, particularly drought tolerance, is intricately linked to carbohydrate metabolism (Basu et al., 1999). Sugars have been shown to modify transcription of many stress-related genes (Gupta and Kaur, 2005). In the present frame work, DHA and F_1 showed a significant accumulation of the raffinose family oligosaccharides (RFOs), mannose-1, 6 phosphate, arabinose, and xylulose as compared to DHC, which might display a protective and adaptive role in DHA and F_1 genotypes under environmental stresses. Such a role has been proposed for *Pseudosuga menziesii* genotypes and environmental stress interaction, resulting in a distinctive accumulation of 19 metabolites that exhibited significant variation, among which

raffinose, arabinose, xylose, malic acid, and alanine were positively identified (Robinson et al., 2007). These forms of sugar are essentially entangled in the plant stress responses, acting as ROS scavengers, cell wall reinforcements, and osmoprotectants that stabilize cellular membranes and maintain turgor (Peshev and Van den Ende, 2013; Keunen et al., 2013). ROFs are synthesized from sucrose, where *GolS* catalyzes the first step in the biosynthesis of RFOs, and *RS* catalyzes the synthesis of raffinose from galactinol (Lehle and Tanner, 1973; Saravitz et al., 1987). Recently, it was reported that the expression of RFO biosynthesis genes is closely associated with the response to environmental stress. The transcriptional levels of *GolS* and *RS* increased under oxidative damage caused by drought, salt, or heat stress (Taji et al., 2002; Peter et al., 2007; Nishizawa et al., 2006). Interestingly, the F_1 genotype in the present study was characterized by high raffinose accumulation and up-regulation of *GolS* and *RS* as compared to DHC. The other important sugar class is sugar alcohols or polyols, which are often referred to compatible solutes; they function as osmoprotectants by forming an artificial sphere of hydration around the macromolecules, thus preventing metabolic inactivation under osmotic stress conditions (Williamson et al., 2002). Mannitol is a common six-carbon sugar alcohol synthesized from mannose-6-phosphates through the action of an *NADPH-mannose-6-P reductase* (*M6PR*) that catalyzes the conversion of mannose-6-P to mannitol-1-P (Rumpho et al., 1983). Mannitol is a potent quencher of ROS generated by abiotic stress (Williamson et al., 2002). The accumulation of mannose-1-P and mannose-6-P in DHA and F_1 would be an intermediate step in the mannitol downstream pathway, adding more insight of stress tolerance for DHA and F_1.

Arabinose and xylulose were specific for DHA and F_1. Arabinose is one of the main components of the pectin polymer side chain and a primary constituent of higher plant cell walls. Moore et al. (2006) reported that *Myrothamnus flabellifolia* leaves composed of an abundance of

arabinose polymer side chains showed extreme flexibility in response to water loss and severe dehydration, suggesting a potential role of pectin-associated arabinose polymers in relation to water deficiency. Coffee plants subjected to heat stress (37ºC) accumulated a higher content of arabinose in their leaf cell-walls, demonstrating the role of the composition cell-wall polymers during heat stress (Lima et al., 2013). Furthermore, Arabinose is a major component of Hyp-rich glycoproteins (HRGPs), which are believed to play a structural role in strengthening cell walls and are expressed in response to pathogen attack (Burget et al., 2003). Transcriptional analysis showed strong up-regulation of *GluE4* and *AraE4* in DHA and F_1, as these genes contribute to the regulation of the monosaccharide pool. The same finding was reported in potato leaves and transgenic *A. thaliana,* with an increase in *GluE4*-conferred tolerance to drought and salt stress (Evers et al., 2010; Liu et al., 2007). Trigonelline the *N*-methyl conjugate of nicotinamide, is often classified as a pyridine alkaloid. It has proven to be a key metabolite that serves as a potent inducer of the defensive metabolism in plants, including the glutathione metabolism, and the accumulation of secondary defense compounds (Berglund, 1994). Trigonelline may function as signal transmitter in response to oxidative stress caused by strong UV-B (Minorsky, 2002). It is interesting to note that trigonelline was remarkably high in DHA and F_1 as compared to DHC. This finding is in accordance with previous reports regarding the accumulation of trigonelline in stress-resistant plant, which supports our hypothesis regarding DHA as a potential resource for stress tolerance. Alfalfa plants undergo a 2-fold increase in trigonelline after salt stress, and potato leaves under drought stress exhibited a 2.39-fold increase, proposing a potential role for trigenolline as an osmoregulator (Evers et al., 2010). Moreover, the metabolic dynamics under cold stress of *Picea sitchensis* populations revealed a 1.7 to 2.6-fold change in relation to the interaction of population and cold stress (Dauwe et al., 2012). The biosynthesis pathway of trigonelline that is

involved in the conversion of nicotinamide into nicotinate and regulated by *PNC* was strongly up-regulated in the DHA and F_1 genotypes. The final step involved the methylation of nicotinate by nicotinate methyltransferase (*NMT*) using an S-adenosyl-methionine pool as a methyl donor, and thus limiting oxidative stress-induced DNA methylation (Wood, 1999).

In the current study we observed an up-regulation of the phospholipid pathway (*CCT*, *LysoPL*, *GPDH,* and *GP-PDE*) and their corresponding metabolites in DHA and F_1. Glycerol-3-phosphate, a characteristic metabolite in DHA and F_1 is an important metabolite that serves as a precursor for the biosynthesis of all plant glycerolipids that contribute to growth and disease-related physiologies (Ghanda et al., 2011). Shen et al. (2006) reported the potential involvement of the glycerol-3-phosphate shuttle in plant redox control that contributes to maintaining the metabolite pools in a relatively constant state under different growth and stress conditions. The metabolic and transcriptic dataset can be clearly linked with abiotic stress factors, as was undertaken in the present study, to establish a revealed picture of the differing metabolisms of onion and shallots that will facilitate our understanding of the biological process in these genotypes and enhance the onion-breeding program's efforts toward a stress-tolerant varieties.

Conclusion

A combination of a broad-scale metabolic and transcript dataset provided insight into the molecular architecture of adaptive variation in DHA and F_1 of tropical origin as compared to DHC genotypes under normal conditions. The increase of certain metabolites and their relevant genes expressed in the DHA and F_1 genotypes gave a better understanding of the molecular genetic determinants of adaptive trait variation with DHA potential as an important resource for an onion-breeding program geared towards environmental stress tolerance.

CHAPTER VII. GENERAL DISCUSSION

Unfavorable environmental growth factors such as drought, high temperatures, nutritional imbalance and diseases severely limits onion production (Enciso et al., 2015); such limitations are expected to increase drastically due to global climate change, affecting the agricultural system in general and, in particular, onion production, yield, quality and the market value of the crop (Lee and Suh, 2009; Enciso et al., 2015; Nakabayashi and Saito, 2015). The need for new genetic resources to meet the requirements of breeders has promoted the study of biosystematic relationship between cultivated and wild *Allium* species (Abdelrahman et al., 2014). Wild and local *Allium* species, such as *A. roylei* and shallot (*A. cepa* L. Aggregatum group), serve as a potential source for onion crop improvements to develop new varieties with some favorable characteristics, including health-enhancing qualities and disease resistance (De Vries et al., 1992b; Currah 2002; Hoa et al., 2012). The goal of our present study is to investigate the *Fusarium* wilt disease resistance mechanism among different *Allium* genotypes by using a metabolite-profiling approach with a strong focus on saponin compounds as prospective chemically based genetic markers. Moreover, to understand the genetic networks and dynamic that are responsible for disease resistance in shallots, next-generation sequencing (NGS) technology of *A. fistulosum*–shallot monosomic additional lines (MALs) was applied. This study is the first to present a large-scale RNAseq data set for different *Allium* genotypes acquired by NGS technology. Candidate genes involved in steroidal saponin upstream and downstream biosynthesis and transcriptional regulators, including *acetyCoA-transferase* (*ACCT*), *cycloartenol-C-24-methyltransferase* (*SMT1*), *glycosyltransferases* (*GTs*), *beta-glucosidase* (*BGLU*), myeloblastosis (MYB), ethylene-responsive factor (ERF), and basic helix-loop-helix (bHLH), were obtained in this study. In addition, intraspecific hybridization between a doubled haploid shallot (DHA) and a doubled haploid bulb onion (DHC) was carried

out to obtain the F_1 hybrid. Targeted metabolite profiling, using liquid chromatography/tandem-mass spectrometry (LC-MS/MS) integrated with RNAseq genotyping using NGS technology, provides insight into the metabolic and genomic architecture of DHA, DHC, and the F_1 hybrid and reflects their adaptability to abiotic stress.

Intraspecific variation within *Allium* species subg. Melanocrommyum and Nectaroscordum

Intraspecific diversity is presumably the result of plant adaptation to different natural environments or of human preference (Alonso-Blanco et al., 2005). *Allium* subgenus Melanocrommyum and Nectaroscordum represents one of the largest subgenera in the genus; the species in this two subgenera are specifically adapted to arid conditions and consist mostly of diploid perennial species (Fritsch, 1992a; Gurushidze et al., 2008). In Chapter II, principal components analyses (PCA) was carried out using the whole metabolites data set to create a scatter view of the species based on their metabolite signatures. The Melanocrommyum species showed traces of cysteine sulfoxides; however, a detectable amount was observed in the Nectaroscordum subgen., represented by *A. siculum*. The same finding was observed in previous reports (Fritsch and Keusgen, 2006). Organosulfur compounds are the most important substances in *Allium* plants in terms of both chemotaxonomic value and biological activity (Rose et al., 2005). Differences between species and cultivars in flavor characteristics probably arise from variability in sulfur uptake and in its metabolism (Kamenetsky and Rabinowitch, 2006). *Allium siculum* could be a selective candidate for breeding experiments, with the goal of purposefully improving aroma, taste, and also pharmacological properties of interspecific *Allium* hybrids. The quantitative and qualitative saponin profiling combined with antifungal activity revealed that each plant has its unique saponin profile that can be translated into a specific bioactivity; this is an important tool for the better selection of wild *Allium* species for improving disease resistance in edible *Allium.*

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Saponin chemically based genetic markers for *Fusarium* wilt disease resistance screening in different *Allium* genotypes

In Chapter III, we extend our previous investigation of the Melanocrommyum species regarding saponin profiling to obtain a deeper understanding of the specific saponin compounds responsible for *Fusarium* disease resistance. The saponin composition of *A. nigrum* was successfully achieved through the chromatographic characterization of *A. nigrum* root extract, which led us to isolate aginoside, a spirostanol saponin compound. The isolated compound was able to inhibit many *Fusarium* pathogens and spore germination of different phytopathogens in a concentration-dependent manner. The potent antifungal activity displayed by aginoside suggests that this compound, either alone or with others, may act as a chemical barrier to protect plants from fungal attacks. This result suggests that aginoside saponin could be a selective chemical marker for *Fusarium* disease resistance screening as well as agrochemical industries. Saponins have been shown to be important secondary metabolites in the antifungal activities of *Allium* species (Lanzotti, 2005; Lanzotti et al., 2012b).

In Chapter IV, we were able to isolate and identify two important spirostanol saponin compounds from shallots (Alliospiroside A and Alliospiroside B), in addition to partially purified furostanol saponin compounds 4 and 5. Of all of the compounds isolated, Alliospiroside A exhibited powerful antifungal activity against *Fusarium* pathogens, which indicates the importance of this compound for disease resistance. Quantitative and qualitative variations were observed in the TLC profiles of the shallot, bulb onion, and F_1 and F_2 hybrids (BC_1, BC_1S_1, and BC_1S_2). There was a clear segregation of the phenotypic expression of Alliospiroside A in the F_1 and F_2 resistance and susceptible progeny. In addition, a detectable amount of furostanol saponin was observed in the resistance lines, especially after pathogen

inoculation. We hypothesized that Alliospiroside A could be an important factor in developing *Fusarium* disease resistance in the onion. Recently, Teshima et al. (2013) reported antifungal activity of Alliospiroside A against different *Fusarium* and other phytopathogens. Furthermore, the transformation of furostanol to a spirostanol saponin via *beta-glucosidase* (*BGLU*) and other related genes can be used as a genetic marker for disease resistance selection. Morant et al. (2008) reported the functional role of *BGLU* though the bioactivation of many plants' phytoanticipin-related defense compounds.

From phenotyping to RNAseq genotyping of *A. fistulosum* with an extra chromosome from the shallot

In our previous studies, using a complete set of *A. fistulosum*-shallot monosomic addition lines, the inheritance of genetic materials from the shallot to the Japanese bunching onion enhanced *A. fistulosum*'s flavonoid profile (Masuzaki et al., 2006), amino acid content, and cysteine sulfoxide production during the summer season (Masamura et al., 2011), sucrose and fructan content in the winter season (Yaguchi et al., 2008), and shallot-specific saponin compounds for *Fusarium* basal rot resistance (Hoa et al., 2012). Our results in Chapter IV suggest an important role of chromosomes 1A and 2A in shallot saponin biosynthesis. In addition, chromosome 2A was characterized by the production of Alliospiroside A and furostanol saponin compounds 4 and 5. Herein, we present the results of the study designed to characterize the transcriptomes of *A. fistulosum* (FF), shallot (AA), and *A. fistulosum*-shallot monosomic addition line (MALs) roots, bulbs, and leaves. Our ultimate goal is to discover candidate genes that encode enzymes in the steroidal saponin biosynthetic pathway and to determine the chromosomal locations of several candidate genes related to saponin biosynthesis. A significant upregulation of many saponin candidate genes was observed in chromosomes 1A, 2A, and AA, as compared with FF. Interestingly, *GTs* and *BGLU* genes were upregulated in the chromosome 2A. Recently, Augustin et al. (2012) and Subramaniyam et al. (2014) showed that *GTs* are considered to be a

candidate involved in the biosynthesis and glucosylation of saponin, which could be a cause for plant resistance . Furthermore, Inoue and Ebizuka (1996) and Morant et al. (2008) intensified the functional role of *BGLU* in the conversion of furostanol to a spirostanol saponin type. In addition, we were able to allocate *GTs* in chromosome 2A, which promotes our hypothesis regarding the potential role of these genes in saponin biosynthesis and subsequent improved disease resistance in the shallot and other related *Allium* species.

Wild *Allium* species as a potential genetic resource for disease resistance

Allium roylei has proven to be a crucial bridge species, enabling introgressions of genetic resources from other related species of section *Cepa* into the onion, allowing disease resistance and other valuable characteristics to be introgressed into the onion (Kofoet et al., 1990). The prospect of exploiting *A. roylei* as a source of disease resistance in onion breeding is very promising, as this wild species has proven to be completely resistant to downy mildew (*Peronospora destructor*) and partially resistant to leaf blight disease caused by *Botrytis squamosa* and basal rot disease caused by *Fusarium oxysporum* f. sp. *Cepae* (De Vries, 1992a). To investigate the involvement of *Allium roylei* metabolites in the plant's defenses, a comprehensive analysis of the content of cysteine sulfoxides, flavonols, polyphenols, ascorbic acid, and saponin was carried out in the various organs of this species. Metabolomic high-performance liquid chromatography (HPLC), spectral-based analyses, and histochemical studies have given important insight to the validity of saponin as a key component involved in plant protection. The root-basal stem, bulb, and leaf extracts exhibited 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The antimicrobial properties of the saponin and flavonoid crude extracts were evaluated. The saponin extracts demonstrated significant antifungal activity depending on the applied concentration and the growth inhibition rate of the tested fungal pathogens. Interspecific hybrids between *A. roylei* and *A. cepa* demonstrated improvement in leaf blight resistance that is conditioned by the *Bs1* dominant gene from *A.*

roylei (De Vries et al., 1992). Downy mildew resistance originating from *A. roylei* Stearn provides complete resistance to onions based on the *Pd1* dominant gene (Scholten et al., 2007). Furthermore, the interspecific hybrids between *A. roylei* and shallot to obtain F_1, allotriploid, and amphidiploid lines revealed a significant variation in the total saponin content and quality. This modification in shallot saponin pool would be derived from *A. roylei* saponin biosynthesis controlled by the introgressed genes located on the extra chromosomes of *A. roylei*. The same tendency of saponin modification was also observed in our recent study of the saponin profile of allotriploid (FFR) and amphidiploid (FFRR) lines of *A. fistulosum-A. roylei* (Ariyanti et al., 2015).

RNAseq genotyping and metabolomic profiling in doubled haploids of *Allium cepa* for abiotic stress prospecting

Environmental stress conditions such as drought, heat, salinity, or pathogen infection can have a devastating impact on plant growth and yield, resulting in a mandate for stress-tolerant crop varieties. In Chapter VI, crossbreeding tropical and cultivated onion species provided a hybrid F_1 possessing genetic and metabolic parental properties. A combination of a broad-scale metabolic and transcript data sets provided insight into the molecular architecture of adaptive variation in DHA and F_1 of tropical origin, as compared to DHC genotypes under normal conditions. The increase of certain metabolites and their relevant genes expressed in the DHA and F_1 genotypes gave a better understanding of the molecular genetic determinants of adaptive trait variation with DHA potential as an important resource for an onion-breeding program geared toward environmental stress tolerance. Substantial variation has been observed among genotypes; many of these transcripts seem to be tangled in different aspects of primary as well as secondary metabolism, including carbohydrates, amino acids, flavonoids, and phospholipid metabolism. Such trends in metabolic accumulation and transcriptional variation were reported in different plant stress tolerances (Parker et al., 2009; Evers et al., 2010; Dauwe et al., 2012).

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LIST OF PAPERS RELATED TO THE THESIS

Mostafa A., Sudisha J., El-Sayed M., Ito S.I., Ikeda T., Yamauchi N., Shigyo M. (2013) Aginoside saponin, a potent antifungal compound, and secondary metabolite analyses from *Allium nigrum* L. Phytochemsitry letters Vol. 6 (2)274-280.

(In relation to Chapter III)

Abdelrahman M., Hirata S., Ito S.I., Yamauchi N., Shigyo M. (2014). Compartmentation and localization of bioactive metabolites in different organs of *Allium roylei.* Bioscience, Biotechnology, and Biochemistry Vol. 78(7)1112-1122.

(In relation to Chapter V)

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SUMMARY

Consumption of the *Allium* species as food and an ethnomedicine is mainly attributed, respectively, to its nutritional and functional properties, including antiasthmatic, antichlolesterolemic, and antimicrobial properties. The genus *Allium* is enormous (850 species) and is broadly spread over the northern hemisphere. A region of exceptionally wide genetic diversity stretches from the Mediterranean Basin to Central Asia. *Allium* species have adapted to diverse ecological niches, leading to the development of an astonishing number of different morphotypes. This is the main reason for the widely recognized difficulties in the taxonomy of *Allium.* The need for disease resistance materials in onion breeding has promoted studies of biosystematics relationships between cultivated and wild *Allium* species. The wild and local *Allium* species, such as *A. roylei* and shallot (*A. cepa* L. Aggregatum group), serve as a potential source for onion crop improvements in order to develop new varieties with some favorable characteristics, including health-enhancing qualities and disease resistance. In the present study, an approach for profiling targeted metabolites and RNAseq genotyping was adopted for a strong focus on saponin compounds as prospective chemically based genetic markers 1) for exploring an intraspecific variation within *Allium* species subg. Melanocrommyum and Nectaroscordium, 2) for isolating, purifying, and evaluating potential saponin compounds related to *Fusarium* disease resistance from *A. nigrum,* 3) for isolating and purifying several saponin compounds from shallots and applying them as chemical marker for selecting *Fusarium* disease resistance within different shallots and bulb onion hybrid populations, 4) to understand the genetic background regulating the saponin biosynthesis in *A. fistulosum*-shallot monosomic additional lines 5) for investigating the involvement of

A. roylei metabolites in the phenotypic expression for disease resistance, as well as saponin profiling of F_1, amphidiploid, and allotriploid lines of *A. roylei* and shallots and 5) for investigating the transcriptome and target metabolome variability in doubled haploids of *A. cepa* for prospective stress responsiveness.

The first part of this study revealed the profile of thin-layer chromatography and the remarkable antifungal activity against different *Fusarium* pathogens of the crude saponins extracted from *Allium* species subg. Melanocrommyum and Nectaroscordium. However, with the exception of *A. siculum*, which shows a distinguished ACSO composition and considerably higher amounts, very low contents of flavor precursors (cysteine sulfoxides, ACSOs) were found in these species. The promising saponin compounds in these subgenera can be used as chemical markers for disease resistance selection and chemotaxonomy of the genus *Allium*.

In the second part, a deep understanding of the metabolites' composition of *A. nigrum* was successfully gained through HPLC and spectral analyses. Phytochemical analyses of ACSOs, total polyphenols (TPs), and total saponins revealed quantitative variations within the different organs of *A. nigrum*. The excessive accumulation of ACSOs was detected in scaly bulbs, of TPs in leaf blades, and of saponins in roots. The chromatographic characterization of *A. nigrum* root extract led us to isolate a spirostanol saponin. The structure was elucidated by spectroscopic analysis (2D NMR, FABMS, HR-ESI-MS). The structure of this compound was identified as 25(R,S)-5a-spirostan-2a,3b,6b-trio1-3-O-b-D-glucopyranosyl-(1,2)-O-[b-D-xylopyranosyl-(1,3)]-O-b-D-glucopyranosyl-(1,4)-b-D-galactopyranoside or aginoside. The highest aginoside content, was detected in the root. The *in vitro* and *in vivo* antifungal activity of aginoside was evaluated for the first time against different phytopathogens, providing a new avenue for saponin application in

the agrochemical industries. The same pattern of research was carried out using shallots. The output of this part was accomplished by the isolation and identification of two important spirostanol saponin compounds, Alliospiroside A and Alliospiroside B. Among all of the compounds isolated, Alliospiroside A exhibited powerful antifungal activity against *Fusarium* pathogens, which indicates the importance of this compound for disease resistance. Quantitative and qualitative variations were observed in the TLC profile of introgressed shallot ('Chaing-Mai') and bulb onion and subsequent hybrid populations (BC_1, BC_1S_1, and BC_1S_2). We clearly observed an intensive accumulation of Alliospiroside A in the resistance lines as compared with those of susceptible lines. Moreover, furostanol saponins can be a reservoir for the biosynthesis of spirotanol saponins, and the gene related to this biotransformation (beta-glucosidase) could be an important genetic marker for disease resistance selection. This study is the first to present a large-scale RNAseq data set for different *Allium* genotypes acquired by NGS technology. Candidate genes involved in steroidal saponin upstream and downstream biosynthesis and transcriptional regulators, including *acetyCoA-transferase* (*ACCT*), *cycloartenol-C-24-methyltransferase* (*SMT1*), *glycosyltransferases* (*GTs*), *beta-glucosidase* (*BGLU*), myeloblastosis (MYB), ethylene-responsive factor (ERF), and basic helix-loop-helix (bHLH), were obtained in this study.

To investigate the involvement of *A. roylei* metabolites in the plant's defenses, the contents of ACSOs, flavonols, polyphenols, ascorbic acid, and saponins in the various organs of this species were comprehensively analyzed. The root-basal stem, bulb, and leaf extracts exhibited 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity with inhibition concentrations (IC_{50}) ranging from 0.649 to 0.757 mg/ml. The antimicrobial properties of the saponin and flavonoid crude extracts were evaluated. The saponin

extracts demonstrated significant antifungal activity dependent on the applied concentration, and the growth inhibition rate of the tested fungal pathogens ranged from 1.07 to 47.76%. No appreciable antibacterial activity was recorded in the same sample. HPLC and spectral-based analyses together with histochemcial studies have provided important insight into the validity of saponins as a key component in plant protection. In addition, crossbreeding between *A. roylei* (RR) and shallot (AA) was carried out and the F_1 hybrid was subjected to chromosomal doubling and then backcross with shallot to obtain the amphidiploid and allotriploid lines. Significant higher saponin content was and antifungal activity observed in the amphidiploid (AARR) and allotriploid (AAR) plant in comparison with the shallot. The observations of chemical modifications in the shallot–*A. roylei* would bring helpful information regarding chromosome manipulation to improve the consumer quality as well as the disease resistance of shallot and bulb onion.

Environmental stress conditions, such as drought, heat, salinity, or pathogen infection, can have a devastating impact on plant growth and yield, resulting in a mandate for stress-tolerant crop varieties. In this study, intraspecific hybridization between a doubled haploid shallot (DHA) and doubled haploid bulb onion (DHC) was carried out to obtain the F_1 hybrid at first. The targeted metabolite profiling, using liquid chromatography/tandem-mass spectrometry (LC-MS/MS) integrated with transcriptional analysis of their relevant genes, provides insight into the metabolic and genomic architecture of DHA, DHC, and the F_1 hybrid. From a complete set of 113 targeted metabolites, 49 metabolites were found to be statistically different among the genotypes; 11 metabolites were characteristic for DHC, 10 for DHA, 14 for F_1, and 14 metabolites were mutual among the three genotypes. Several key genes and metabolites introgressed in abiotic stress responsiveness have been up-regulated in DHA and F_1 hybrids, as

compared with DHC, even in non-stressed conditions. Principal component analysis (PCA) and Volcano plot analysis revealed that metabolic traits (*i.e.*, amino acid, carbohydrate, flavonoid, and phospholipid biosynthesis) and their relevant genes were mostly over-expressed with the existence of shallot genome (s) in DHA and F_1, reflecting the adaptability to abiotic stress of these two genotypes as compared with that of DHC.

All of the results obtained create prospects for developing new varieties of *Allium* vegetables and carrying out further omics approaches, both in cultivated and wild species of this genus.

ネギ属における機能性代謝物の生化学分析：作物ストレス耐性の改変に関する将来戦略

ネギ属植物が有する栄養的特性や抗喘息，抗コレステロール，抗菌などの機能的特性は，それらの食用および薬用植物種としての利用価値を高めている．ネギ属には多くの種が含まれ（800～850 種），その生息地域は北半球に全域に広がっている．また，非常に広い遺伝的多様性をもつことでも知られており，特に中央アジアから地中海盆地までの一帯が多様性のホットスポットとなっている．ネギ属植物は様々な生態学的ニッチを獲得し，それにより広範な形態型を発展させてきた．一方で，このことは，良く知られているように，ネギ属植物の分類をより一層困難なものにしている．ネギ属の栽培種と野生種の種間関係に関する研究は，タマネギ育種における病害抵抗性素材の重要性と相まって発展してきた．野生種の *Allium roylei* や熱帯地方の在来種のシャロット（*A. cepa* L. Aggregatum group）は病害抵抗性や健康機能性を有しており，タマネギ品種改良に利用できる育種素材として注目されている．本研究では，有用性が見込める化学的遺伝マーカーとしてサポニン化合物に注目し，1）Melanocrommyum および Nectaroscordium 亜属が生産するサポニン類の化学・抗菌性分析により多様性を探索するため，2）*A. nigrum* からフザリウム病害抵抗性に関連する化合物を単離・精製し，生物農薬としての可能性を評価するため，3）シャロットからサポニン化合物の単離・精製を行い，シャロットとタマネギや *A. roylei* とシャロットの間の交雑系統におけるフザリウム病害抵抗性の選抜指標を獲得するため，4）*A. roylei* における代謝物と病害抵抗性の関係を調査するため，および 5）*A. cepa*

の倍加半数体におけるトランスクリプトームとターゲットメタボロームの統合解析を実施し，シャロットが有するストレス応答に係る代謝経路や遺伝子を明らかにするために，代謝物をターゲットとした各種分析をそれぞれ実施した．

先ず，Melanocrommyum および Nectaroscordium から抽出したサポニン類を薄層クロマトグラフィー(TLC)により化学的に分離して多寡を把握するとともに，フザリウムに対する抗菌活性を調査した．さらに，辛味や風味に関係する硫黄化合物の前駆体（システインスルホキシド，ACSOs）に関する定性・定量解析を実施したところ， *A. siclum* を除いた全ての植物種で低含量となっていた．何種類かの化合物について，病害抵抗性の選抜マーカーとしての有効性を検証したところ，サポニンが最も有望であることが示唆された．

次に，HPLC および分光分析により，*A. nigrum* の各器官における代謝産物の比較解析を行った．器官毎に ACSOs，総フェノールおよび総サポニン含量を比較したところ，ACSOs は鱗茎部(0.367 mg/g fw)で，フェノールは葉身部(116.05 mg CE/100 g fw)で，また，サポニンは根部（ 19.38 mg/g dw ）で，それぞれ蓄積量が多かった .*Allium nigrum* 根部の抽出物より各化合物の精製を試み，さらに，スペクトル分析 (2D NMR, FABMS, HR-ESI-MS) による構造決定によりスピロスタン型グリコシドであるアジノシドの同定に成功した．その構造は，25(R,S)-5a-spirostan-2a,3b,6b-trio1-3-O-b-D-glucopyranosyl-(1,2)-O-[b-D-xylopyranosyl-(1,3)]-O-b-D-glucopyranosyl-(1,4)-b-D-galactopyranoside と同定された．また，最も高いアジノシド含量（ 2.9 mg/g dw ）が根部でみられた．生体内や生体外でのアジノシドの抗真菌活性を様々な植物病原体に対して評価したところ，生物農薬としてのサポニンの可能性を示唆する結果が得られた．

上記と同様の研究がシャロットを植物材料として用いて行われ，2種類の主要なスピロスタノールサポニン化合物である Alliospiroside A および Alliospiroside B の単離・同定に成功した．単離された全ての化合物の中で，Alliospiroside A はフザリウム菌に対する強力な抗真菌活性を示し,植物体内の病害抵抗性の発現に強く関与することが示唆された．シャロット（‘チェンマイ’）とタマネギから得られた TLC プレート上で検出されたサポニン類の量的および質的多寡を交雑集団（BC_1, BC_1S_1 および BC_1S_2）について観察したところ，抵抗性個体のみで Alliospiroside A の明確な蓄積がみられた．さらに，シャロットやタマネギが生産するフルスタノールサポニンはスピロスタノールサポニンの生合成における前駆物質となっており，前者から後者への物質変換に関連する酵素遺伝子（β-グリコシダーゼ）は耐病性選抜用遺伝的マーカーとなる可能性がある．

植物防御応答と A *roylei* の代謝産物の関係を調査するために，ACSOs，フラボノイド，ポリフェノール，アスコルビン酸およびサポニンの化学分析が組織毎に実施された．根の茎基部，球および葉の抽出物は 0.649 から 0.757mg/mL の範囲の阻害濃度（IC_{50}）を伴う 2,2-diphenyl-1-picrylhydrazyl（DPPH）ラジカル捕捉活性を示した．また，同植物種から部分精製したサポニンおよびフラボノイドの抗菌活性を調査したところ，サポニン抽出物は適用した濃度において顕著な抗真菌活性を示し，供試した菌類病原体の成長抑制率は 1.07 から 47.76%の範囲であった．一方で，いずれの物質も抗細菌活性は有していなかった．一連の HPLC 分析，分光分析および組織化学的解析より，サポニンが植物防御物質として真

菌病抵抗性に直接関与している可能性が示された．

乾燥，高温，高塩濃度および菌感染のような環境ストレスは植物の成長および収量に多大な影響を与えることから，ストレス耐性品種の獲得が求められる．本研究では，先ず，シャロット倍加半数体(DHA)とタマネギ倍加半数体(DHC)の種内交雑により F_1 個体を作出した．次に，液体クロマトグラフィー - タンデム質量分析(LC-MS/MS)を駆使してターゲット代謝産物のプロファイリングを行うことにより，それらと関連遺伝子のトランスクリプトーム解析結果が統合され，DHA，DHC と F_1 個体におけるメタボロ・ゲノム構造に関する新たな知見が得られた．113 種類のターゲット代謝産物のうち，49 種類の生産量が植物材料間で有意に異なっていた．すなわち，DHC に特有の代謝物は 11 種類，DHA は 10，F_1 は 14 であり，さらに 14 の代謝産物は 3 つに共通のものであった．非ストレス条件下の DHA と F_1 における非生物的ストレスに反応して誘導される鍵酵素の遺伝子および関連の代謝物の発現量は同条件下の DHC と比較して軒並みに上昇していた．さらに，主成分分析（PCA）および Volcano Plot 分析を行ったところ，ストレス関連代謝物（アミノ酸，炭水化物，フラボノイド，リン脂質生合成など）や関連遺伝子（群）がシャロットゲノムを有する DHA や F_1 で過剰発現していることがわかり，これらの 2 種類の植物の非生物的ストレスに対する適応性は DHC を上回ると予測できた．

本研究で得られた成果は，ネギ属野菜の耐病性育種や栽培種・野生種を用いたオミクス統合解析を実施する際の礎になるといえよう．