RECOVERY FROM AUTOTOXICITY IN LISIANTHUS AND STRAWBERRY BY AMINO ACIDS APPLICATION

（アミノ酸施与によるトルコギキョウおよびイチゴの自家中毒回避）

Md. Fuad Mondal

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by

Md. Fuad Mondal

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Professor Toshiki ASAO, PhD

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Allelopathy in crop plants

Allelopathy is a phenomenon of direct or indirect, beneficial or adverse effects of a plant on the other through the release of chemicals in the environment. Rice (1974, 1979) also indicated that allelopathy might contribute to patterning densities and the distribution of species which in specific instances could severely limit diversity of the plant community. There are three basic features of allelopathy: firstly, the object of interaction is the plants. Secondly, the material of interaction is the secondary metabolites of plants, and must have the suitable way getting into the environment, but not the secondary metabolites had changes within plants. Thirdly, allelochemicals are used for influencing the growth and the development of its own or neighboring plants. Allelochemicals can be found in different concentrations in several parts of plants (leaves, stems, roots, rhizomes, seeds, flowers and even pollen) (Bertin et al., 2003; Gatti et al., 2004; Kruse et al., 2000) and their pathway of release into the environment varies among species. The following are known pathways: (1) Exudation and deposition on the leaf surface with subsequent washing off by rainfall; (2) Exudation of volatile compounds from living green parts of the plant; (3) Decay of plant residues (e.g., litter fall or dead roots); and (4) Root exudation (Chon et al., 2006; Olofsdotter et al., 2002). In the last few decades extensive research have done on different aspect of allelopathy of crops. These include symptom and severity of adverse effects of living plants and their residues upon the higher plants and crop yield, interactions among the organism, ecological significance of allelopathy in plants communities, replanting problems with

crop rotation, autotoxicity and the synthesis, isolation and identification in agro ecosystem.

1.2. Autotoxicity in monoculture system

Allelopathy has two forms, interspecific: when the donor and the recipient belong to different species; however, if the donor and the recipient belong to same species it becomes intraspecific allelopathy and the term used is autotoxicity. Thus, autotoxicity occurs when a plant releases toxic chemical substances into the environment that inhibit germination and growth of same plant species (Miller, 1996). It has been reported to occur in a number of crop plants in agro ecosystem causing serious problems such as growth reduction, yield decline and replant failures (Singh et al., 1999; Pramanik et al., 2000; Asao et al., 2003). The chemicals responsible for bringing about such effects are known as allelochemicals or autochemicals or simply phytotoxins. The varieties of such chemicals were found in plants and are either secondary metabolites or the waste products of the primary metabolic processes (Swain, 1977). The chemical nature of these compounds can be simply organic acids, straight chain alcohols, aldehydes or ketones, unsaturated lactones, fatty acids, naphthaquinones, complex quinones, simple phenols, phenolic acids, tannins, terpenoids (monoterpenes, sesquiterpene lactones, and diterpene lactones), amino acids, polypeptides, alkaloids, glucosinates, purines, and nucleotides, etc. (Gross and Parthier, 1994; Einhellig, 1995; Seigler, 1996). The release of these chemicals from the plant is facilitated by many processes such as leachation (Overland, 1966), volatilization (Petrova, 1977), root exudation (Tang and Young, 1982), crop residue decomposition (Putnam, 1985), and pollen spreading in some plants (Cruz-Ortega et al., 1988). The released chemical compounds create problems in

monoculture and/or closed hydroponic culture systems as they can accumulate and inhibit the growth of the actual crop. Previously our research group has studied the phenomenon of autotoxicity in several vegetables crops such as cucumber (Yu and Matsui, 1994; Asao et al., 1998, 1999, 2000), taro (Asao et al., 2003) several leafy vegetables (Asao et al., 2004a), some ornamentals (Asao et al., 2007) and strawberry (Kitazawa et al., 2005) at the glasshouse and plant factory supported research facility of Experimental Research Center for Biological Resources Science, Shimane University, Japan using hydroponic culture.

1.3. Autotoxicity in lisianthus [*Eustoma grandiflorum* (Raf.) Shinn.] in closed hydroponics

Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn.] is a seed propagated ornamental flower native to the central and southern regions of the United States of America and inhabits moist prairies ranging from Nebraska to Colorado and Texas (Halevy and Kofranek, 1984; Ohkawa, 1987). This cut flower has emerged as one of the top ten cut flower crops in international flower trade during the past few years due to its vast charming colour shades, flower shapes, growing feasibility under varying agro climatic conditions and good vase life. It is a prominent cut flower from Japan where it has been in cultivation since 1960 (Ohkawa and Sasaki, 1997). The growth of lisianthus seedlings is very slow, requiring 50 to 140 d from germination to transplanting (Harbaugh, 1995; Matsuo and Shirasaki, 1990; Tsukada et al., 1991a, 1991b). Growth inhibitors such as maleic and benzoic acid were detected in root exudates of lisianthus when it was grown in closed hydroponic system (Asao et al., 2007). Benzoic acid is the potential autotoxic chemical which is responsible for the growth and yield reduction in

many crops (Kitazawa et al., 2005, Asao et al., 2003).

1.4 Autotoxicity in strawberry in closed hydroponics

In strawberry this phenomenon is typically characterized by the black rot diseases of roots (Strong and strong, 1931). Black root rot is a worldwide disease that limits the yield of strawberry has been studied extensively (Yuen et al., 1991; Wing et al., 1994, Asad-Uz-Zaman et al., 2015). Root symptoms include the deterioration of the root cortex of perennial roots and loss of feeder roots. Many organism including fungi (Martin and Bull, 2002, Zhao et al., 2005, Zhu et al., 1994), nematode (LaMondia, 2004), or physical factors like soil compactness, soil texture, higher rates of herbicidal application, age of the seedlings etc. are responsible for this complex disorder (Wing et al., 1995). Exudate chemicals from strawberry plants also interference in the rhizosphere soil is associated with this disease. Like lisianthus, strawberry plants roots also release benzoic acid, when it grows in closed hydroponic culture (Kitazawa et al., 2005). When root exudates accumulated in their growing medium, the growth and metabolism of strawberry roots were inhibited, which resulted in an increase in the percentages of electrolytes in cells, a decrease in the free radical scavenging activity of roots, and an increased in root lipid peroxidation (Zhen et al., 2003). Damaged strawberry roots hampers water and mineral nutrient uptake. As a result, the growth of shoot and root, number of flowers and harvested fruit per plant, and fruit enlargement greatly reduced. Reduction or removal of these inhibitory allelochemicals from the culture solution would lead to normal growth and yield. Activated charcoal has been used to adsorb the accumulated phytotoxic chemicals for the culture solution and improve the growth and yield in strawberry (Kitazawa et al., 2005). In several other

researches, such as supplementation of auxin in strawberry (Kitazawa et al., 2007) or electro-degradation of phytotoxic chemicals in strawberry (Asao et al., 2008; Asaduzzaman et al., 2012) were also found to be effective for recovering the autotoxic effect in closed hydroponics. However, finding suitable method for controlling autotoxicity in strawberry would be of great help for the commercial production of strawberry in a recycled hydroponics.

1.5 Amino acids in plants

Amino acids are biologically important organic compounds composed of amine ($-NH_2$) and carboxylic acid (-COOH) functional groups, along with a side-chainspecific to each amino acid. The key elements of an amino acid are carbon, hydrogen, oxygen, and nitrogen, though other elements are found in the side-chains of certain amino acids. These are the basic component of all living cells. In plants, amino acids are functioning in different ways. From cell wall structure to cell functioning, in everywhere amino acids are found. For example, Hydroxy-proline (Hyp) present in cell wall structural named as Hyp rich glycoproteins (HRGPs) which ubiquitous components of the plant extracellular matrix and comprise up to 10% (w/w) of cell wall in dicots (Cassab and Verner, 1998). Cystine (Cys) participates in the synthesis of essential bio-molecules like antioxidants, vitamins and co-factors (Noctor et al., 1998; Leustek et al., 2000; Droux, 2004; Saito, 2004). Like Cys, free as well as protein bound Methionine (Met) has ubiquitous functions in plants. It plays a role in the initiation of mRNA translation and is the precursor of essential bio-molecules trough S-adenosylmethionine (AdoMet) (Ravanel et al., 1998; Leustek et al., 2000; Lu, 2000). In plants, cytokinins signals are mediated by multi-component phosphorylation system composed of a Histidine

(His)-Protein kinase (Kakimoto, 2003). In *Arabidopsis thaliana*, intercellular signaling by cytokinin is referred to as His-to-Aspartate phosphorelay system (Oka et al., 2002). Tryptophan (Trp) is a pivotal precursor of secondary metabolites in plants, including indole glucosinolates and indole phytoalexins in brassicaceous species (Hull et al., 2000; Mikkelsen et al., 2000; Glawischnig et al., 2004), GABA, as a signal molecule and provides further insights into the role of the GABA metabolic pathway in response to stress and C/N metabolism. (Bouche and Fromm, 2004). Arginine (Arg) is a basic amino acid can represent a significant part of the stored nitrogen in protein, as free amino acid in seeds, bulbs, or other parts of plants (Micallef and Shelp, 1989). Besides these, lots of research articles are available which revealed the importance of amino acids in plants.

1.6 Foliar application of amino acids during stresses

One of the most common stress responses in plants is over production of amino acids, (de Souza et al., 2013). Generally, amino acids protect plants from stresses through different courses, including contribution to cellular osmotic adjustment, detoxification of reactive oxygen species, protection of membrane integrity, and stabilization of enzymes/proteins (Yancey et al., 1982; Bohnert and Jensen, 1996). Proline accumulation has been reported during conditions of drought (Choudhary, et al., 2005), high salinity (Yoshiba, et al., 1995), high light and UV irradiation (Saradhi, et al., 1995), heavy metals (Schat, et al., 1997) and in response to biotic stresses (Fabro, et al., 2004; Haudecoeur et al., 2009). As plants recover their growth during stresses by over production of amino acids, many researchers suggested applying amino acids

exogenously for improving the growth and yield of crop plants in stress condition (Maini et al., 1999; Heuer, 2003; SH Sadak et al., 2015). Recent researches revealed that, amino acids can be absorbed by leaf exogenously (Furuya and Umemiya, 2002; Stiegler et al., 2013). In addition, exogenously foliar applied amino acids have positive effects on the growth, yield and quality of marigold (Sorwong and Sakhonwasee, 2015), *Urtica pilulifera* (Wahba et al., 2015), alfalfa (Pooryousef and Alizadah 2014), *Codiaeum variegatum* (Mazher et al., 2011), grape (Garde-Cerdán et al., 2015; Portu et al., 2015). In this regards, amino acids might have the potentialities to recover the autotoxic effects in lisianthus and strawberry.

1.7 Objectives of the study

Autotoxic effects of root exudates of lisianthus and strawberry plants on their growth and development is to be caused by impairment of nutrient and water absorption by injured roots. Supply of nutrients alternatively other than root uptake can sustain plant growth during this allelochemical stress. The availability and uptake of nitrogen is considered as the major factor affecting growth (Lea and Azevedo, 2006). Amino acids are rich in nitrogen which forms the basic component of all living cells. In addition, amino acids are low molecular weight organic compounds, highly soluble and nontoxic at high cellular concentrations. Recently amino acids have been used exogenously as bio-stimulants in plants under abiotic and biotic stresses (Maini et al., 1999; Heuer, 2003; SH Sadak et al., 2015). Moreover, those are also used as foliar spray to improve the growth, yield and quality of crops (Mazher et al., 2011; Takeuchi et al., 2008). In this regard, amino acid has a great potentiality of using under managed culture techniques. As the accumulated allelochemicals in closed culture become stressful to

plant, spraying of amino acid to lisianthus and strawberry plants would be positive. Therefore the aims of the present study were:

- To evaluate the performance of amino acids on the growth and flowering of lisianthus under autotoxic condition in closed hydroponic culture.
- To evaluate the performance of amino acids on the recovery of growth and yield of strawberry plants under autotoxicity in closed hydroponic culture.

CHAPTER 2

Effects of amino acids on the growth and flowering of lisianthus [*Eustoma grandiflorum* (Raf.) Shinn.] under autotoxicity in closed hydroponic culture

1. Introduction

Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn.] is a seed propagated herbaceous annual ornamental plant which native to the central and southern regions of the United States of America, and was introduced into Japan more than 70 years ago (Ohkawa et al., 1991). In Japan, the production of cut lisianthus flowers increased by about 3-fold from 1986 to 2007, and it has become an important cut flower in Japan, ranking fifth in the production value of cut flowers in 2004. Still commercial producer are facing different aspects of production problem of lisianthus. One of them is the slow growth at seedling stage (Harbaugh, 1995; Matsuo and Shirasaki, 1990) which ultimately hampers the cut flower production. Growth inhibitors such as maleic and benzoic acid were detected in root exudates of lisianthus when it was grown in closed hydroponic system (Asao et al., 2007). Benzoic acid is the potential allelochemical which is responsible for the growth and yield reduction in many crops such as strawberry (Kitazawa et al., 2005), taro (Asao et al., 2003), leafy vegetables (Asao et al., 2004a). Allelochemicals play a multitude of ecological and physiological roles as they alter mineral uptake (Baziramakenga et al., 1994), disrupt membrane permeability (Baziramakenga et al., 1995), cause stomatal closure and induce water stress (Barkosky and Einhellig, 1993). These allelochemicals also influence respiration (Penuelas et al., 1996), affect photosynthesis and protein synthesis (Mersie and Singh, 1993; Rohn et al., 2002), impair hormonal balance (Holappa and Blum, 1991) and alter enzyme activities (Rohn

et al., 2002; Doblinski et al., 2003). During autotoxicity, ion uptake and hydraulic conductivity (i.e., water uptake) were worse affected processes since root was the first organ to come into contact with autotoxins in the rhizosphere (Blum et al., 1999). Autotoxic compounds might induce a secondary oxidative stress manifested as enlarged production of reactive oxygen species (ROS) (Weir et al., 2004). Toxic ROS can affect membrane permeability, cause damage to DNA and protein, induce lipid peroxidation, and ultimately lead to programmed cell death. Therefore, autotoxic effects of root exudates in lisianthus on its growth and development is likely to be caused by impairment of nutrient and water absorption by injured roots.

Foliar application of nutrients has been recognized by many researchers, as a very efficient method of plant nutrition (Roosta and Hamidpur, 2011, Stiegler et al., 2013). Supply of mineral nutrient alternative to roots uptake can sustain lisianthus growth even during this allelochemical stress. In plants, nitrogen is the main mineral nutrient that is required in the largest quantities and represents up to 2% of plant dry matter. As a result of its important role in metabolism, the availability of nitrogen (N) is one of the key factors that limit crop productivity (Masclaux-Daubresse et al., 2010, Lea and Azevedo, 2006, Warner et al., 2004). Therefore, it can be sprayed on the leaves as a source of nutrient during autotoxicity. Foliar spray of urea is very common (Bowman and Paul, 1992) where it increased the leaf photosynthetic rates and leaf urease enzyme activities (Peltonen, 1993). Recent research focuses on developing foliar spray programs of amino acids. Amino acids are the nitrogenous compound that forms the basic component of all living cells. It can be absorbed by leaf exogenously (Furuya and Umemiya, 2002; Stiegler et al., 2013). Amino acids are the building block of proteins and serve in a

variety of important pathways. They can also act as parts of co-enzymes or as precursors for biosynthesis such as Glutamine and Ornithine which are precursors for nucleotides and polyamines respectively (Alcázar et al., 2010). Foliar application of amino acids has positive effects on the growth, yield and quality of *Urtica pilulifera* (Wahba et al., 2015), alfalfa (Pooryousef and Alizadah 2014), chinese cabbage (Cao et al., 2010); leafy radish (Liu et al., 2008); *Codiaeum variegatum* (Mazher et al., 2011) and Japanese pear (Takeuchi et al., 2008), grape (Garde-Cerdán et al., 2015; Portu et al., 2015). Apart from this, the role of amino acids to act as bio-stimulants in plants under abiotic and biotic stress conditions has been demonstrated (Maini et al., 1999; Heuer, 2003, SH Sadak et al., 2015). As the accumulated allelochemicals in closed culture become stressful to plants, spraying of amino acids to lisianthus plants would be positive in closed hydroponic culture. In our previous study, we found the positive effect of Glutamic acid and Hydroxy-proline on the autotoxicity experienced strawberry plants in the closed hydroponic (Mondal et al., 2013). Therefore the purpose of the present study was to evaluate the performance of amino acids on the growth of lisianthus under autotoxic condition in closed hydroponic culture.

2. Materials and Methods

2.1. Seedling growth bioassay

2.1.1 Expt. I. Effects of amino acids on the lisianthus seedlings grown in the renewed nutrient solution

Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn cv. Ichiban-boshi] seeds (Sakata Co. Ltd. Yokohama, Japan) were sown on May 28, 2010 in cell trays (3 cm × 3 cm × 4 cm, 28 cell/tray) containing moisten horticultural soil substrate (Takii, Kyoto, Japan) covering with vermiculites. Cell trays were kept at 10 ºC for 4 weeks cold treatment and

then transferred to growth chamber at 20/15 ºC (day/night) under fluorescent light with intensity of 74-81 μmol $m^{-2}s^{-1}$ and a 12 hour photoperiod. Germination was started on July 2, 2010. 25% Enshi nutrient solution (pH 7.25 and EC 0.8 dS m^{-1}) was used as fertilizer during the growth of seedlings in the cell tray. The full strength Enshi nutrient solution contains the following amount of salts per 1000 L of tap water: 950 g Ca $(NO_3)_2 \cdot 4H_2O$; 810 g of KNO_3; 500 g of $MgSO_4 \cdot 7H_2O$; 155 g of $NH_4H_2PO_4$; 3 g of H_3BO_3; 2 g of $ZnSO_4 \cdot 7H_2O$; 2 g of $MnSO_4 \cdot 4H_2O$; 0.05 g of $CuSO_4 \cdot 5H_2O$; 0.0 2g of Na_2MoO_4; 25 g of NaFe-EDTA (Hori, 1966). After four weeks on July 30, 2010 similar vigor seedlings were selected and transplanted to plastic containers (17 cm × 29 cm × 9.5 cm) after slightly shaking the cubic substrates enclosed roots in the tap water into a bucket to easily separate the substrate from the roots and kept in the growth chamber at 25/20 ºC (day/night) under fluorescent light with the intensity of 74-81 μmol $m^{-2}s^{-1}$ and a 12-h photoperiod. Each container was filled with 3 L of 25% Enshi solution. The solution in the container was renewed every two weeks. Ten seedlings were planted in each container in such a way that the roots were inserted into the nutrient solution inside the container keeping shoot outside. Three containers (10 seedlings ×3 = 30 seedlings) were used for one treatment. In this experiment total 30 seedlings × 25 treatments = 750 seedlings were used simultaneously. Urethane foam blocks (23 mm × 23 mm × 27 mm) were used for holding the plant tight with a floating board on the nutrient solution. No aeration system was used in this experiment. One day after transplanting, 23 water soluble amino acids viz., Alanine (Ala), Arginine (Arg), Asparagine (Asn), Aspartic acid (Asp), Cysteine (Cys), Glutamatic acid (Glu), Glutamine (Gln), Glycine (Gly), Hydroxy-proline (Hyp), Lysine (Lys), Ornithine (Orn), Proline (Pro), Serine (Ser), Threonine (Thr), Tryptophan (Trp), Methionine (Met), Leucine (Leu), Isoleucine (Ile),

Citrulline (Cit), Histidine (His), Phenylalanine (Phe), Valine (Val), G*amma*-aminobutyric acid (GABA) (Special Grade chemical, Nacalai Tesque, INC. Kyoto, Japan); urea (Otsuka agrio Co, Ltd, Tokyo, japan) and distilled water as control were applied as droplets by a micro-pipette (Gilson S. A. S, France) applied on the leaves and stem of lisianthus seedlings at 0.5 ml per plant two times in a week. The surfactant Approach BI (Kao, Osaka, Japan) was added to the amino acid and urea solutions in the proportion of 0.02% (v/v). The concentrations of urea and amino acids were adjusted to nitrogen content of Pro at 200 mg L^{-1} to maintain the same concentration level. After ten weeks of amino acids application on October 2, 2010, the number of leaves, maximum leaf width and length and maximum root length of lisianthus seedlings were measured. Then the lisianthus seedlings were dried in a constant temperature oven (DKN 812, Yamato Scientific Co., Ltd. Japan) at 80 °C for 72 h. Dry weight was measured when the dry matter reaches at constant weight.

2.1.2. Expt. II. Effects of amino acids on the lisianthus seedlings grown in the non-renewed nutrient solution

In this experiment, the materials and methods from sowing to transplanting were similar to those described above for Expt. I with the difference in cell tray size (4 cm × 4 cm × 4 cm, 72 cell/tray) (Fig. 1). Sowing and germination were occurred on September 5 and October 8, respectively. On December 28, 2012, seedlings were transplanted into 3 L plastic container (Fig. 2). Three containers (5 seedlings × 3 = 15 seedlings) were used for one treatment and total 15 seedlings × 26 treatments = 390 seedlings were used simultaneously. Nutrient solutions were either renewed or non-renewed entirely, and amino acids and urea were applied in later case. Renewed culture solutions were

changed with new nutrient solutions whereas non-renewed nutrient solutions were analyzed for major nutrients and adjusted as close as possible to initial concentrations at every two weeks on the basis of chemical analyses with Compact NO_3^- meter (B-343, Horiba, Ltd. Kyoto, Japan) for NO_3^-, Spectrophotometer (U-2900, Hitachi, Tokyo, Japan) for PO_4^{3-} and Polarized Zeeman Atomic Absorption Spectrophotometer (Z-2310, Hitachi, Tokyo, Japan) for K^+, Ca^{2+}, Mg^{2+} and Fe^{3+}. From January 5, 2013, twenty three water soluble amino acids viz., Ala, Arg, Asn, Asp, Cys, Glu, Gln, Gly, Hyp, Lys, Orn, Pro, Ser, Thr, Trp, Met, Leu, Ile, Cit, His, Phe, Val and GABA; urea and distilled water as control were applied by the same methods as mentioned in Expt. I three times a week. After 4 weeks of amino acids application, on February 2, 2013, growth parameters and the chlorophyll content of leaf by SPAD (Konica Minolta, Tokyo, Japan) were measured. Dry weight of *Lisianthus* seedlings were measured after oven drying the seedling as described for Expt. I.

Fig. 1. Lisianthus seedlings grown in the cell tray with horticultural soil inside the growth chamber.

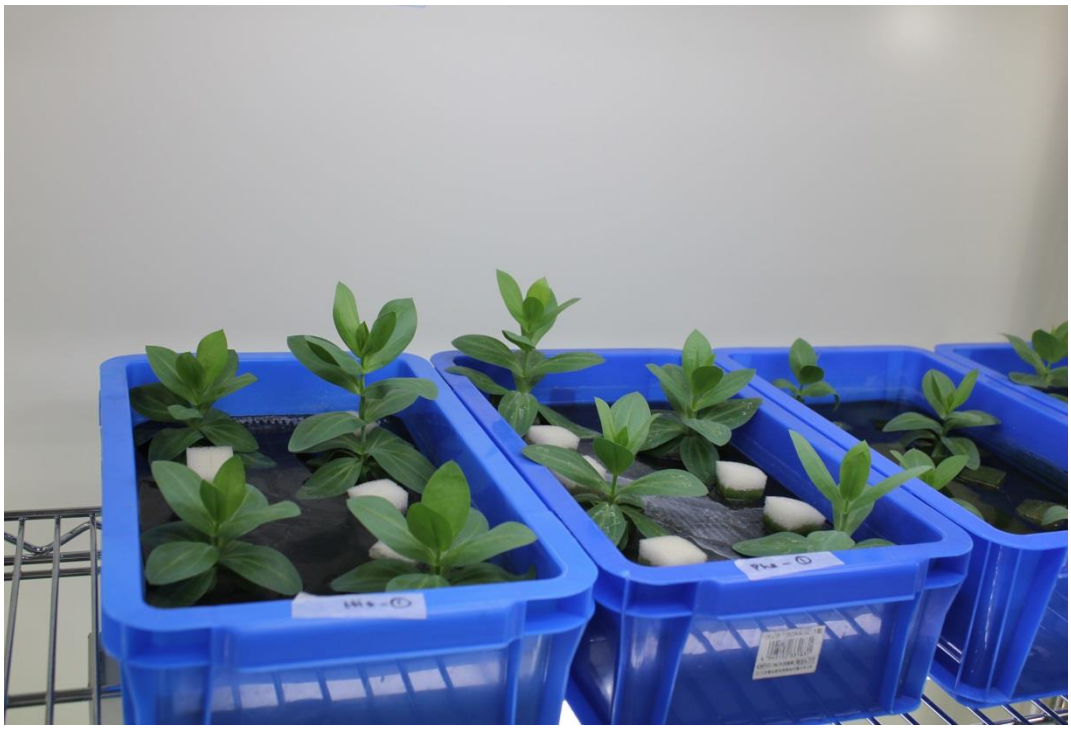

Fig. 2. Lisianthus seedlings into the hydroponic system inside the growth chamber.

2.2.1. Expt. III (a) Effects of seven amino acids on the lisianthus seedlings grown in the horticultural soil

In this experiment, sowing and germination conditions of lisianthus were similar to those described above for Expt. II with the difference in light condition. Hybrid Electrode Fluorescent Light (HEFL) was used as a light source in the controlled growth chambers (Fig. 3). Sowing and germination were occurred on June 5 and July 10, 2013, respectively. On August 7, 2013, thirty seedlings (10 plants × 3 replications) of similar vigor and good growth were selected from each cell tray for each treatment. In this experiment total 30 seedlings × 9 treatments = 270 seedlings were used simultaneously. In this experiment, six amino acids (Gln, Gly, Pro, Met, Leu and His), selected for their better performance in seedlings growth bioassay in Expt. II and Betaine (Bet) (Special Grade chemical, Nacalai Tesque, INC. Kyoto, Japan) as a new amino acid, urea and distilled water as control were applied on lisianthus seedlings by the similar methods

mentioned for Expt. II. Amino acids were applied for four weeks from the August 12, 2013 to September 9, 2013. After 4 weeks of amino acids application on September 9, 2013 growth parameters such as number of leaves, plant height, leaf length, leaf width, root length, fresh weight of shoot and the chlorophyll content of leaf by SPAD were measured. In this experiment substrates along with the roots were separated according to the methods mentioned in Expt. I.

Fig. 3. Lisianthus seedlings grown in the horticultural soil under Hybrid Electrode Fluorescent Light (HEFL) inside the growth chamber.

2.2.2. Expt. III (b) Effects of seven amino acids on the lisianthus plants grown in closed hydroponics in the greenhouse

After growth measurement, lisianthus seedlings from the seedlings growth bioassay were transplanted to plastic container (54 cm × 35 cm × 20 cm) with 30 L of 25% Enshi nutrient solution under greenhouse condition. Five seedlings from the each amino acid treatment were planted in each container supported by four urethane blocks (Fig. 4 and 5)

with three replications and 18 treatments (5×3× 18=270 seedlings). Nutrient solutions were either renewed or non-renewed and circulated for 24 hours by pumps (KP-101, Koshin, Kyoto, Japan) for 5 min at 10 min intervals using an automatic timer (KS-1500, Iuchi, Osaka, Japan). In greenhouse setting, amino acids treated seedlings were continued with either amino acid {Gln (A), Gly (A), Pro (A), Met (A), Leu (A), His (A), and Bet (A)} or not {Gln (B), Gly (B), Pro (B), Met (B), Leu (B), His (B), Bet (B)} in non-renewed nutrient solution. Non-renewed nutrient solutions were analyzed for major nutrients and adjusted as close as possible to initial concentrations at every two weeks on the basis of chemical analyses. One day after transplanting, water, urea and selected seven amino acids used in Expt. III (a) were applied by the same methods as mentioned in Expt. I. The dates of anthesis were recorded for each plant to check whether there any influence of amino acids on flowering of lisianthus. At the first anthesis, plants were harvested. Leaf number, leaf length and width, root length, fresh weight of shoot, numbers of flower buds were measured. After measuring, lisianthus shoots with flowers were kept in a bucket with 4 liter of water at the control room condition with 20 ºC temperature and 70% relative humidity to check the effects of amino acids on the vase life of lisianthus flowers (Fig. 6). Water was changed in each 3 days. Data of vase life was taken until the first petal of each flower was wilted. Shoots and roots dry weight were measured following the similar methods for Expt. I.

Fig. 4. Lisianthus plants grown in closed hydroponic system in the greenhouse.

Fig. 5. Lisianthus plants supported by urethane blocks in closed hydroponic container.

Fig. 6. Lisianthus cut flowers kept into the bucket with water at their first anthesis.

2.3. Statistical analysis

A randomized complete block design with three replicates was used for culture of lisianthus in container based hydroponics in the greenhouse whereas, complete block design was performed in culture of lisianthus seedlings in the control room condition. Analysis of variance was performed to test for statistical differences among the treatments, and means were statistically analyzed using Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$ level of significance by IBM SPSS Statistic v22.0 (IBM SPSS, 2014. Chicago IL, USA) and Tukey's test by Statcel 2 statistical software (OMS publication, Tokorozawa, Saitama, Japan). Number of plants per treatment (n) were 30, 15, 30 and 15 in Expt. I. in Expt. II. Expt. III (a) and Expt. III (b), respectively.

3. Results

3.1. Expt. I. Effects of amino acids on the lisianthus seedlings grown in the renewed nutrient solution

In the first seedlings growth bioassay 23 water soluble amino acids and urea were applied on the leaves and stem of lisianthus seedlings grown in renewed hydroponic solution. Amino acids application showed a significant effect on the lisianthus seedlings growth. Compared to the control, longer root was evidenced in Cys treated seedlings (Table 1). Ala, Glu, Hyp, and Lys treated plants reduced their leaf numbers against control plants. The application of His and GABA significantly increased the dry matter of lisianthus seedlings compared to control plants (Fig. 7). However, Ala, and Ser showed negative effects on the dry matter production in lisianthus seedlings.

3.2. Expt. II. Effects of amino acids on the lisianthus seedlings grown in the non-renewed nutrient solution

Compared to the control (NRW) seedlings, amino acids treated seedlings did not show any significant difference on the seedling growth (Table 2). However, among the amino acids Pro, Gln; and Ala treated seedlings produced higher and lower dry matter, respectively (Fig. 8, Fig. 9). Compared to the control higher numbers of leaves were found in Cys treated seedlings. Among the amino acids treated seedlings, highest plant height was measured in Gln and Gly treated seedlings. The Chlorophyll content of leaf measured by SPAD, leaf length and root length did not show any significant difference among the treatments.

Table 1

Effects of twenty three amino acids on the growth of lisianthus seedlings grown by the renewed nutrient solution in closed hydroponic system.

Amino acids[z]	No. of leaves[y]	Leaf width (mm)	Leaf length (mm)	Root length (mm)
Control	16.2 ab[x]	25.0 ab	45.8 ab	183.2 c
Urea	20.0 a	23.0 ab	42.1 ab	225.0 bc
Ala	15.7 c	16.4 c	23.7 c	167.0 d
Arg	16.2 ab	25.3 ab	39.5 ab	256.3 b
Asn	16.3 ab	25.0 ab	40.5 ab	214.7 bc
Asp	17.1 ab	21.9 ab	33.8 bc	236.0 bc
Cys	15.8 bc	20.1 bc	29.7 bc	286.6 a
Glu	14.5 c	20.4 bc	34.1 bc	178.0 cd
Gln	17.4 ab	25.2 ab	43.6 ab	250.7 bc
Gly	15.5 bc	24.3 ab	44.6 ab	199.2 bc
Hyp	14.7 c	21.5 bc	36.3 abc	215.2 bc
Lys	14.3 c	23.4 ab	44.5 ab	190.8 c
Orn	16.8 ab	26.0 ab	42.1 ab	218.4 bc
Pro	16.7 ab	24.8 ab	39.5 ab	233.9 bc
Ser	15.2 bc	19.7 bc	33.0 bc	202.4 bc
Thr	15.2 bc	24.0 ab	41.9 ab	216.4 bc
Trp	18.2 ab	24.9 ab	37.4 ab	236.3 bc
Met	17.5 ab	23.8 ab	43.1 ab	232.0 bc
Leu	16.1 bc	23.3 ab	39.8 ab	206.5 bc
Ile	16.6 ab	24.9 ab	44.2 ab	216.2 bc
Cit	17.0 ab	23.6 ab	40.9 ab	211.2 bc
His	19.7 a	30.4 a	52.1 a	272.3 b
Phe	18.3 ab	27.7 a	39.8 ab	264.3 b
Val	17.1 ab	27.4 a	45.6 ab	227.9 bc
GABA	19.2 a	29.4 a	52.2 a	220.0 bc
	*	*	*	*

[z] Lisianthus seedlings grown in renewed nutrient solution with amino acids and urea supplementation.

[y] Parameters were measured on per plant basis.

[x] Mean values within the column followed by different letters varied significantly according to the Tukey`s test (n = 30).

[*]Significant at $P < 0.05$.

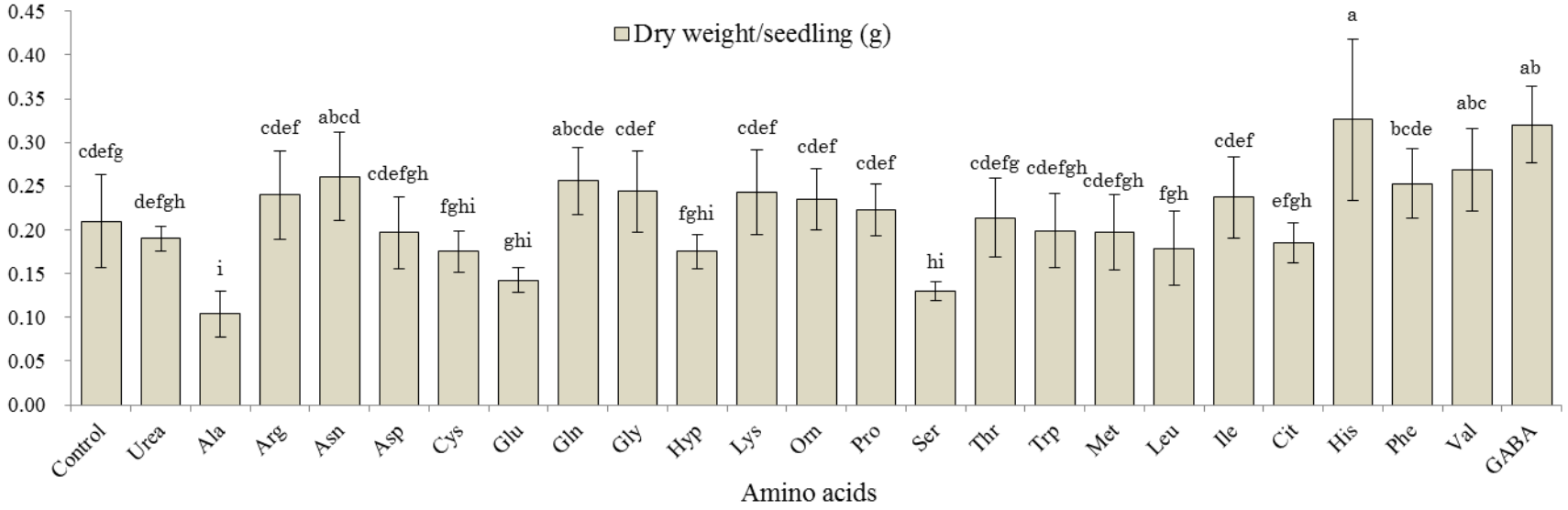

Fig. 7. Effects of twenty three amino acids and urea on the dry matter production of lisianthus seedlings grown in the renewed nutrient solution in closed hydroponics. Control (water supply), amino acids are presented as their three letter abbreviation. Mean±SD within the column followed by different letters varied significantly according to the Tukey`s test at $P < 0.05$ ($n = 30$).

Table 2. Effects of twenty three amino acids on the growth of lisianthus seedlings grown by the non-renewed nutrient solution in closed hydroponic system.

Amino acids[z]	No. of leaves	SPAD	Plant height (mm)	Leaf length (mm)	Leaf width (mm)	Root length (mm)
NRW	13.2 b[x]	50.7	69.9 ab	47.7	25.1 ab	186.4
RW	14.3 ab	51.0	78.1 ab	52.2	23.8 ab	166.2
Urea	14.9 ab	51.0	79.8 ab	51.4	25.0 ab	180.0
Ala	14.7 ab	47.0	60.4 ab	44.8	20.1 b	169.4
Arg	15.6 ab	50.9	77.8 ab	48.3	23.1 ab	190.5
Asn	15.8 ab	49.8	75.4 ab	50.4	23.8 ab	168.4
Asp	16.2 ab	50.9	77.8 ab	47.0	24.6 ab	170.7
Cys	16.5 a	47.8	61.1 b	44.8	22.0 ab	209.8
Glu	15.1 ab	49.0	71.1 ab	47.5	20.2 b	160.7
Gln	15.8 ab	49.9	89.5 a	54.3	26.2 a	177.8
Gly	15.0 ab	52.8	88.6 a	51.9	24.3 ab	159.7
Hyp	14.4 ab	51.2	79.8 ab	50.5	23.1 ab	162.6
Lys	15.1 ab	50.0	82.4 ab	49.2	22.3 ab	174.0
Orn	14.8 ab	50.4	80.8 ab	51.3	25.2 ab	183.2
Pro	15.6 ab	51.8	85.3 ab	48.1	25.8 a	180.5
Ser	16.3 ab	45.9	73.5 ab	49.1	25.3 ab	180.0
Thr	14.7 ab	48.2	74.4 ab	47.7	22.2 ab	150.6
Trp	15.1 ab	46.0	77.5 ab	49.0	21.3 ab	168.8
Met	14.8 ab	48.9	76.1 ab	49.6	23.2 ab	154.1
Leu	14.9 ab	48.9	85.3 ab	54.2	24.6 ab	164.9
Ile	15.7 ab	46.9	74.3 ab	49.0	21.6 ab	151.4
Cit	15.8 ab	45.8	78.2 ab	48.6	23.8 ab	154.5
His	14.3 ab	48.8	76.9 ab	52.3	23.4 ab	156.4
Phe	14.5 ab	49.5	80.9 ab	50.8	22.0 ab	158.7
Val	14.8 ab	46.1	79.4 ab	51.7	23.4 ab	169.6
GABA	13.5 ab	46.6	66.4 ab	43.1	20.5 ab	142.9
	*	ns	*	ns	*	ns

[z] Lisianthus seedlings in renewed (RW), and non-renewed (NRW), and non-renewed nutrient solution with amino acids and urea supplementation.

[x]Mean values within the column followed by different letters varied significantly according to the Tukey`s test (n = 15).

ns: non-significant or *significant at $P < 0.05$.

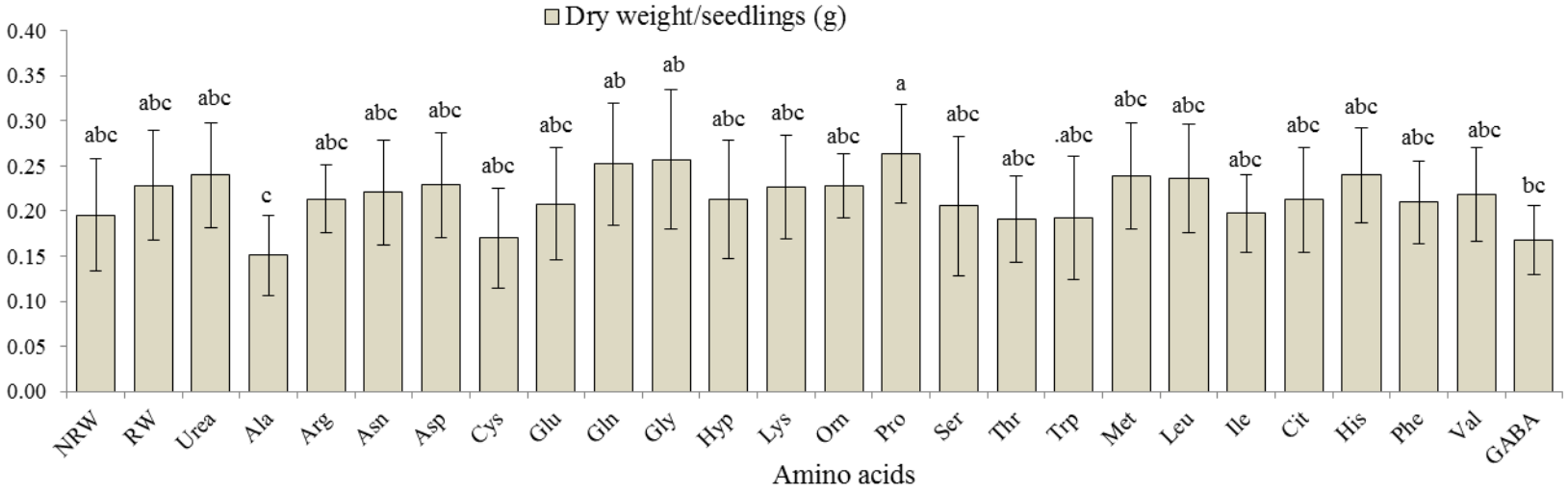

Fig. 8. Effects of twenty three amino acids and urea on the dry matter production of lisianthus seedlings grown in the non-renewed nutrient solution in closed hydroponics. RW: renewed, NRW: non-renewed, amino acids are presented as their three letters abbreviation. Mean±SD within the column followed by different letters varied significantly according to the Tukey`s test at $P < 0.05$ ($n = 15$).

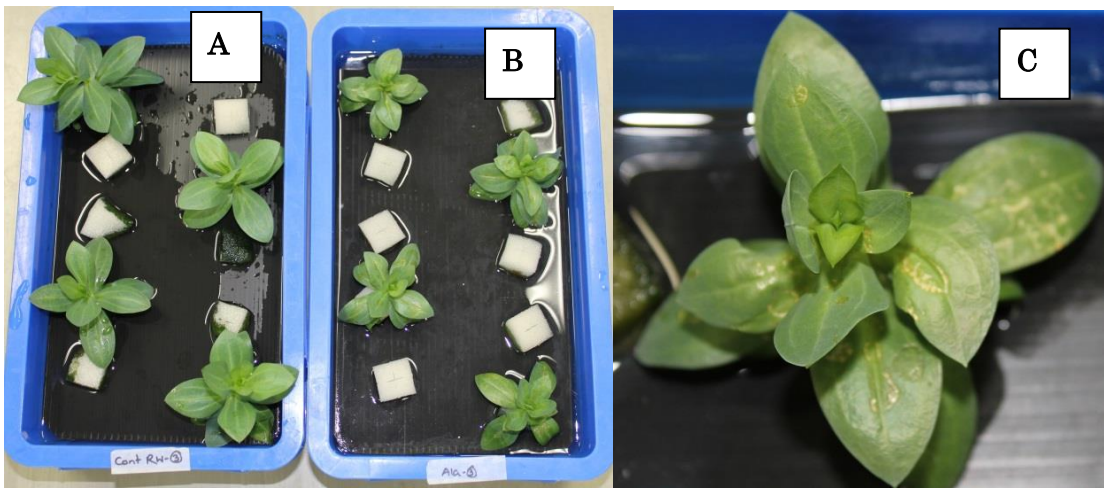

Fig. 9. Lisianthus seedlings grown in renewed nutrient solution (A) and non- renewed nutrient solution with foliar application of Ala (B). Injured leaves with yellowish spots due to Ala application (C).

3.3.1. Expt. III. (a) Effects of amino acids on the lisianthus seedlings grown in the horticultural soil

Based on the seedling growth performance, Gln, Gly, Pro, Met, Leu and His from the experiment II and Bet were applied on the lisianthus seedlings grown in horticultural soils in the cell trays. Growth parameters were measured before transplanting the seedling in the hydroponic solution in the greenhouse. Compared to the control, amino acids application exhibited a significant effect on the growth of the seedlings (Table 3, Fig. 10 and 11). Higher numbers of leaves were found in His treated seedlings. Leaf length and leaf width were increased when the seedlings were treated with Gln, Gly, Pro and His; Gly, Pro and Leu, respectively. Root length of the seedlings within the substrate was also increased by the application of Pro. Compared to the control, all the amino acids increased the plant height in lisianthus seedlings. The chlorophyll content of Leaf measured by SPAD was increased in Urea and Gly applied seedlings. The shoot fresh weight of the lisianthus seedlings was also increased by the amino acids

application. Compared to the control, higher shoot fresh weight was measured in Pro and treated seedlings.

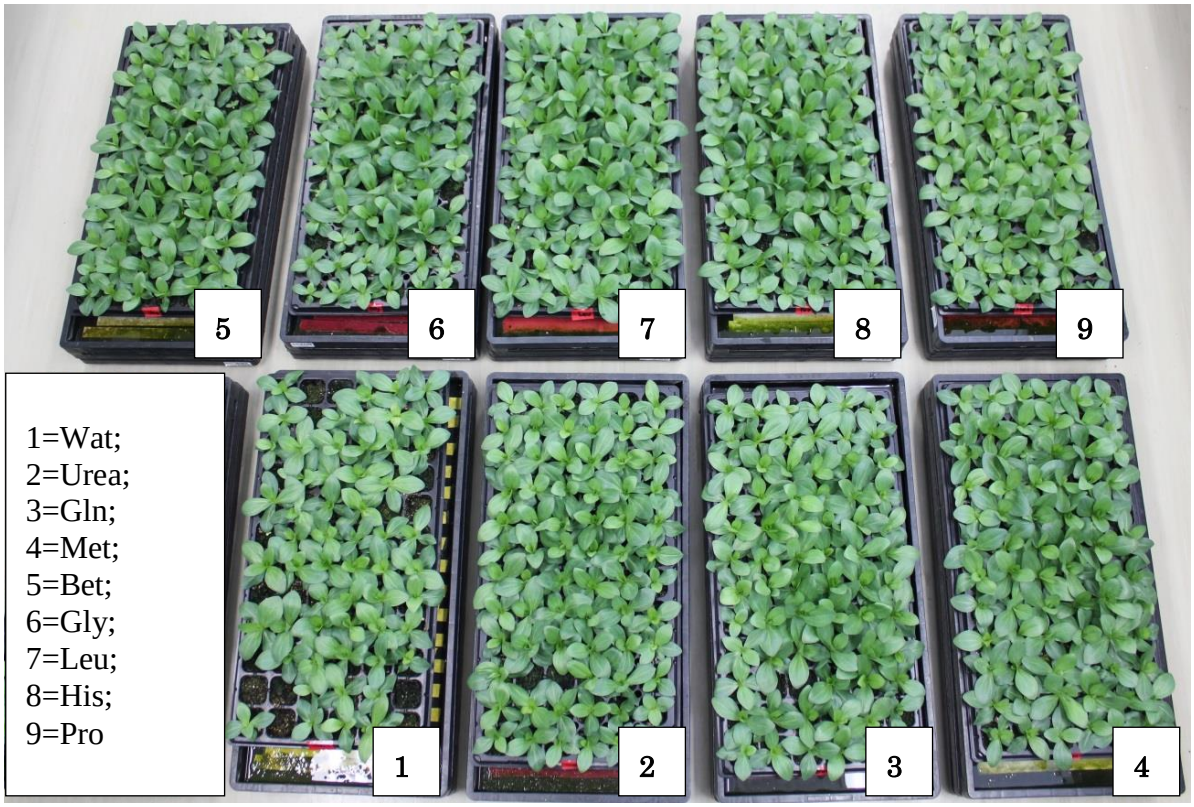

Fig. 10. Amino acids applied lisianthus seedlings grown in cell tray with horticultural soil.

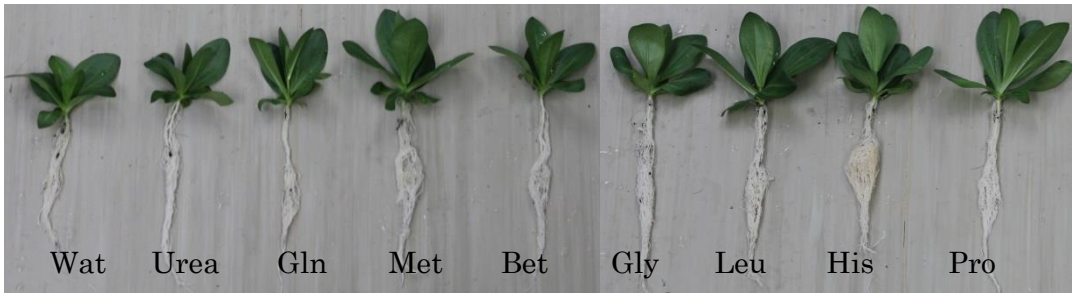

Fig. 11. Amino acid applied lisianthus seedlings.

Table 3
Effects of seven amino acids on the growth of lisianthus seedlings grown in horticultural soil under growth chamber condition.

Amino acids[z]	No. of leaves[y]	SPAD	Plant height (mm)	Leaf length (mm)	Leaf width (mm)	Root length (mm)	Shoot fresh weight (g)
Water	7.6 b[x]	52.8 b	57.2 c	51.3 b	26.8 b	168.8 bc	2.4 b
Urea	7.7 b	56.2 a	64.9 b	55.2 ab	26.9 b	142.7 c	2.7 ab
Gln	8.0 ab	52.2 b	68.1 ab	61.8 a	29.0 ab	189.5 ab	3.1 ab
Gly	8.7 ab	57.2 a	71.9 a	59.2 a	30.6 a	157.0 c	3.0 ab
Pro	8.8 ab	55.0 ab	72.5 a	61.0 a	32.5 a	205.2 a	3.4 a
Met	8.7 ab	51.2 b	64.2 b	53.8 ab	28.8 ab	174.5 ab	3.0 ab
Leu	8.3 ab	53.1 b	71.5 a	58.0 ab	31.8 a	201.7 ab	3.3 a
His	9.0 a	51.8 b	67.8 ab	61.8 a	28.2 ab	197.7 ab	3.2 ab
Bet	8.6 ab	53.1 b	66.7 ab	57.5 ab	28.7 ab	197.5 ab	2.6 ab
	*	*	*	*	*	*	*

[z] Lisianthus seedlings grown in horticultural soil substrate with amino acids and urea supplementation; water supply as control.
[y] Parameters were measured on per plant basis.
[x] Mean values within the column followed by different letters varied significantly according to the Tukey`s test (n = 30).
*Significant at $P < 0.05$.

3.3.2. Expt. III. (b) Effects of amino acids on the growth and the flowering of the lisianthus plants grown in hydroponics in greenhouse

Amino acids treated seedling were categories into two groups in the greenhouse, amino acids application from seedling to anthesis (A) and only seedling stage (B). Either amino acid applied or not, lisianthus plants height was increased compared to the control (Fig. 12 and Table 4). Higher numbers of leaves were evidenced in plants grown in renewed nutrient solution. Leaf size, Chlorophyll content measured by SPAD and root length did not show any significant difference among the treatments (Data not shown here). Shoot dry weight was significantly increased in urea (A), urea (B), Leu (A). Leu (B), His (B), Bet (A), Bet (B) treated plants. Amino acids have effects on the flowering of lisianthus. Compared to the control, His (A) treated plants were recorded with early flowering. Results showed that His treated lisianthus plants with continuous supply from seedling to reproductive stage, started their anthesis 20 days before against the control plants. Numbers of bud, flower size and vase life of lisianthus flowers did not show any significant difference among the treatments (Table 5).

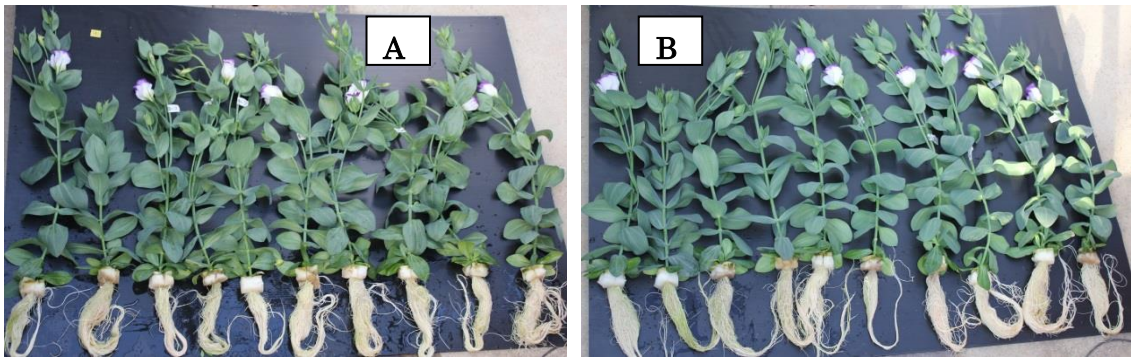

Fig. 12. Harvested lisianthus plants from the greenhouse culture. A= Continuous supplied of amino acids from seedling to anthesis stage; B= Amino acids supplied only in the seedling stage. RW, NRW, Urea, Gln, Gly, Pro, Met, Leu, His and Bet treated lisianthus plants from the left to the right in both A and B.

Table 4

Effects of amino acids on the growth and anthesis of lisianthus plants grown in the non-renewed nutrient solution in closed hydroponic culture under greenhouse condition.

Amino acids[z]	No. of leaves[y]	Plant height (cm)	Shoot dry weight (g)	Days of anthesis
NRW	23.1 b[x]	51.9 b	6.1 b	109 ab
RW	26.4 a	66.6 a	8.7 ab	93 bc
Urea (A)	25.0 ab	66.1 a	9.0 a	102 ab
Urea (B)	23.9 ab	64.8 a	9.3 a	100 ab
Gln (A)	25.5 ab	65.7 a	8.9 ab	97 ab
Gln (B)	24.8 ab	62.0 a	8.6 ab	102 ab
Gly (A)	25.2 ab	62.6 a	8.4 ab	94 bc
Gly (B)	24.6 ab	65.4 a	8.7 ab	99 ab
Pro (A)	26.0 ab	64.1 a	8.9 ab	104 ab
Pro (B)	25.2 ab	66.9 a	8.7 ab	96 bc
Met (A)	25.2 ab	65.1 a	8.1 ab	92 bc
Met (B)	24.0 ab	62.7 a	7.6 ab	98 ab
Leu (A)	25.7 ab	64.4 a	9.8 a	104 ab
Leu (B)	24.9 ab	63.4 a	9.1 a	103 ab
His (A)	25.0 ab	63.0 a	8.5 ab	89 c
His (B)	25.3 ab	66.2 a	9.8 a	91 bc
Bet (A)	25.5 ab	67.4 a	9.1 a	102 ab
Bet (B)	24.9 ab	63.3 a	9.3 a	113 a
	*	*	*	*

[z] Lisianthus seedlings grown in renewed (RW), non-renewed (NRW), and non-renewed nutrient solution with amino acids and urea supplementation; (A) = amino acids supplementation from seedling stage to anthesis; (B) = amino acids supply on the seedling stage only,

[y] Parameters were measured on per plant basis.

[x] Mean values within the column followed by different letters varied significantly according to the Tukey`s test (n = 15).

*significant at P< 0.05.

Table 5. Effects of amino acids on the flower characteristics of lisianthus plants grown by non-renewed nutrient solution in closed hydroponic culture under greenhouse condition.

Amino acids[z]	No. of buds	Flower length (mm)	Flower width (mm)	Vase life (days)
NRW	6	54	42	20
RW	7	55	45	24
Urea (A)	7	54	40	20
Urea (B)	7	53	41	20
Gln (A)	7	56	45	18
Gln (B)	6	54	43	21
Gly (A)	9	54	47	21
Gly (B)	7	56	42	20
Pro (A)	8	55	46	17
Pro (B)	7	56	44	24
Met (A)	7	57	43	21
Met (B)	6	56	45	20
Leu (A)	7	56	42	19
Leu (B)	7	54	42	21
His (A)	8	57	45	21
His (B)	7	56	42	20
Bet (A)	7	57	45	23
Bet (B)	6	55	44	22
	ns	ns	ns	ns

[z] Lisianthus seedlings grown in renewed (RW), non-renewed (NRW), and non-renewed nutrient solution with amino acids and urea supplementation; (A) = amino acids supplementation from seedling stage to anthesis; (B) = amino acids supply on the seedling stage only.

[y] Parameters were measured on per plant basis.

ns: non-significant according to the Tukey`s test at $P < 0.05$ ($n = 15$).

4. Discussion

Amino acids have profound effects on the growth of lisianthus. In our first experiment among the twenty three water soluble amino acids, foliar application of His, GABA increased the dry mass of lisianthus seedlings grown in the renewed nutrient (Fig.7). Ashrafuzzaman et al., (2010) found that foliar application of GABA increased the growth of bitter gourd plant. Several researchers also found that foliar application of amino acids increased the biomass of leafy radish, wheat, lemon grass, bean and onion plants (Liu et al., 2008; Gupta et al., 2003; Gamal El-din et al., 1997; Nassar et al., 2003; Amin et al., 2011). N contents of the amino acids might induce the growth of lisianthus seedlings. N as an essential nutrient plays crucial roles in different aspects of plant growth and development. Primary metabolites where N is the main component such as amino acids, the building blocks in the synthesis of proteins, are involved in plant growth and development (Hounsome et al., 2008). Foliar application of amino acids increased the protein content in mulberry plants (Das et al., 2002). However, among the twenty three amino acids Ala, and Ser showed negative effects on the lisianthus seedlings by decreasing the leaf size and numbers. These amino acids have negative effects on the growth of lisianthus seedlings.

In non-renewed container based hydroponics, higher dry matter was produced by Pro, Gln application and lower dry matter was produced by Ala application. (Fig. 8). Gln plays an important role to regulate the N status of plants (Glass et al., 2002). Amino acids are important in many biological molecules, such as forming parts of coenzymes, or as precursors for the biosynthesis of molecules such as Gln and Orn, which are precursors for nucleotides and polyamines, respectively (Alcázar et al., 2010). In our

third experiment, when Gln, Gly, Pro, Met, Leu, His and Bet were applied on the lisianthus seedlings, fresh weight of Pro treated seedlings were significantly increased (Table 3) whereas seedlings applied with water did not increase fresh weight. Declined seedlings growth might be due to the autotoxic effects of lisianthus root exudates (Asao et al., 2007). When plants experience autotoxicity, ion uptake and hydraulic conductivity are inhibited since root is the first organ to come into contact with autotoxins in the rhizosphere (Blum et al., 1999). Several researchers found positive impacts of amino acids as foliar spray under stress condition for example Pro to wheat, Pro, Ala, Ser, and Asp to maize under osmotic stress (Rajagopal and Sinha, 1980; Thakur and Rai, 1985) and Pro, Phe to maize and board bean under salinity stress (Abd El-Samad et al., 2011). Pro is the most widely studied because of its considerable importance in the stress tolerance as compatible osmolyte (MacCue and Hanson, 1990; Samras et al., 1995, Delauney and Verma, 1993). There are a number of reports that exogenous application of Pro increases its endogenous levels in plant tissues subjected to water stress conditions (Ali et al., 2007; Ashraf and Foolad, 2007; Hoque et al., 2007) which contribute to osmotic adjustment in plant tissues (Bajji et al., 2000). The improved growth in Pro supplied plants might be achieved by preserving the osmotic balance, stabilizing subcellular structures, such as membranes and proteins, and scavenging ROS (Heuer, 2003; Ashraf and Foolad, 2007). Moreover, Pro acts as a reserve source of carbon, nitrogen and energy during recovery from stress (Zhang et al., 1997).

When amino acids treated seedlings were transferred to the greenhouse in closed hydroponic system with either continuous application of same amino acids or water; in both cases, lisianthus plants increased growth against the control (Table 4). Dry weight

of shoot was increased in urea (A), urea (B), Leu (A), Leu (B), His (B), Bet (A) and Bet (B) treated plants. Early flowering of lisianthus plants was evidenced in the His (A) treated plants. Several hypotheses have been proposed to explain the role of amino acids in plant growth, Hashimoto and Yamada (1994) suggested several alternative routes of IAA synthesis in plants, all starting from amino acids. In plants, cytokinins signals are mediated by multi-component phosphorylation system composed of a His Protein kinase (Kakimoto, 2003). In *Arabidopsis thaliana*, intercellular signaling by cytokinin is referred to as Histidine-to-Aspartate phosphorelay system (Oka et al., 2002). There is evidence for the plant cytokinin hormones having a central role in signaling plant N status (Inoue et al., 2001). Vogt (2010) found that aromatic amino acids (AAA) derived specialized metabolites play important roles in various aspect of plant life such as growth, development, reproduction, defense and environmental responses. Several His kinase genes have also been reported to be involved in drought response in Arabidopsis (Tran et al., 2007; Wang et al., 2012; Muñiz et al., 2010). From this reports and results, His might induce synthsis of cytokinins in lisianthus and resulted in growth improvements. In plants, Leu-rich repeat receptor kinase (LRR-RKs) regulate a wide variety of developmental and defense-related processes including cell proliferation, stem cell maintenance, hormone perception, host specific as well as non-host specific defense responses, wounding response and symbiosis (Torii, 2004; De Smet et al., 2009; Wang et al., 2010). Yang et al. (2003) found that *Sorghum bicolor* accumulates Glycine betaine during dehydration stress to recover the stress effects. Tanaka et al., (1987) found that amino acids have positive and negative effect on flowering of *Lemna pausicostata* by controlling the nutrient uptake. These results indicated lisianthus seedling growth and its subsequent growth after transplanting are influenced by amino

acids application. Some amino acids recovered their growth only by the application of amino acids during the seedling stage, and others needed to apply until the anthesis. Numbers of bud, flower size and vase life of lisianthus flowers did not show any significant difference among the treatments. Presenting results indicate that amino acids have no effects on the flower characteristics and vase life.

5. Summary

Foliar application of amino acids was investigated for the recovery of the growth of lisianthus (*Eustoma grandiflorum* (Raf.) Shinn cv. Ichiban-boshi) under autotoxicity developed in the closed hydroponic system. Twenty three water soluble amino acids were applied on lisianthus seedlings grown in either renewed or non-renewed nutrient solution under controlled environment facility of Shimane University. The concentrations of all amino acids were adjusted to nitrogen content of Proline at 200 mg L^{-1}. Compared to the water control, His and GABA application increased the dry matter contents in renewed nutrient solution. In non-renewed nutrient solution, higher dry matter was produced by the Pro and Gln treated seedlings whereas Ala treated seedlings produced the lowest dry matter. Based on the seedling growth in non- renewed nutrient solution six amino acids namely Gln, Gly, Pro, Met, Leu and His were selected for further investigation along with Bet as a new amino acids following lisianthus seedling grown in horticultural soil substrate and the same seedlings were transferred to the container based closed hydroponic system in the greenhouse. All amino acids application increased the seedling height in horticultural soil substrate condition. Higher shoot fresh weight and root length were measured in Pro treated seedlings. Amino acids treated seedlings were continued under solution culture with either foliar application of

amino acids or water in the greenhouse. All amino acids treated plants height was increased either continued with amino acids application or water. His application only in seedling stage and urea, Leu and Bet application either in seedling or seedling to reproductive stage increased the shoot dry weight at final harvest. Early flowering of lisianthus was evidenced in the His treated (from seedling to anthesis) plants. Therefore, foliar spray of His can recover the growth with early flowering of lisianthus during autotoxicity in closed hydroponic system.

CHAPTER 3

Recovery from autotoxicity in strawberry by supplementation of amino acids

1. Introduction

Autotoxicity from the root exudates of strawberry in closed hydroponic culture has been investigated (Kitazawa et al., 2005). During this phenomenon strawberry plant's roots secreted allelochemicals mainly benzoic acid to the culture solution causing damage to the root cells, which in turns hamper water and mineral nutrient absorption. As a result, the growth of shoot and root, number of flowers and harvested fruit per plant, and fruit enlargement reduced greatly. Removal of these inhibitory allelochemicals from the culture solution would lead to normal growth and yield. In this regards, activated charcoal has been used to adsorb the accumulated phytotoxic chemicals for the culture solution and improve the growth and yield in strawberry (Kitazawa et al., 2005), taro (Asao et al., 2003), cucumber (Asao et al., 1998, 1999, 2000), several leafy vegetables (Asao et al., 2004a), and some ornamentals (Asao et al., 2007). Other means such as degradation of growth inhibitors by microbial strain in cucumber (Asao et al., 2004b), supplementation of auxin in strawberry (Kitazawa et al., 2007) or electro-degradation of phytotoxic chemicals in strawberry (Asao et al., 2008; Asaduzzaman et al., 2012) were also found to be effective for recovering the autotoxic effect in closed hydroponics. However, finding suitable method for controlling autotoxicity in strawberry would be of great help for the commercial production of strawberry in non-renewed hydroponics.

Allelopathic compounds may induce a secondary oxidative stress manifested as enlarged production of reactive oxygen species (ROS) (Weir et al., 2004). Toxic ROS

can affect membrane permeability, cause damage to DNA and protein, induce lipid peroxidation, and ultimately lead to programmed cell death. Zhen et al. (2003) found that, when strawberry root exudates accumulated in their growing medium, the growth and metabolism of roots were inhibited, which resulted in an increase in the percentages of electrolytes in cells, a decrease in the free radical scavenging activity of roots. Supply of mineral nutrient alternatively other than by root uptake can sustain plant growth during this allelochemical stress. The availability and uptake of nitrogen is considered as the major factor affecting growth which can be sprayed on the leaves as a source of nutrients (Lea and Azevedo, 2006). Use of foliarly applied urea as a nitrogen source is common (Bowman and Paul, 1992; Vasilas et al., 1980). For example, in wheat, foliarly applied urea produced positive effects; these were attributed to higher leaf photosynthetic rates and higher leaf urease enzyme activities (Peltonen, 1993).

Amino acids are the nitrogenous compound which forms the basic component of all living cells. It can absorb by leaf exogenously (Furuya and Umemiya, 2002). Therefore, it has a great potentiality of using under managed culture techniques. Recently they are used as foliar spray to improve the growth, yield and quality of crops (Mazher et al., 2011; Takeuchi et al., 2008). Several researchers found positive impacts of amino acids as foliar spray under stress condition for example Proline to wheat (Rajagopal and Sinha, 1980), Proline, Alanine, Serine, and Asparagine to maize (Thakur and Rai, 1985) under osmotic stress and Proline, Phenylalanine to maize and board bean under salinity stress (Abd El-Samad et al., 2011). As the accumulated allelochemicals in closed culture become stressful to plant, spraying of amino acid to strawberry plants would be positive. So far, spraying amino acids in recovering strawberry plant growth during autotoxicity

has not been studied. Therefore, the purpose of the present study was to evaluate the performance of amino acids on the recovery of growth and yield of strawberry plants under autotoxicity in closed hydroponic culture.

2. Materials and Methods

2.1. Culture of strawberry plants in container based hydroponics

Strawberry (*Fragaria* × *ananassa* Duch. cv. Toyonoka) plantlets reproduced through plant tissue culture were used for this experiment. The study was conducted in 100 m^2 glasshouse of Experimental Research Center at Biological Resources Science, Shimane University. Initially strawberry plantlets at four to five leaves stage were transplanted to plastic container (20 × 54 × 34 cm) with 55 L of 25% Enshi nutrient solution (pH 7.25 and EC 0.8 dS m^{-1}) (Fig. 13 and 14). The full strength Enshi nutrient solution contains the following amount of salts per 1000 L of tap water: 950 g $Ca(NO_3)_2 \cdot 4H_2O$; 810 g of KNO_3; 500 g of $MgSO_4 \cdot 7H_2O$; 155 g of $NH_4H_2PO_4$; 3 g of H_3BO_3; 2 g of $ZnSO_4 \cdot 7H_2O$; 2 g of $MnSO_4 \cdot 4H_2O$; 0.05 g of $CuSO_4 \cdot 5H_2O$; 0.0 2g of Na_2MoO_4; 25 g of NaFe-EDTA (Hori, 1966). Five plantlets were planted in each container in such a way that the roots were inserted into the nutrient solution inside the container keeping shoot outside. Urethane foam block (23 mm × 23 mm × 27 mm) was used for holding the plant tight with a floating board on the nutrient solution. Nutrient solutions were circulated 24 h by pumps (KP-101, Koshin, Kyoto, Japan) with automatic timer (KS-1500, Iuchi, Osaka, Japan) which were either renewed or non-renewed entirely and non-renewed with amino acids and urea application. Renewed culture solutions were changed biweekly with new nutrient solutions and non-renewed nutrient solutions were analyzed for major nutrients and adjusted as close as possible to initial concentrations at every two weeks on the

basis of chemical analyses with Compact NO_3^- meter (B-343, Horiba, Ltd. Kyoto, Japan) for NO_3^-, Spectrophotometer (U-2900, Hitachi, Tokyo, Japan) for PO_4^{3-} and Polarized Zeeman Atomic Absorption Spectrophotometer (Z-2310, Hitachi, Tokyo, Japan) for K^+, Ca^{2+}, Mg^{2+} and Fe^{3+}. Two day after transplanting, twenty two water soluble amino acids viz., Alanine (Ala), Arginine (Arg), Asparagine (Asn), Aspartic acid (Asp), Cysteine (Cys), Glutamatic acid (Glu), Glutamine (Gln), Glycine (Gly), Hydroxy-proline (Hyp), Lysine (Lys), Ornithine (Orn), Proline (Pro), Serine (Ser), Threonine (Thr), Tryptophan (Typ), Methionine (Met), Leucine (Leu), Isoleucine (Ile), Citrulline (Cit), Histidine (His), Phenylalanine (Phe), and Valine (Val) and urea were individually sprayed as foliar application at 2 mL per plant by a 500 mL sprayer three times a week on the strawberry plants grown in non-renewed nutrient solution. The concentrations of urea and amino acids were adjusted to nitrogen content of Pro at 200 mg L^{-1} to maintain the same concentration level. The dates of anthesis were recorded for each plant to check whether any influence of amino acids on flowering of strawberry among the treatments. Pollination was aided by a soft brush at two days intervals. Fruits were harvested when those became about 80% red in colour. At each harvest fresh weight of fruits were recorded and gathered for final yield calculation. At final harvest, leaf number, leaf length and width, root length, crown diameter, fresh weight of leaf, crown and inflorescence were recorded. Then strawberry plant parts were separated into leaf, crown, inflorescence and root and dried in a constant temperature oven (DKN 812, Yamato Scientific Co., Ltd. Japan) for 72 h at 80 °C. When the dry matter reaches constant weight, dry weight of different plant parts was measured.

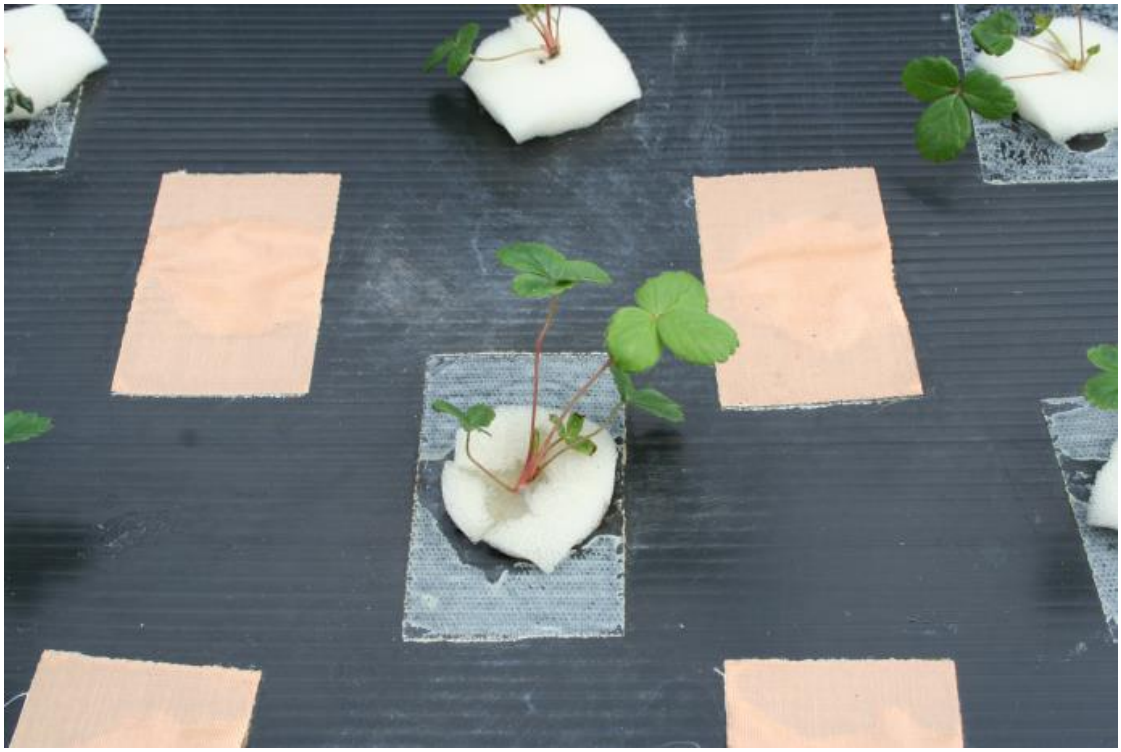

Fig. 13. Four to five leaves stage strawberry plantlets supported by urethane blocks grown in container based closed hydroponic system.

Fig. 14. Strawberry plants grown in container based hydroponic system.

2.2. Culture of strawberry plants in Wagner’s pot based hydroponics

Seven amino acids were selected for their better growth and yield performance in the container based hydroponics. These short listed amino acids were further investigated in the glasshouse following Wagner's pot hydroponics using strawberry cultivar 'Toyonoka'. Healthy plantlets obtained through micro propagation with five to six leaves were planted into Wagner’s pot (ten plants in one line) connected with a plastic reservoir (63 × 48 × 22 cm) containing 60 L of 25% Enshi nutrient solution in closed hydroponic system (Fig. 15). The system includes main inlet pipes (15 mm diameter) for supply and drainage of nutrient solution between reservoir and pots, Wagner's pot (1/5000a, NF-5, AsOne, Osaka, Japan) with 3 L capacity for planting, inlet tubes (4 mm diameter) to supply solution to the pots, and 60 L capacity nutrient solution container with a pump (KP-101, Koshin, Kyoto, Japan). The culture solution was not renewed during the entire growth period and it was recycled through the pipes for 5 min at 10 min intervals using an automatic pump timer (KS-1500, Iuchi, Osaka, Japan). One month after transplanting, the selected amino acids viz., Ala, Glu, Hyp, Thr, His, Phe, including g*amma*-aminobutyric acid (GABA) and water as control were sprayed on leaves of strawberry plants. The concentrations of all amino acids were adjusted to nitrogen content of Pro at 200 mg L^{-1}. Water and amino acids were applied at 1.4 mL per plant by a 100 mL sprayer three times a week. The major nutrients in the non-renewed culture solution were analyzed and adjusted following methods and instruments used in container based hydroponics at every two weeks. Other cultural practices were done as described in the previous culture. Fruits were harvested when those became about 80% red in colour. The harvested fruits were grouped into three stages based on their harvesting date and gathered for final yield calculation. The relative amount of

chlorophyll in strawberry leaves were measured (SPAD-502 plus, Konica Minolta Sensing, Inc. Osaka, Japan) at final harvest. Growth and yield of strawberry plants were measured following the methods as described in the previous culture.

Fig. 15. Wagner's pot based hydroponics system for strawberry culture in greenhouse.

2.3. Determination of fruit qualities of strawberry

After harvest fruits were composited and were frozen at –30 °C for subsequent analysis of soluble solids, titratable acid and ascorbic acid content. Fruit samples were kept out of freezer before analysis to obtain juice for determining the above qualities of strawberry fruits. The soluble solid content of fruit collected from container based culture was determined using a digital refract meter (AsOne, SpittzIPR-101α, Osaka, Japan) whereas, fruits of Wagner's pot culture was determined using a pocket digital refractometer (PAL-1, Atago Ltd., Japan). Titratable acid contents were determined by diluting each 2 mL aliquot of strawberry juice to 10 mL with 8 mL distilled water and added 2–3 drops of phenolphthalein then adjusted the pH to 8.2 using 0.1 N NaOH.

Then the titratable acid was converted into % citric acid. The ascorbic acid content was measured with 2,4-dinitrophenylhydrazine (DNP) colorimetry. Strawberry fruit juice (0.5 mL) were taken in 50 mL test tube then 0.5 mL of 10% meta-phosphoric acid solution, 1 mL of distilled water, 1 mL of 0.03% 2,6-dichlorophenol-indophenol (DCP), 2 mL of thiourea, and 1 mL of 2,4-DNP was added to the samples following 3 h incubation at 37 °C in water bath (BW400, Yamato Scientific Co. Ltd. Japan). After incubation samples 5 mL of 85% H_2SO_4 were added keeping in water cooled with iced water. After 30 min cooling ascorbic acid content was measured at 520 nm by spectrophotometer (U-2900, Hitachi High Technologies Corporation, Tokyo, Japan).

2.4. Culture of strawberry plantlets under *in vitro* condition

In order to control the effects of environmental factors and also microbial degradation of amino acid, strawberry plantlets were cultured under *in vitro* condition at Plant Factory supported Research Laboratory of Shimane University. Strawberry cv. Toyonoka plantlets of similar vigor were transferred into a culture box (100 ×110 ×100 mm) with 100 mL substrate (Fig. 16). The plant boxes were capped with bio-filter (for aeration) and placed in growth chamber at 20/15 ºC (day/night) under florescent light with intensity of 74-81 μmol m^{-2} $s^{-1}$and 12 h photoperiod. The substrates were prepared using 25% Enshi nutrients solution (EC 0.8 dS m^{-1}) with agar (9 g L^{-1}) as solidified agent and sucrose (30 g L^{-1}) as carbon source. One plantlet was planted in each plant box and three plant boxes were used for each treatment with three replications. The seven amino acids (Ala, Glu, Hyp, Thr, His, Phe and GABA) used in the Wagner's pot hydroponics were also used in this experiment with water, urea and renew of substrate as control. In case of renew of substrate, it was changed to new substrate biweekly and

water, urea, amino acids were sprayed on the strawberry leaves at 3.0 mL per plant using 0.45 µm syringe filter (Toyo Roshi kaisha, Ltd. Japan) inside the clean bench. The concentrations of urea and amino acids used were adjusted to nitrogen content of Pro at 200 mg L^{-1}. After eight weeks, growth variables and relative amount of chlorophyll content in strawberry plants were measured and compared among the treatments.

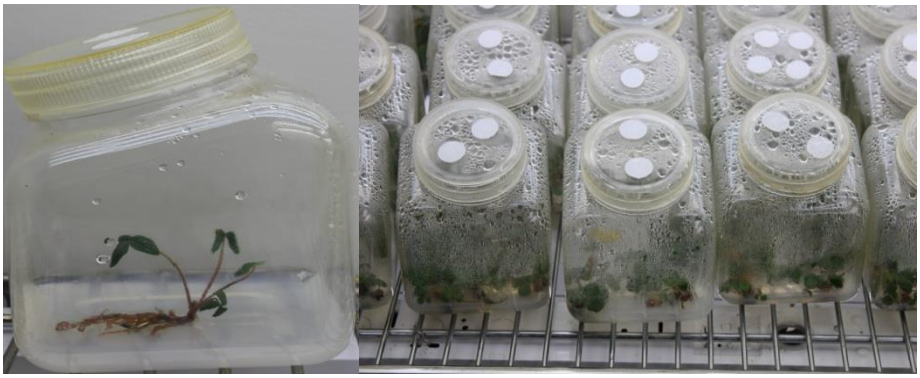

Fig. 16. *In vitro* culture of strawberry plantlets into the plant box.

2.5. Determination of nutrient contents of *in vitro* cultured strawberry plantlets

Strawberry plant parts were separated into leaves, stem and root sand kept in a constant temperature oven (DKN 812, Yamato Scientific Co. Ltd., Japan) for at least 72 h at 80◦C. When the dry matter reached the constant weight, it was ground into powder with a mixer machine (National MX-X53, Japan). Samples weighing 0.5 g were mixed with 8 ml of HNO3 and digested by microwave sample preparation system (ETHOS1, Milestone S.r.l, Bergamo, Italy). After digestion, samples were measured up to 50 ml of volumetric flask and then filtered with qualitative filter paper (Grade no. 131, Toyo Roshi Co. Ltd, Tokyo, Japan). The filtered sample solutions were analyzed for K, Ca, Mg, Fe and Na by Zeeman Atomic Absorption Spectrophotometer (Z-2310, Hitachi, Tokyo, Japan).

2.6. Statistical analysis

A randomized complete block design with three replicates was used for both culture of strawberry in container based hydroponics and Wagner's pot hydroponics in the greenhouse whereas, complete block design was performed in culture of strawberry plantlets *in vitro* condition. Analysis of variance was performed to test for statistical differences among the treatments and mean separations were performed by Tukey's Honestly Significant Difference (HSD) and Least Significant Difference (LSD) test at $P < 0.05$ level of significance by MSTATC statistical software.

3. Results

3.1.1. Evaluation of twenty two amino acids on the growth of strawberry plants under autotoxicity in container based hydroponics

Foliar application of twenty two amino acids showed significant influence on the growth of the strawberry plants grown in non-renewed culture solution in hydroponics (Fig. 17 and Table 6). Plants grown in renewed nutrient solution produced bigger leaves compared to plants grown in non-renewed nutrient solution. Leaf size was not significantly increased by foliar spray with Arg, Asp, Gln, Gly, Orn, Pro, Ser, Met, Leu, Ile, Cit and Val. Spray of Ala, Cys, Glu, Hyp, Lys, Thr, Trp, His and Phe on the leaves of strawberry plants grown in non-renewed nutrient solution increased leaf length and width compared with water as control. Number of leaves did not differ significantly in plants grown in non-renewed nutrient solution with or without supplementation of amino acids. Longer roots were recorded in plants sprayed with urea, Ala, Cys, Lys, Trp and grown in renewed nutrient solution compared to other amino acids. Smaller crowns were found in Gly, Pro, Ser, Met, Leu and Val sprayed plants compared to plants grown in renewed culture solution. Higher leaf fresh weight was measured in Cys, Glu, Trp and Phe treated plants compared to plants grown in non-renewed solution. Crown fresh weight was increased in plants sprayed with urea, Ala, Cys, Glu, Gln, Hyp, Lys, Thr, Trp, Cit, His, Phe and Val whereas, fresh weight of flowering bud was increased in plants sprayed with Cys, Glu, Hyp, Lys, Thr, Trp, His and Phe. Among the amino acids applied Cys, Glu, Lys, Trp, His and Phe supplemented plants produced higher dry weight in leaves, crown, inflorescence and root compared to plants in non-renewed nutrient without amino acid application (Fig. 18). Ala, Hyp and Thr also produced higher dry matter in all parts except leaves. From above results it is evident that growth

variables were reduced when strawberry plants were grown in non-renewed nutrient solutions compared to renewed solution but these were improved in plants grown in non-renewed nutrient solution with the supplementation of Ala, Cys, Glu, Hyp, Lys, Thr, Trp, His and Phe.

Fig. 17. Effects of different amino acids on the growth of strawberry plants in container based closed hydroponic system.

Table 6. Effect of twenty two amino acids on the growth of strawberry plants grown by non-renewed nutrient solution in closed hydroponic system.

Amino Acids[z]	Number of leaves[y]	Leaf length (cm)	Leaf width (cm)	Root length (mm)	Crown diameter (mm)	FW of leaves (g)	FW of crown (g)	FW of flowering bud (g)
RW	42.4 ab[x]	37.4 a	21.7 a	372.5 ab	49.5 a	194.6 a	11.7 a	14.6 a
NRW	24.0 ab	17.0 c	13.3 bc	283.7 bc	39.8 ab	26.3 c	4.5 b	5.1 bc
Urea	28.1 ab	27.0 b	18.7 ab	401.5 a	39.5 ab	73.9 bc	8.7 ab	6.6 bc
Ala	38.4 ab	29.5 ab	20.2 ab	334.0 ab	36.5 ab	121.2 bc	9.1 ab	8.9 b
Arg	33.0 ab	17.9 c	12.9 bc	234.5 c	38.0 ab	36.0 c	5.4 b	6.4 bc
Asn	30.0 ab	26.3 b	17.9 ab	297.5 bc	35.5 ab	56.5 c	4.5 b	6.7 bc
Asp	33.6 ab	25.4 bc	18.1 ab	281.9 bc	32.1 ab	73.9 bc	6.1 b	8.2 bc
Cys	40.2 ab	33.3 ab	20.3 ab	335.0 ab	41.2 ab	147.2 ab	9.5 ab	10.9 ab
Glu	37.8 ab	33.2 ab	21.5 ab	314.0 b	38.5 ab	146.7 ab	9.2 ab	12.8 ab
Gln	25.3 ab	25.5 bc	17.0 b	320.5 b	34.6 ab	56.7 c	6.9 ab	6.3 bc
Gly	23.4 ab	15.8 c	12.5 bc	282.3 bc	29.7 b	28.2 c	5.8 b	3.3 bc
Hyp	36.0 ab	30.6 ab	19.5 ab	317.5 b	41.5 ab	105.7 bc	8.7 ab	11.1 ab
Lys	34.8 ab	33.3 ab	21.6 ab	336.0 ab	43.5 ab	128.3 b	9.0 ab	11.5 ab
Orn	24.5 ab	23.7 bc	15.4 bc	306.5 b	39.8 ab	40.1 c	5.4 b	4.8 bc
Pro	23.6 b	20.1 bc	15.3 bc	300.5 bc	23.9 b	39.5 c	4.3 b	4.0 bc
Ser	25.8 ab	19.8 bc	16.3 bc	292.1 bc	23.5 b	48.6 c	5.6 b	5.9 bc
Thr	34.9 ab	32.0 ab	20.5 ab	304.5 b	43.5 ab	100.8 bc	8.9 ab	9.9 ab
Trp	43.7 a	35.1 ab	21.4 ab	374.5 ab	47.0 ab	139.5 ab	11.4 ab	10.9 ab
Met	28.2 ab	19.0 bc	13.7 bc	293.0 bc	28.6 b	32.5 c	4.3 b	3.5 bc
Leu	31.2 ab	10.1 c	11.8 c	276.3 bc	27.5 b	29.4 c	3.6 b	2.6 c
Ile	32.9 b	21.2 bc	14.1 bc	297.0 bc	36.0 ab	49.5 c	5.0 b	5.7 bc
Cit	22.7 ab	15.8 c	13.7 bc	283.5 bc	33.0 ab	23.1 c	6.6 ab	3.9 bc
His	39.5 ab	32.3 ab	21.0 ab	319.0 b	36.6 ab	123.4 b	11.1 ab	10.4 ab
Phe	41.4 ab	33.4 ab	21.8 a	329.0 b	39.3 ab	145.3 ab	9.2 ab	11.4 ab
Val	27.4 ab	20.1 bc	14.6 bc	279.5 bc	28.8 b	37.3 c	7.8 ab	4.8 bc
	*	*	*	*	*	*	*	*

[z]Strawberry plants grown in renewed (RW), non-renewed (NRW), and non-renewed nutrient solution with amino acids and urea application.
[y]Parameters were measured on per plant basis; Fresh weight (FW), Dry weight (DW).
[x] Means within column followed by different letters are significant according to Tukey's test at $P < 0.05$ ($n = 15$).
[*] Significant at $P < 0.05$.

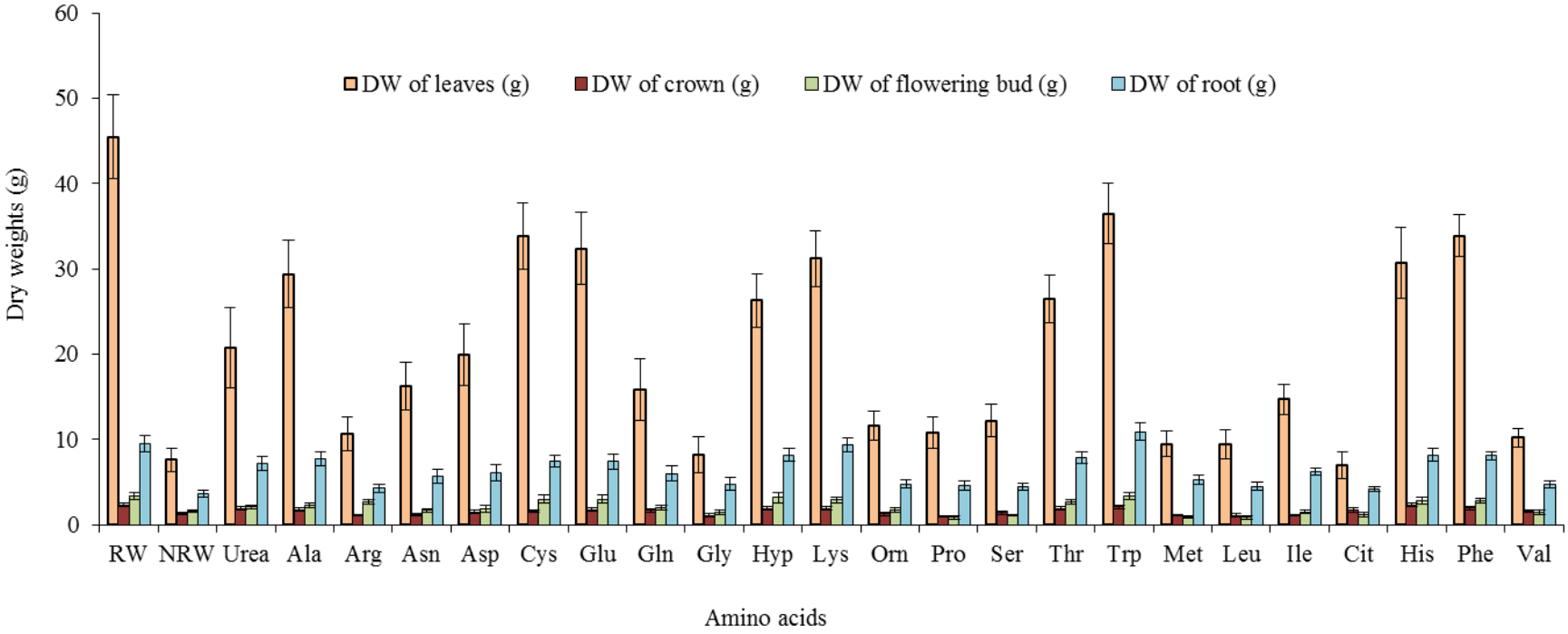

Fig. 18. Effects of twenty two amino acids on the dry matter production of strawberry plants grown in non-renewed nutrient solution in closed hydroponic system. RW: Renewed, NRW: Non-renewed, amino acids are presented as their three letters abbreviation.

3.1.2. Evaluation of twenty two amino acids on the fruit yield and quality of strawberry plants under autotoxicity in container based hydroponics

Amino acids lead a positive effect on the yield attributes and fruit quality of strawberry grown in container based hydroponics (Fig. 19, 20, 21; Table 7). Anthesis date was influenced by the application of amino acids on strawberry plant leaves and it was found that about 23 days earlier flowering in Ala, Arg, Asn and Phe sprayed plants. Fruit yield per plant was decreased about 74% in plants grown in non-renewed nutrient solution against the plants grown in renewed nutrient solution. Application of urea, Ala, Asn, Asp, Cys, Glu, Gln, Hyp, Lys, Orn, Thr, Trp, His, Phe and and Val improved fruit yield in plants grown in non-renewed nutrient solution which was attributed by number of flowers and number of mature fruits. Average fruit weight also correspond the yield in these amino acid supplemented plants. There were no significant differences among the amino acid applied in terms of strawberry fruit qualities such as soluble solids, citric acidity and ascorbic acid.

Fig. 19. Strawberry fruits harvested from container based hydroponic system.

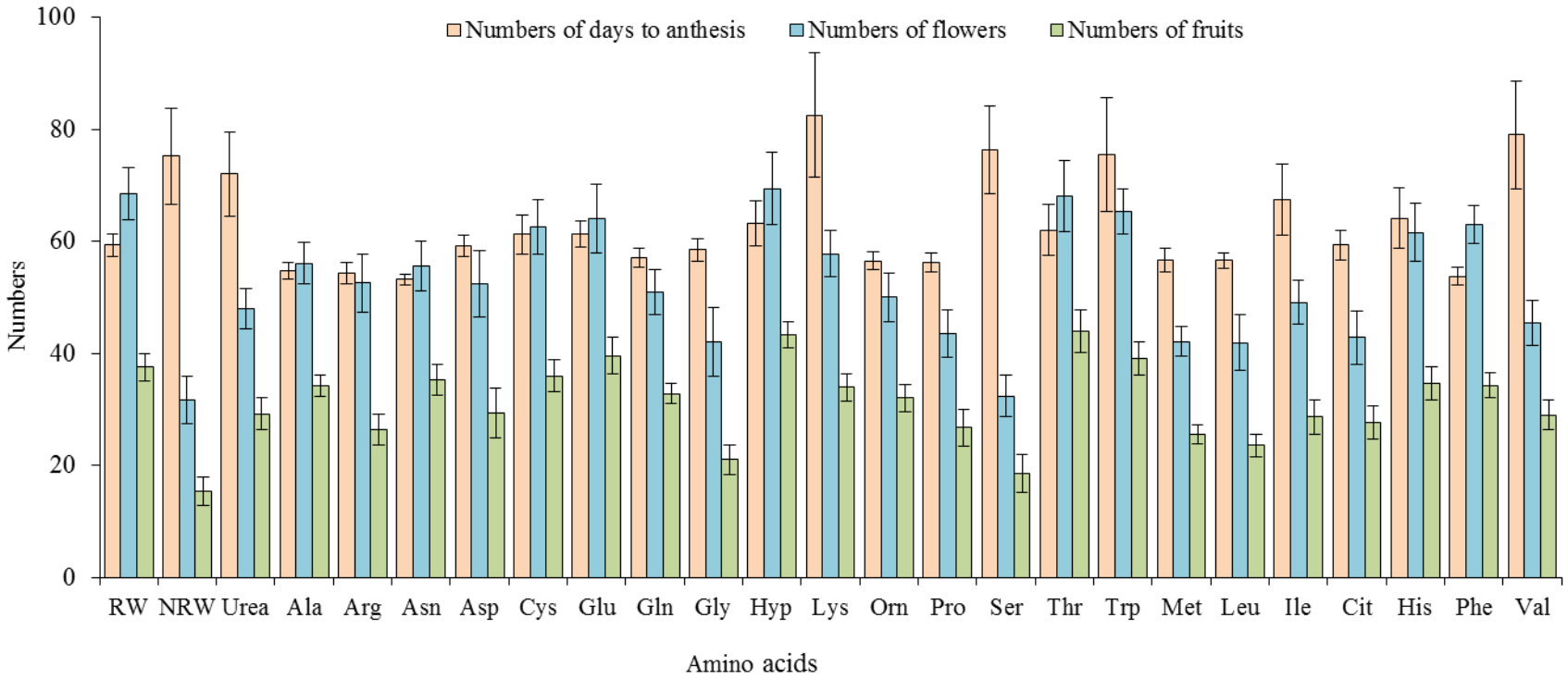

Fig. 20. Effect of twenty two amino acids on the flowering and fruit setting of strawberry plants grown in non-renewed nutrient solution in closed hydroponic system. RW: Renewed, NRW: Non-renewed, amino acids are presented as their three letters abbreviation.

Fig. 21. Effect of twenty two amino acids on the yield of strawberry plants grown in non-renewed nutrient solution in closed hydroponic system. RW: Renewed, NRW: Non-renewed, amino acids are presented as their three letters abbreviation.

Table 7. Effect of twenty two amino acids on fruit quality of strawberry plants grown by non-renewed nutrient solution in closed hydroponic system.

Amino acids[z]	Soluble solids[y] (%)	Citric acidity (%)	Ascorbic acid (mg/100g)
RW	7.5	0.42	61.3
NRW	5.8	0.38	69.3
Urea	7.2	0.45	55.9
Ala	7.6	0.45	57.3
Arg	8.3	0.45	54.2
Asn	7.8	0.48	55.1
Asp	6.5	0.42	54.5
Cys	6.7	0.38	74.1
Glu	7.2	0.38	60.8
Gln	7.2	0.42	64.1
Gly	7.0	0.35	57.5
Hyp	6.9	0.35	55.4
Lys	7.1	0.26	55.0
Orn	6.0	0.38	58.3
Pro	6.4	0.32	50.1
Ser	7.8	0.35	44.5
Thr	7.5	0.32	50.8
Trp	8.0	0.35	58.4
Met	7.1	0.42	76.3
Leu	7.8	0.42	64.6
Ile	8.0	0.54	74.9
Cit	7.3	0.48	60.9
His	7.4	0.35	78.6
Phe	7.5	0.42	69.3
Val	7.4	0.35	72.0
	ns	ns	ns

[z] Strawberry plants grown in renewed (RW), non-renewed (NRW), and non-renewed nutrient solution with amino acids and urea application.

[y] Parameters were measured on per plant basis.

[ns] Non-significant according to Tukey's test at $P < 0.05$ ($n = 15$).

3.2.1. Effects of selected seven amino acids on the growth of strawberry plants under autotoxicity in Wagner's pot based hydroponics

The effects of seven amino acids were investigated on the growth of strawberry plants grown in recycled culture solution in Wagner's pot closed hydroponic system. Result showed a significant difference in plants growth supplemented with amino acids (Table 8). Among the amino acids, Ala, Glu, Phe and GABA supplemented plants increased their leaf fresh weight by about 25, 22, 25, and 23%, against water spray as control, respectively whereas leaf dry weight was significantly increased only in Glu and His treated plants. There was no significant difference among the treatments in terms of number of leaves, relative amount of chlorophyll content and crown fresh weight. Bigger crown was found in Glu (22.0 mm), Hyp (21.8 mm) and GABA (18.9 mm) treated plants. Foliar spray of Hyp significantly increased the crown dry weight. Amino acids have influence on the root growth which was evidenced in Glu, Phe and Hyp supplemented plants where these amino acids increased by 33, 41 and 59% dry weight of root, respectively compared with control.

Table 8. Effect of seven amino acids on the growth of strawberry plants grown by non-renewed nutrient solution in Wagner's pot based hydroponic system.

Amino acids[a]	No. of leaves[b]	SPAD	FW of leaves (g)	FW of crown (g)	Crown diameter (mm)	Root length (mm)	DW of leaves (g)	DW of crown (g)	DW of root (g)
Water	72.6	38.8	223.2 b[c]	38.9	15.9 d	32.6 ab	51.3 b	7.5 b	10.5 c
Ala	91.3	38.7	278.1 a	44.0	17.0 cd	30.2 ab	63.0 ab	8.5 ab	12.0 bc
Glu	84.5	38.4	272.8 a	45.9	22.0 a	32.5 ab	67.9 a	10.2 ab	14.0 ab
Hyp	87.8	39.5	269.6 ab	49.8	21.8 ab	32.0 ab	64.5 ab	11.3 a	16.7 a
Thr	60.2	38.5	256.1 ab	49.0	17.4 cd	28.4 b	63.4 ab	8.8 ab	11.8 bc
His	88.5	38.4	268.1 ab	47.6	16.5 cd	32.0 ab	66.9 a	8.2 b	12.2 bc
Phe	94.1	39.2	279.0 a	48.2	18.3 cd	30.5 ab	64.2 ab	10.2 ab	14.8 ab
GABA	94.8	39.2	274.0 a	49.2	18.9 bc	34.3 a	63.9 ab	9.8 ab	13.5 abc
	ns	ns	*	ns	*	*	*	*	*

[a] Strawberry plants grown in non-renewed nutrient solution with amino acids application.

[b] Parameters were measured on per plant basis; Fresh weight (FW), Dry weight (DW).

[c] Means within column followed by different letters are significant according to LSD test at $P < 0.05$ ($n = 15$).

[ns, *] Non-significant or significant at $P < 0.05$, respectively.

3.2.2. Effects of seven amino acids on the fruit yield and quality of strawberry plants under autotoxicity in Wagner's pot based hydroponics

Application of amino acids greatly influenced the yield in strawberry plants following recycled Wagner's pot hydroponics (Table 9). Ala, His, Thr, Hyp and Glu treated plants increased fruit yield in 30, 38, 40, 50 and 51% in comparison to control. In these treatments the highest numbers of fruits were recorded. Spraying of Phe and GABA did not influenced on the fruit yield of strawberry. Greater numbers of fruits were recorded from Ala, Thr, His, Glu and Hyp treated plants. Average fruit weight was not significantly improved by the amino acids under investigation. Application of amino acids did not left any effects on the soluble solid content at different stages of harvested strawberry fruits, however, citric acid content (%) in fruits significantly varied in all three stages (Table 10). In the stage I, Glu, Hyp and GABA treated strawberry plants produced fruits with high citric acid content whereas in the stage II it was higher in Glu and GABA supplemented plants fruits. In the stage III, the higher citric acid levels were found in fruits with, Ala, Hyp and GABA application. Although ascorbic acid content in the strawberry fruits was varied in the early harvested fruits but in the mid and later harvested fruits did not differ it significantly.

Table 9. Effect of seven amino acids on the fruit yield of strawberry in Wagner's pot based hydroponic system.

Amino acids[z]	Number of fruits[y]	Average fruit weight (g)	Fruit yield (g)
Water	63.3 c[x]	6.7	432.2 d[z]
Ala	88.2 ab	6.8	560.2 abc
Glu	90.4 ab	7.3	654.2 a
Hyp	97.5 a	6.8	649.8 a
Thr	90.5 ab	6.7	606.2 ab
His	89.2 ab	7.0	594.7 ab
Phe	77.3 bc	6.9	474.1 cd
GABA	72.8 bc	7.3	498.2 bcd
	**	ns	**

[z] Strawberry plants grown in non-renewed nutrient solution with amino acids application.

[y] Parameters were measured on per plant basis; Fresh weight (FW), Dry weight (DW).

[x] Means within column followed by different letters are significant according to LSD test at $P < 0.05$ ($n = 15$).

[ns, **] Non-significant or significant at $P < 0.01$, respectively.

Table 10. Soluble solid content, citric acidity and ascorbic acid content in fruits of strawberry plants supplemented with amino acids in Wagner's pot based hydroponic system. Fruits harvested at stage I (2011/3/25–2011/4/30), stage II (2011/5/1–2011/5/25) and stage III (2011/5/26–2011/6/20).

Amino acids[z]	Soluble solid content (%)			Citric acidity (%)			Ascorbic acid (mg/100g)		
	Stage I	Stage II	Stage III	Stage I	Stage II	Stage III	Stage I	Stage II	Stage III
Water	7.6	7.3	7.9	0.22 b[y]	0.22 c	0.26 b	42.1 ab	41.0	40.3
Ala	6.7	7.1	7.0	0.35 ab	0.26 bc	0.45 a	45.6 a	39.6	39.3
Glu	7.9	7.3	7.1	0.51 a	0.38 ab	0.35 ab	41.6 ab	38.0	36.2
Hyp	6.8	5.8	7.3	0.45 a	0.35 abc	0.41 a	40.9 bc	38.4	35.7
Thr	6.0	5.6	6.8	0.38 ab	0.32 abc	0.35 ab	37.8 bc	41.2	38.4
His	7.4	7.3	7.2	0.38 ab	0.29 bc	0.22 b	40.9 bc	42.6	38.6
Phe	6.8	6.8	6.7	0.38 ab	0.29 bc	0.35 ab	37.0 c	41.2	37.4
GABA	7.4	6.0	7.8	0.58 a	0.45 a	0.45 a	41.7 ab	38.8	40.7
	ns	ns	ns	**	**	**	*	ns	ns

[z] Strawberry plants grown in non-renewed nutrient solution with amino acids application.

[y] Means within column followed by different letters are significant according to LSD test at $P < 0.05$ ($n = 15$).

[ns, *, **] Non-significant or significant at $P < 0.05$, 0.01%, respectively.

3.3.1. Effects of seven amino acids on the growth of strawberry plantlets under *in vitro* condition

Growth of strawberry plantlets was evaluated under *in vitro* condition with the supplementation of seven amino acids (Fig. 22 Table 11). Leaf number was increased in amino acids supplied plantlets compared to water control. Higher numbers of leaves were counted in plantlets cultured in renewed substrates as control. Urea, Ala, Glu, Phe and GABA treated plantlets increased their relative amount of chlorophyll content against water control. The longest leaf was found in renewed substrate plantlets and all the amino acids supplied plantlets increased their leaf length over water control. Compared with water and urea, Thr improved the leaf width. Ala, Glu, Hyp and GABA treated plantlets resulted in longer roots against water and urea while the longest root was found in renewed substrate plantlets. The results showed that only Hyp application improved the crown diameter. Treated with Hyp produced bigger crown compared to other amino acids. Higher fresh weight of leaf was obtained when plantlets were sprayed by Glu and Hyp as compared to water spray as control. Hyp treated plantlets increased their crown and root fresh weight against water control. All the amino acids treated plantlets improved their root fresh weights against water control. Hyp treated plantlets also produced significantly higher root fresh weight than plantlets grown in renewed substrates. In case of leaf dry weight, Hyp and urea treated plantlets gained higher weight which are similar. Crown dry weight did not showed any significant difference among the amino acid treatments. Under *in vitro* condition strawberry plantlets improved the root dry matter against water and renewed control. Highest root dry weight was found in Glu treated plant and other amino acids such as Ala, Thr, Hyp and GABA; as well as urea also improved.

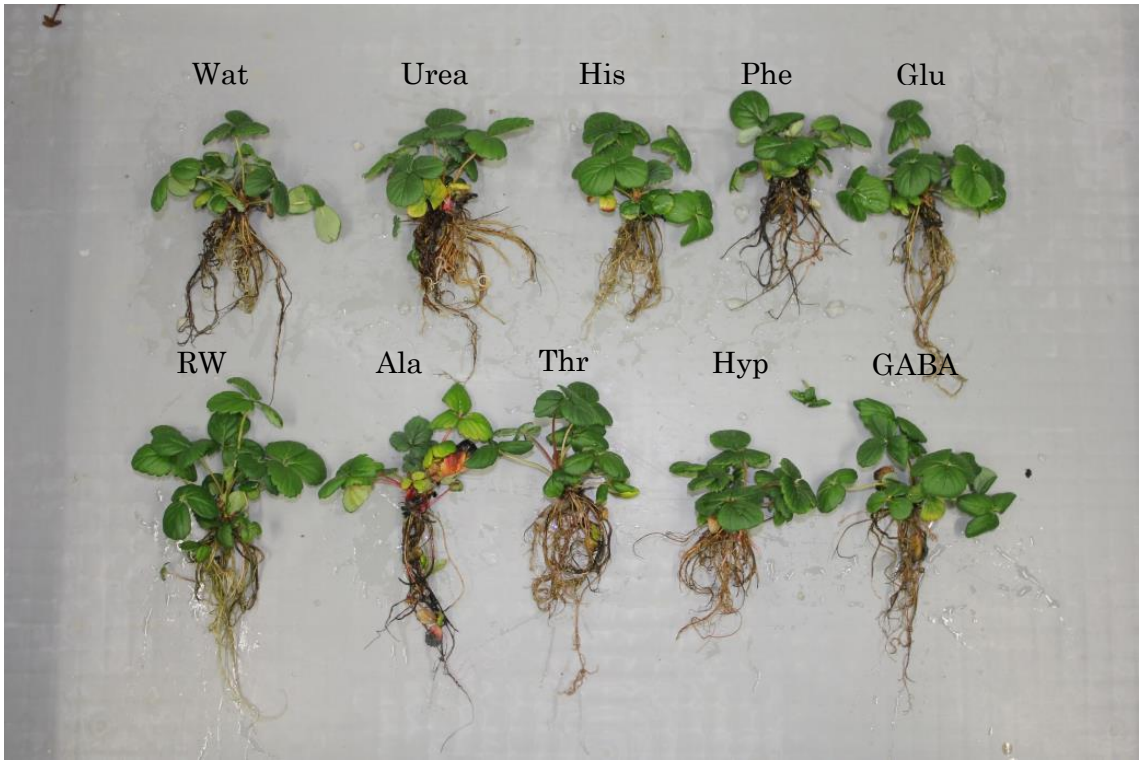

Fig. 22. Amino acids treated 8 weeks *in vitro* strawberry plantlets (Wat=water as control, RW= Renewed substrate treated plantlets).

Table 11. Effect of seven amino acids on the growth of strawberry plantlets under *in vitro* condition.

Amino acids[z]	Number of leaves[y]	SPAD	Leaf length (mm)	Leaf width (mm)	Root length (mm)	Crown diameter (mm)	FW of leaves (mg)	FW of crown (mg)	FW of root (mg)	DW of leaves (mg)	DW of crown (mg)	DW of root (mg)
RW[x]	12.6 a[c]	40.7 bc	58.2 a	45.8 a	181.2 a	3.5 b	1480.0 a	154.0 b	962.0 c	284.0 a	28.0	102.0 c
Water	6.9 c	42.7 c	43.2 c	37.8 c	81.4 e	3.4 b	967.8 c	125.6 b	691.1 d	201.1 c	27.8	97.8 c
Urea	9.3 b	48.0 ab	47.6 b	38.0 c	86.0 de	3.8 b	1185.6 bc	114.4 b	1470.0 a	270.0 ab	31.1	164.4 a
Ala	8.7 b	48.6 a	48.9 b	40.8 bc	116.8 bc	3.7 b	1049.2 bc	147.0 b	1126.7 bc	220.0 bc	30.0	153.3 ab
Glu	8.6 bc	48.1 ab	52.3 ab	41.5 bc	130.1 b	3.9 b	1194.7 b	169.7 b	1112.8 bc	228.3 bc	34.4	167.8 a
Hyp	9.6 b	44.7 bc	46.9 b	41.0 bc	114.3 bc	4.7 a	1218.3 b	255.0 a	1304.2 ab	267.5 ab	39.2	150.8 ab
Thr	8.9 b	44.2 bc	47.3 b	42.4 ab	103.3 cd	3.8 b	1048.0 bc	142.9 b	1137.5 bc	220.0 bc	29.2	154.2 ab
His	9.3 b	46.4 bc	43.9 b	37.8 c	91.3 de	4.0 ab	1049.2 bc	140.0 b	1005.8 c	218.6 bc	35.0	124.2 bc
Phe	9.3 b	47.8 ab	45.2 b	38.9 bc	101.1 cde	3.9 ab	1078.9 bc	142.2 b	1060.6 bc	224.8 bc	28.3	123.3 bc
GABA	9.4 b	46.8 ab	47.8 b	40.9 bc	122.2 bc	4.0 ab	1179.2 bc	175.8 ab	1228.3 abc	233.3 abc	32.5	155.8 ab
	*	*	*	*	*	*	*	*	*	*	ns	*

[z] Strawberry plants cultured in renewed (RW) and non-renewed substrate with amino acids and water application.

[y] Parameters were measured on per plant basis; Fresh weight (FW), Dry weight (DW).

[c] Means with column followed by different letters are significant according to Tukey's test at $P < 0.05$ ($n = 9$).

[ns, *] Non-significant or significant at $P < 0.05$, respectively.

3.3.2. Effects of seven amino acids on the nutrient contents of *in vitro* cultured strawberry plantlets

K, Ca, Mg and Fe contents of strawberry plant parts were determined. Compared to the control, amino acids applied plantlets increased the mineral contents in their different plant parts (Table 12). K contents of roots were increased when Glu was applied as foliar on the plantlets. Higher K, Ca, Mg and Fe contents were evidenced in the plantlets grown in renewed substrate. Compared to the control, Ca content increased in His treated crown and Glu, Hyp, Thr treated roots. Among the amino acids treated plantlets, higher level of Mg were measured in Hyp treated roots. Leaf Fe content was increased by the application of GABA.

Table 12. Effects of amino acids on the nutrient contents in the strawberry plantlets grown in *in vitro* condition

Amino acids[z]	K (mg/g)			Ca (mg/g)			Mg (mg/g)			Fe (mg/g)		
	Leaf[y]	Crown	Root	Leaf	Crown	Root	Leaf	Crown	Root	Leaf	Crown	Root
RW	521.5 a[x]	25.4 ab	43.6 ab	74.7 a	2.1 c	9.5 bc	55.0 a	1.1	9.7 ab	5.2 a	0.11	1.1 b
Water	218.3 b	22.4 ab	36.6 b	35.5 b	6.0 bc	18.5 b	25.1 b	3.3	6.6 b	2.4 b	1.59	6.5 a
Urea	179.7 b	25.1 ab	62.0 ab	47.8 ab	8.7 abc	32.1 ab	27.6 b	4.3	13.5 ab	2.5 ab	0.12	7.9 a
Ala	247.2 b	32.2 ab	62.4 ab	58.8 ab	13.2 ab	27.8 ab	31.1 b	6.0	10.6 ab	3.7 ab	0.52	5.8 ab
Glu	262.2 b	38.1 a	88.0 a	50.7 ab	13.2 ab	35.5 a	31.6 b	7.4	16.7 ab	3.4 ab	0.54	8.5 a
Hyp	245.2 b	15.8 ab	80.4 ab	62.9 ab	7.5 abc	41.7 a	29.5 b	3.7	20.0 a	3.3 ab	0.19	7.4 a
Thr	243.8 b	24.2 ab	83.8 ab	59.3 ab	13.3 ab	37.8 a	32.9 b	5.4	15.8 ab	3.7 ab	0.44	8.1 a
His	207.4 b	36.1 a	59.2 ab	53.6 ab	17.4 a	24.7 ab	25.7 b	7.3	11.2 ab	1.6 b	1.53	2.9 b
Phe	185.5 b	10.6 ab	40.2 ab	52.0 ab	7.2 abc	21.1 ab	27.7 b	2.4	9.9 ab	2.9 ab	0.13	5.2 ab
GABA	246.7 b	3.9 b	82.2 ab	62.8 ab	7.2 abc	34.6 ab	34.7b	3.0	20.3 a	5.0 a	0.21	7.3 a
	*	*	*	*	*	*	*	ns	*	*	ns	*

[z] Strawberry plants cultured in renewed (RW) and non-renewed substrate with amino acids and water application.
[y] Parameters were measured on per plant basis.
[x] Means with column followed by different letters are significant according to Tukey's test at $P < 0.05$ ($n = 9$).
[ns, *] Non-significant or significant at $P < 0.05$, respectively.

4. Discussion

When plants experiences autotoxicity, ion uptake and hydraulic conductivity (i.e., water uptake) are worse affected processes since root is the first organ to come into contact with autotoxins in the rhizosphere (Blum et al., 1999). Alternative means of supplying mineral nutrient other than absorption by roots can overcome this problem for sustainable growth and yield of strawberry. Studies on the effects of amino acids on the growth and yield of strawberry plants in closed hydroponics would be interesting. In container based hydroponics when twenty two amino acids were sprayed, dry weight of strawberry plants were increased (Fig. 18) which accord with the results of Nassar et al. (2003) and Amin et al. (2011) where foliar application of amino acids increased the dry weight of bean and onion plants respectively. As amino acids are the precursor of chlorophyll synthesis, it plays active role in dry matter production in plants (Yaronskaya et al., 2006). Moreover foliar application of amino acid increased plant protein content which ultimately increased the dry matter (Das et al., 2002). The regulatory effects of certain amino acids, like Phe and Orn, on plant development through their influence on gibberellins has been suggested by Waller and Nowacki (1978). Plants grown in non-renewed nutrient solution showed growth and yield declined but when plants were supplied with Hyp, it produced higher growth and fruit yield all three cultures. The possible reason might be its presence in the cell wall as Hyp-rich glycoproteins, is an extra-cellular structural protein of plant cell walls and extra-cellular matrix during normal development and in response to stress, autotoxicity in this case (Kieliszewski, 2001; Kieliszewski and Shpak, 2001). The higher fruit yield in amino acid supplemented plants than water sprayed plants were due to greater vegetative growth. This positive effect on growth and yield might be due to the assimilation and

metabolism of nitrogen in strawberry plants. For example, Glu is known to have a central role in nitrogen metabolism and is the preferential amino-donor for the different aminotransferase reactions for subsequent amino acid interconversions (Lea and Ireland, 1999). Therefore, greater fruit yield was contributed by vigorous growth, number of flowers, number of mature fruits per plant, and average fruit weight.

In Wagner's pot hydroponics, results revealed that the total dry weight (data not shown) was higher in plants sprayed with Glu, Hyp, and Phe than plants grown in non-renewed nutrient solution with water spray. This accord with Mazher et al. (2011) who reported foliar application of Glu increased the growth and the content of total carbohydrate, nitrogen, and phosphorus and potassium percentages of *Codiaeum variegatum* L. plant. In another study, spraying of Pro or Phe on maize and broad bean increased the amount of dry matter and water content (Abd El-Samad et al., 2011). Higher yield was recorded in Ala, Glu, Hyp, Thr, and His treated plants (Table 9) which were attributed by their better vegetative growth and higher numbers of mature fruits per plant. Moreover, amino acids might have some influence on the pollination and fruit setting. Hyp was found to be localized in growing tips in lily which could elongate the pollen tube enhancing the fertilization and fruit setting (Dashek and Harwood, 1974).

In vitro culture of strawberry plantlets was conducted to exclude the effects of environmental factors like temperature, light intensity, relative humidity and also microbial degradation of amino acids. Therefore, this experiment under control condition can confirm whether there or not any effects of amino acid on the strawberry plant growth in non-renewed nutrient condition. Total dry matter production was greater

in urea, Hyp, Glu and GABA treated plantlets compared to water as control (data not shown) which primarily due to meeting the nitrogenous demand from amino acid source. Recent studies found the positive impact of amino acids *in vitro* condition as organic source of nitrogen in alfalfa, maize, sorghum, pineapple, rice and sugarcane (Skokut et al., 1985; Claparols et al., 1993; Rao et al., 1995; Hamasaki et al., 2005; Grewel et al., 2006 and Asad et al., 2009).

In *in vitro* condition, strawberry plantlets were transferred biweekly in the new substrate from the old substrate. Amino acids were applied on the plantlets with old substrate. Renewed substrate treatment and amino acids application both significantly increased the mineral nutrient contents of strawberry plantlets against the water supply on the plantlets with old substrate (Table 12). Mineral uptake in plantlets grown in non-renewed substrate with water supply was inhibited by the accumulation of autotoxic chemicals (Kitazawa et al., 2005; Zhen et al., 2004). Asaduzzaman et al. (2012) found that Ca uptake in strawberry plants were inhibited by the when the plants were grown in the non-renewed nutrient solution. Glu increased the K contents in roots. Khanna (1998) showed that exogenous Pro promote K^+ uptake by 15% in *Raphanus* seedlings. Cuin and Shabala (2007) also found that 21 (of 26) amino acids caused a signicant mitigation of the NaCl-induced K^+ efflux, while valine and ornithine substantially enhanced the detrimental effects of salinity on K^+ homeostasis. Amino acids treatment increased the Ca content in His treated leaf and Glu, hyp and Thr treated plants. Rana and Rai (1996) found that exogenous application of amino acids increased the Ca contents in *Phaseolus* seedlings. Hyp and GABA increased the Mg contents, respectively. Amino acids have the capability to increase the mineral contents in strawberry plants during autotoxicity.

5. Summary

Amino acids application was investigated in recovering growth and yield of strawberry plants under autotoxicity developed in closed hydroponic systems. In greenhouse setting, a total of twenty two water soluble amino acids were sprayed on strawberry plants at 2 mL per plant three times a week. The concentrations of all amino acids were also adjusted to nitrogen content of Proline at 200 mg L^{-1}. It was found that growth and yield of strawberry plants grown in non-renewed nutrient solution was significantly reduced compared to plants grown in renewed nutrient solution. When plants were grown in non-renewed solution and sprayed Ala, Cys, Glu, Hyp, Lys, Thr, Trp, His and Phe, the growth improved whereas, yield were improved by spraying of Ala, Asn, Asp, Cys, Glu, Gln, Hyp, Lys, Orn, Thr, Trp, His, Phe and Val. Based on growth and yield performance, Ala, Glu, Hyp, Thr, His and Phe were selected for further investigation along with GABA following Wagner's pot based hydroponic system and also *in vitro* condition. Glu and Hyp sprayed plants produced about 50% greater fruit yield compared to water spray as control in Wagner's pot hydroponic system.

Effects of amino acids on strawberry plant growth improvement during autotoxicity were confirmed following *in vitro* culture. Results showed that, leaf dry weight of Hyp treated plants and root dry weight of Ala, Glu, Hyp, Thr and GABA treated plants were improved against control. When the mineral nutrient contents were analyzed from the dry matter of strawberry plant parts, it revealed that amino acids especially Glu and Hyp improved the K, Ca and Mg contents. Therefore, foliar spray of Glu and Hyp on strawberry plants can recover the growth and yield during autotoxicity in closed hydroponic system.

CHAPTER 4

GENERAL SUMMARY

Autotoxicity is an intraspecific allelopathy when a plant releases toxic chemical substances into the environment that inhibit germination and growth of same plant species. It has been reported to occur in a wide number of plant species causing serious autotoxic problems. Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn] and strawberry (*Fragaria* × *ananassa* Duch.) is grown in closed hydroponic culture. Under such conditions plant's roots are able to release allelochemicals mainly benzoic acid causing damage to the root cells, which in turns hamper water and mineral nutrient absorption. As a result, the growth and yield are reduced greatly. Supply of nutrients alternatively other than root uptake, can sustain plant growth during this allelochemical stress. Recently, amino acids have been used as foliar application on many plant species in order to improve the growth and yield during different abiotic and biotic stresses. In this study, foliar application of amino acids was investigated for the recovery of plant growth and yield of lisianthus and strawberry under autotoxicity developed in the closed hydroponic system.

For lisianthus and strawberry, water soluble amino acids were investigated through several experiments. The concentrations of all amino acids were adjusted to nitrogen content of Proline (Pro) at 200 mg L^{-1}. In case of lisianthus, twenty three water soluble amino acids were applied on seedlings which grown under controlled environmental condition either in renewed or non-renewed nutrient solution. Compared to the control, Histidine (His) and *Gamma*-aminobutyric acid (GABA) application increased the dry matter contents in renewed nutrient solution. In non-renewed nutrient solution, higher

dry matter was produced by the Pro and Glutamine (Gln) treated seedlings, meanwhile Alanine (Ala) treated seedlings produced the lowest dry matter.

Based on the seedling growth in non- renewed nutrient solution six amino acids Gln, Glycine (Gly), Pro, Methionine (Met), Leucine (Leu) and His were selected for further investigation along with Betaine (Bet) as a new amino acid following seedling grown in horticultural soil. The application of the aforementioned amino acids increased seedling height in horticultural soil. Higher shoot fresh weight and root length were observed in Pro treated seedlings. Amino acids treated seedlings experiment was continued under solution culture with either foliar application of amino acids or water in the greenhouse. Plant height was increased in all amino acids treated plants. Unlike urea, Leu and Bet, continuous application of His did not improved shoot dry weight but earlier flowering of lisianthus was evidenced.

In case of strawberry, a total of twenty two water soluble amino acids were sprayed in greenhouse setting. It was found that, growth and yield of strawberry plants grown in non-renewed nutrient solution was significantly reduced compared to plants that grown in renewed nutrient solution. When plants grown in non-renewed solution and were sprayed with Ala, Cysteine (Cys), Glutamic acid (Glu), Hydroxy-proline (Hyp), Lysine (Lys), Threonine (Thr), Tryptophan (Trp), His and Phenylalanine (Phe), the growth improved whereas, yield were improved by spraying of Ala, Asparagine (Asn), Aspartic acid (Asp), Cys, Glu, Gln, Hyp, Lys, Ornithine (Orn), Thr, Trp, His, Phe and Valine (Val). Regarding to growth and yield performance, Ala, Glu, Hyp, Thr, His and Phe were selected for further investigation along with GABA following Wagner’s pot based

hydroponic system and also *in vitro* condition. Glu and Hyp sprayed on plants increaced over 50% fruit yield compared to control in Wagner's pot based hydroponic system.

Our results confirmed the effects of amino acids on strawberry plant growth during autotoxicity, following *in vitro* culture. Compared to control, leaf dry weight was increased in Hyp treated plantlets and root dry weight of Ala, Glu, Hyp, Thr and GABA treated plants were increased against control. When nutrient contents were analyzed from the dry matter, it was revealed that Glu and Hyp improved the K, Ca and Mg contents in different parts of the plants.

Considering the growth and yield improvement, foliar application of His on lisianthus and Glu and Hyp on strawberry plants is suggested to recover the decreased growth and yield during autotoxicity in closed hydroponic culture.

SUMMARY IN JAPANESE

自家中毒とは, 植物が同一種の植物の発芽や生育を抑制する有害物質を周りの環境に発生させる時に種内で起きるアレロパシーの一種である。トルコギキョウとイチゴが閉鎖系養液栽培された場合，植物の根は細胞にダメージを与えられ、養水分吸収阻害を引き起こすことになる、主に安息香酸などのアレロパシー物質を放出する。その結果、その成長や収量を著しく減少させることになる。その代わりに、根以外からの養分供給は、アレロパシー物質によるストレス下でも植物の成長を継続させることが出来ると考えられる。近年、様々な無生物および生物由来のストレス下での生育や収量を改善するために，多くの植物でアミノ酸が散布処理されている。本研究では、閉鎖系養液栽培により自家中毒が発生している条件下で、トルコギキョウとイチゴの生育および収量がアミノ酸散布により回復するかどうか検討した。

トルコギキョウおよびイチゴのために、水溶性アミノ酸が実験を通して検討された。すべてのアミノ酸濃度は 200 mg / L プロリンに含まれる窒素分と同等になるように調整した。トルコギキョウの場合、環境制御された状態で交換および非交換培養液で生育させた苗に 23 種の水溶性アミノ酸を与えた。水のみを散布した対照区と比べて、ヒスチジン（His）および 4-アミノ酪酸（GABA）を散布した区では培養液を交換した場合、乾物重が増加した。培養液を非交換した場合、アラニン（Ala）散布により苗の乾物重が低くなったにも係わらず、プロリン（Pro）およびグルタミン（Gln）散布では苗の乾物重が高くなった。

培養液非交換で生育した苗について検討されたグルタミン（Gln）、グリシン（Gly）、プロリン（Pro）、メチオニン（Met）、ロイシン（Leu）、およびヒスチジン（His）の 6 種のアミノ酸が新たなベタイン（Bet）と共に、園芸培土で育てられたトルコギキョウ苗についての今後の検討のために選ばれた。前述のアミノ酸散布が園芸培土で育てられた苗の高さを増加させた。プロリン処理区で苗の地上部生体重と最大根長の増大が観察された。苗に対するアミノ酸処理はガラス室において、アミノ酸もしくは水の散布によって、養液栽培で継続された。草丈はすべてのアミノ酸処理区で増加した。尿素、ロイシンおよびベタインとは違い、ヒスチジンの継続散布によって地上部乾物重は変わらなかったが、トルコギキョウの開花が早まることが明らかになった。

イチゴの場合、22 種の水溶性アミノ酸がガラス室で散布された。培養液非交換条件下で栽培されたイチゴの生育および収量は, 培養液交換条件下と比べて有意に減少することが明らかになった。培養液非交換で栽培され、アラニン、システイン、グルタミン酸、ヒドロキシプロリン、リジン、スレオニン、トリプトファン、ヒスチジンおよびフェニールアラニンを散布した場合、イチゴの生育が改善されたにも係わらず, 収量についてはアラニン、アスパラギン、アスパラギン酸、システイン、グルタミン酸、グルタミン、ヒドロキシプロリン、リジン、オルニチン、スレオニン、トリプトファン、ヒスチジン、フェニールアラニンおよびバリンを散布した場合に改善した。生育と収量に関する実験結果より、アラニン、グルタミン酸、ヒドロキシプロリン、スレオニン、ヒスチジンおよびフェニールアラニンが 4-アミノ酪酸と共に、今後のワグネルポットでの養液栽培と *in vitro* においての検討のために選ばれた。ワグネルポットでの養液栽培において、グルタミン酸およびヒドロキシプロリンの散布は対照区(水のみ散布)と比べて 50%以上の果実収量増加となった。

我々の実験結果より、自家中毒下でのイチゴの生育に及ぼすアミノ酸の効果が *in vitro* においても確かめられた。対照区と比べて、葉の乾物重がヒドロキシプロリンを処理することにより増加した。また、アラニン、グルタミン酸、ヒドロキシプロリン、スレオニンおよび 4-アミノ酪酸を処理した根の乾物重は対照区と比べて増加した。植物体の乾物により養分含量を測定すると、グルタミン酸およびヒドロキシプロリンが植物の異なった部位におけるカリウム、カルシウムおよびマグネシウム含量を増大させた。

生育および収量増加より、トルコギキョウのヒスチジン散布, およびイチゴのグルタミン酸およびヒドロキシプロリン散布は閉鎖系養液栽培において引き起こされる自家中毒による生育および収量の減少を回復することを示唆した。

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LIST OF PUBLICATIONS

1. **Md. Fuad Mondal,** Md. Asaduzzaman, Hideyuki Tanaka, Toshiki Asao. 2015. Effects of amino acids on the growth and flowering of *Eustoma grandiflorum* under autotoxicity in closed hydroponic culture. *Scientia Horticulturae*. (In press) (The corresponding content is presented in Chapter 2).

2. **Md. Fuad Mondal**, Md. Asaduzzaman, Yutaro Kobayashi, Takuya Ban, Toshiki Asao. 2013. Recovery from autotoxicity in strawberry by supplementation of amino acids. *Scientia Horticulturae*.Vol. 164, 137–144 (The corresponding content is presented in Chapter 3).

LIST OF SUBPUBLICATIONS

1. **Md. Fuad Mondal**, Md. Asaduzzaman, Toshiki Asao. 2015. Adverse Effects of Allelopathy from Legume Crops and Its Possible Avoidance. *American Journal of Plant Sciences*, 6, 804-810.

2. **Md. Fuad Mondal**, Md. Asaduzzaman, Md. Hafizur Rahman Hafiz, Toshiki Asao. 2015. Overcoming the autotoxic effects in hydroponic strawberry by amino acids supplementation on leaves and electro-degradation of nutrient solution (Accepted as a book chapter in “New Developments in Allelopathy Researches”, NOVA Science Publisher, USA.