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Author(s)

Md. Fuad Mondal, Md. Asaduzzaman, Hideyuki Tanaka, Toshiki Asao

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Effects of amino acids on the growth and flowering of *Eustoma grandiflorum* under autotoxicity in closed hydroponic culture

Md. Fuad Mondal[a, b, c], Md. Asaduzzaman[a], Hideyuki Tanaka[a] and Toshiki Asao[a, *]

[a]Department of Agriculture, Faculty of Life and Environmental Science, Shimane University, 2059 Kamihonjo, Matsue, Shimane 690-1102, Japan

[b]United Graduate School of Agricultural Sciences, Tottori University, Koyama-cho, Minami Tottori, Tottori 680-8553, Japan

[c]Faculty of Agriculture, Sylhet Agricultural University, Sylhet-3100, Bangladesh

E-mail address: Md. Fuad Mondal[a, b, c] (fuadentom@yahoo.com), and Toshiki Asao[a,*] (asao@life.shimane-u.ac.jp)

***Corresponding author.** Tel: +81 852 34 1817; Fax: +81 852 34 1823. *E-mail address:* asao@life.shimane-u.ac.jp (T. Asao).

Abstract

Foliar application of amino acids was investigated for the recovery of the growth of *Eustoma* (*Eustoma grandiflorum* (Raf.) Shinn cv. Ichiban-boshi) under autotoxicity developed in the closed hydroponic system. Twenty three water soluble amino acids were applied on *Eustoma* 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 *Eustoma* 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 *Eustoma* was evidenced in the His treated plants. Therefore, foliar spray of His can recover the growth with early flowering of *Eustoma* during autotoxicity in closed hydroponic system.

Key words

Root exudates, autotoxicity, amino acids, Histidine (His), foliar application, seedling bioassay, closed hydroponic culture

1. Introduction

Eustoma grandiflorum 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 *Eustoma* 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 eustoma. 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 eustoma 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) are worse affected processes since root is the first organ to come into contact with autotoxins in the rhizosphere (Blum et al., 1999). Autotoxic 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. Therefore, autotoxic effects of root exudates in *Eustoma* 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 (Li, 2001; Roosta and Hamidpur, 2011, Stiegler et al., 2013). Supply of mineral nutrient alternative to roots uptake can sustain *Eustoma* 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 *Eustoma* 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 *Eustoma* 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 *Eustoma* seedlings grown in the renewed nutrient solution

Eustoma grandiflorum (Raf.) Shinn cv. Ichiban-boshi seeds (Sakata no tane, 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 *Eustoma* 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 *Eustoma* seedlings were measured. Then the *Eustoma* 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 *Eustoma* seedlings grown in the non-renew 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). Sowing, germination and transplanting were occurred on September 5, October 8 and December 28, 2012, respectively. 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 *Eustoma* seedlings were measured after oven drying the seedling as described for Expt. I.

2.2. Expt. III (a) Effects of seven amino acids on the *Eustoma* seedlings grown in the horticultural soil substrate

In this experiment, sowing and germination conditions of *Eustoma* 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. 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 *Eustoma* 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.

2.2. Expt. III (b) Effects of seven amino acids on the *Eustoma* plants grown in closed hydroponics in the greenhouse

After growth measurement, *Eustoma* 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 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 (+), Gly (+), Pro (+), Met (+), Leu (+), His (+), and Bet (+)} or water supply {Gln (–), Gly (–), Pro (–), Met (–), Leu (–), His (–), Bet (–)} in non-renewed nutrient solution. Water treated seedlings were continued in both renewed and 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 *Eustoma*. At the first anthesis, plants were harvested. Leaf number, leaf length and width, root length, fresh weight of shoot, numbers of flower buds was measured. After measuring, *Eustoma* 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 effect of amino acids on the vase life of *Eustoma* flowers. Waters was changed in each 3 days. Data of vase life were taken until the first petal of each flower was wilted. Shoots and roots dry weight was measured following the similar methods for Expt. I.

2.3. Statistical analysis

A randomized complete block design with three replicates was used for culture of *Eustoma* in container based hydroponics in the greenhouse whereas, complete block design was performed in culture of *Eustoma* 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 *Eustoma* 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 *Eustoma* seedlings grown in renewed hydroponic solution. Amino acids application showed a significant effect on the *Eustoma* seedlings growth. The application of His and GABA significantly increased the dry matter of *Eustoma* seedlings compared to control plants. However, Ala, and Ser showed negative effects on the dry matter production in *Eustoma* seedlings. (Fig. 1). Compared to the control, longer root was evidenced in Cys treated seedlings. (Supplementary Table S1). Ala, Glu, Hyp, and Lys treated plants reduced their leaf numbers against control plants.

3.2. Expt. II. Effects of amino acids on the *Eustoma* 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. However, among the amino acids Pro, Gln; and Ala treated seedlings produced higher and lower dry matter, respectively (Fig. 2). Compared to the control higher numbers of leaves were found in Cys treated seedlings (Supplementary Table S2). 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.

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

Based on the seedling growth performance, Gln, Gly, Pro, Met, Leu and His from the experiment II and Bet were applied on the *Eustoma* 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 1). 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 *Eustoma* seedlings. The chlorophyll content of Leaf measured by SPAD was increased in Urea and Gly applied seedlings. The shoot fresh weight of the *Eustoma* seedlings was also increased by the amino acids application. Compared to the control, higher shoot fresh weight was measured in Pro and treated seedlings.

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

Amino acids treated seedling were categories into two groups in the greenhouse, one was continuous application of respective amino acids (+) and another was application of water instead of amino acids (–). Either amino acid applied or not, *Eustoma* plants height was increased compared to the control (Table 2). 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 (+), urea (–), Leu (+). Leu (–), His (–), Bet (+), Bet (–) treated plants (Table 2).

Amino acids have effects on the flowering of *Eustoma* (Table 2). Compared to the control, His (–) treated plants were recorded with early flowering. Results showed that His treated *Eustoma* 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 *Eustoma* flowers did not show any significant difference among the treatments (Supplementary Table S3)

4. Discussion

Amino acids have profound effects on the growth of *Eustoma*. In our first experiment among the twenty three water soluble amino acids, foliar application of His, GABA increased the dry mass of *Eustoma* seedlings grown in the renewed nutrient (Fig.1). 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 *Eustoma* 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 *Eustoma* seedlings by decreasing the leaf size and numbers. These amino acids have negative effects on the growth of *Eustoma* seedlings.

In non-renewed container based hydroponics, among the amino acids Pro, Gln; and Ala treated seedlings produced higher and lower dry matter, respectively (Fig. 2). 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 *Eustoma* seedlings, fresh weight of Pro treated seedlings were significantly increased (Table 1) whereas seedlings applied with water did not increase fresh weight. Declined seedlings growth might be due to the autotoxic effect of *Eustoma* 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, *Eustoma* plants increased growth against the control (Table 2). Dry weight of shoot was increased in urea (+), urea (–), Leu (+), Leu (–), His (–), Bet (+) and Bet (–) treated plants. Early flowering of *Eustoma* plants was evidenced in the His (+) 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 *Eustoma* 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 that amino acids had effects on seedling stage as well as in vegetative stage. Some amino acids recovered their growth only by the application of amino acids during the seedling stage, and others needed supply until the anthesis. Numbers of bud, flower size and vase life of *Eustoma* 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.

Conclusion

Amino acids have some effects on the *Eustoma* seedlings both in vegetative and reproductive stages during autotoxicity. When twenty three water soluble amino acids were applied on *Eustoma* seedlings grown in either renewed or non-renewed nutrient solution, both positive and negative effects were found on the seedling growth. Twenty three amino acids were short listed to Gln, Gly, Pro, Met, Leu, and His on the basis of their better performance on seedling growth in non-recycled hydroponics. In the following experiment, selected amino acids along with Bet were further investigated following *Eustoma* seedlings grown in horticultural soil as substrate and after measuring the seedling growth parameters those were transferred to the container based closed hydroponic system in the greenhouse. In greenhouse setting, some amino acids recovered their growth only by the application of amino acids during the seedling stage, and others needed supply until the anthesis. 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 was evidenced in the His treated plants. Considering the effects of amino acids on the growth and flowering, His can be used as foliar application to recover the growth of *Eustoma* in closed hydroponic culture. Further investigation is necessary to determine the timing and doses of amino acids application for more efficient utilization by the *Eustoma*.

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Table captions:

Table 1. Effects of seven amino acids on the growth of *Eustoma* seedlings grown in horticultural soil substrate under growth chamber condition.

Table 2. Effects of amino acids on the growth and anthesis of *Eustoma* plants grown in the non-renewed nutrient solution in closed hydroponic culture under greenhouse condition.

Supplementary Table S1. Effects of twenty three amino acids on the growth of *Eustoma* seedlings grown in the renewed nutrient solution in closed hydroponic system.

Supplementary Table S2. Effects of twenty three amino acids on the growth of *Eustoma* seedlings grown in the non-renewed nutrient solution in closed hydroponic system.

Supplementary Table S3. Effects of amino acids on the flower characteristics of *Eustoma* plants grown in the non-renewed nutrient solution in closed hydroponic culture under greenhouse condition.

Table 1

Effects of seven amino acids on the growth of *Eustoma* seedlings grown in horticultural soil substrate 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
	*	*	*	*	*	*	*

*Significant at $P < 0.05$.

[z] *Eustoma* 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 at $P < 0.05$ ($n = 30$).

Table 2

Effects of amino acids on the growth and anthesis of *Eustoma* 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 (+)	25.0 ab	66.1 a	9.0 a	102 ab
Urea (-)	23.9 ab	64.8 a	9.3 a	100 ab
Gln (+)	25.5 ab	65.7 a	8.9 ab	97 ab
Gln (-)	24.8 ab	62.0 a	8.6 ab	102 ab
Gly (+)	25.2 ab	62.6 a	8.4 ab	94 bc
Gly (-)	24.6 ab	65.4 a	8.7 ab	99 ab
Pro (+)	26.0 ab	64.1 a	8.9 ab	104 ab
Pro (-)	25.2 ab	66.9 a	8.7 ab	96 bc
Met (+)	25.2 ab	65.1 a	8.1 ab	92 bc
Met (-)	24.0 ab	62.7 a	7.6 ab	98 ab
Leu (+)	25.7 ab	64.4 a	9.8 a	104 ab
Leu (-)	24.9 ab	63.4 a	9.1 a	103 ab
His (+)	25.0 ab	63.0 a	8.5 ab	89 c
His (-)	25.3 ab	66.2 a	9.8 a	91 bc
Bet (+)	25.5 ab	67.4 a	9.1 a	102 ab
Bet (-)	24.9 ab	63.3 a	9.3 a	113 a
	*	*	*	*

*significant at $P < 0.05$.

[z] *Eustoma* seedlings grown in renewed (RW), non-renewed (NRW), and non-renewed nutrient solution with amino acids and urea supplementation; (+) = amino acids supplementation from seedling stage to anthesis; (–) = 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 at $P < 0.05$ ($n = 15$).

Supplementary Table 1

Effects of twenty three amino acids on the growth of *Eustoma* seedlings grown in 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
	*	*	*	*

[*] Significant at P < 0.05

[z] *Eustoma* 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 at P< 0.05 (n = 30).

Supplementary Table 2

Effects of twenty three amino acids on the growth of *Eustoma* seedlings grown in the non-renewed nutrient solution in closed hydroponic system.

Amino acids[z]	No. of leaves[y]	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

ns: non-significant or *significant at $P < 0.05$.
[z] *Eustoma* seedlings grown in renewed (RW), non-renewed (NRW), and non-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 at $P < 0.05$ ($n = 15$).

Supplemtary Table 3. Effects of amino acids on the flower characteristics of *Eustoma* plants grown in the 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 (+)	7	54	40	20
Urea (−)	7	53	41	20
Gln (+)	7	56	45	18
Gln (−)	6	54	43	21
Gly (+)	9	54	47	21
Gly (−)	7	56	42	20
Pro (+)	8	55	46	17
Pro (−)	7	56	44	24
Met (+)	7	57	43	21
Met (−)	6	56	45	20
Leu (+)	7	56	42	19
Leu (−)	7	54	42	21
His (+)	8	57	45	21
His (−)	7	56	42	20
Bet (+)	7	57	45	23
Bet (−)	6	55	44	22
	ns	ns	ns	ns

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

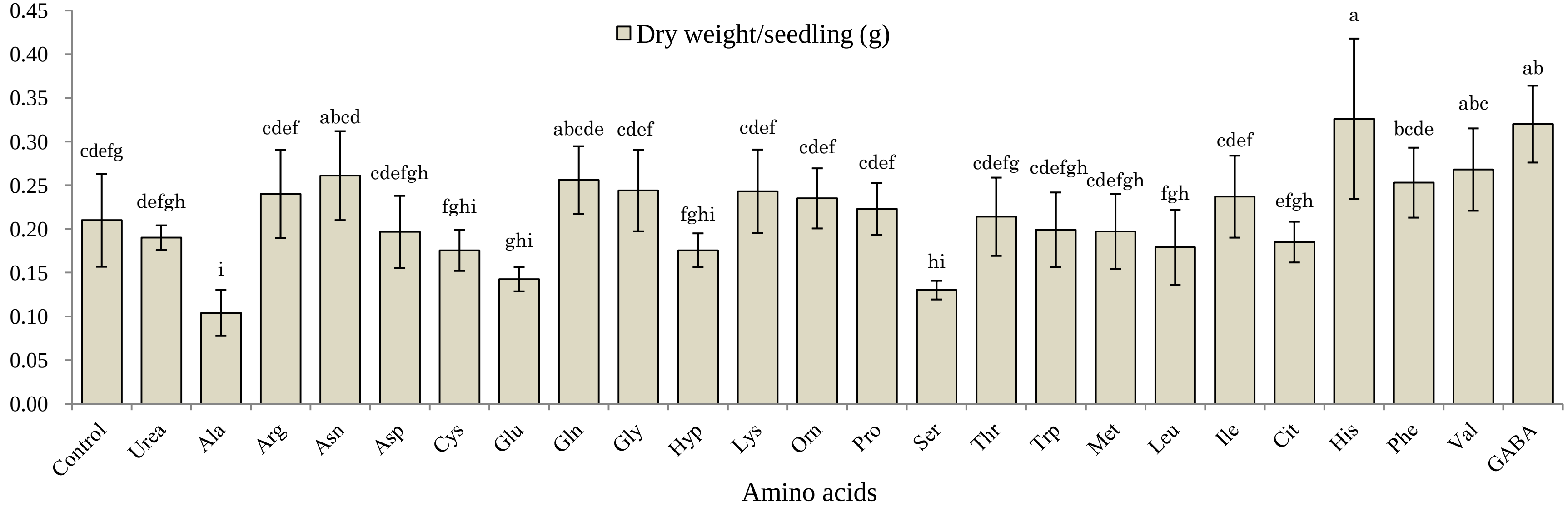
[z] *Eustoma* seedlings grown in renewed (RW), non-renewed (NRW), and non-renewed nutrient solution with amino acids and urea supplementation; (+) = amino acids supplementation from seedling stage to anthesis; (–) = amino acids supply on the seedling stage only.

[y] Parameters were measured on per plant basis.

Figure captions

Fig. 1. Effects of twenty three amino acids and urea on the dry matter production of *Eustoma* 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 $P < 0.05$ ($n = 30$).

Fig. 2. Effects of twenty three amino acids and urea on the dry matter production of *Eustoma* 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 $P < 0.05$ ($n = 15$).



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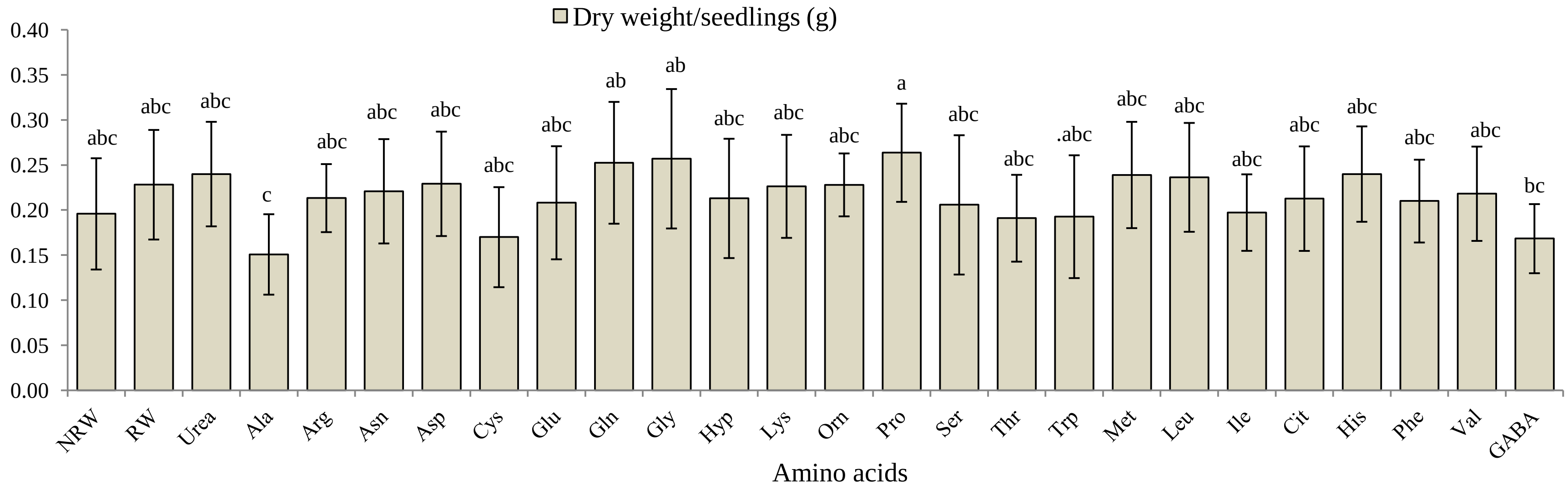

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