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Application of Amino Acids Improves Lettuce Crop Uniformity and Inhibits Nitrate Accumulation Induced by the Supplemental Inorganic Nitrogen Fertilization

Pavlos Tsouvaltzis*, Athanasios Koukounaras and Anastasios S. Siomos
Department of Horticulture, Aristotle University, 54124 Thessaloniki, Greece
*For correspondence: ptsouv@agro.auth.gr

Abstract

Lettuce plants (*cv.* 'Simpson') were grown for 10 weeks during winter under greenhouse to investigate the possibility of replacing the supplemental nitrogen (N) fertilization by using Amino16®. Amino16® is a hydrolyzed protein solution containing 11.3% L-amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine), 4% total N and 25% organic matter, produced of plant origin raw materials. Three weeks after transplanting, supplemental fertilization with ammonium nitrate or Amino16® was applied. Ammonium nitrate solution was applied to the soil, while Amino16® either to the soil or foliage. Two and 4 weeks later, the foliar application of Amino16® was repeated. Plants without any supplemental fertilization were used as control. The results indicated that crop uniformity was substantially increased when plants received supplemental fertilization with ammonium nitrate and even more when Amino16® was applied to soil. Nutritional quality of lettuce plants, in terms of dry matter, total soluble solid, ascorbic acid and total phenol contents, was not affected by supplemental fertilization either with ammonium nitrate or Amino16®. On the other hand, the application of Amino16® prevented the accumulation of nitrates observed in plants fertilized with ammonium nitrate. Moreover, the foliar application of Amino16® resulted in significant increase of antioxidant capacity, in comparison to the control. According to the above, the use of Amino16® as alternative of inorganic supplemental fertilization is of high importance, in order to improve lettuce crop uniformity, minimize nitrate content in plants, without negative effect on other nutritional components or yield.

Keywords: Amino16®; Antioxidant; Fertilization; Foliar; Nutritional quality

Introduction

Nitrogen is the most essential mineral nutrient that promotes sufficient plant growth and consequently yield. It is absorbed by roots either as ammonium (NH_4^+) or nitrate (NO_3^-) ion and incorporated in amino acids and eventually proteins (Blom-Zandstra, 1989).

Frequently farmers use excessive rates of nitrogen in vegetables to avoid N-deficiency (Porto *et al.*, 2008), ignoring environmental pollution, increase in production cost as well produce quality deterioration problems (Blom-Zandstra, 1989; Wang *et al.*, 2008; Montemurro, 2010). For example, Wang *et al.* (2008) reported that over-application of N decreased vitamin C, soluble sugar, Mg and Ca concentrations, as well as soluble solids in tomato fruit, while increasing titratable acidity and the acid: sugar ratio, lead to decrease in commercial and nutritional quality. Excessive nitrogen supplement may pollute ground water (Gonzalez *et al.*, 2010). Additionally, abundant NO_3^- availability leads to excessive absorption by the roots in larger quantities converted into NH_4^+ via nitrate reductase (Blom-Zandstra, 1989; Wang *et al.*, 2008), resulting to NO_3^- accumulation in the vacuoles of the cells. This frequently takes place in lettuce plants to continue N uptake until harvest (Salomez and Hofman, 2009) and accumulate excessive concentrations of NO_3^- in their tissues. This is regarded as negative qualitative aspect from a nutritional standpoint, because NO_3^- intake by humans has been associated with various health hazards (Blom-Zandstra, 1989).

Accumulation of NO_3^- in field grown lettuce depends on nitrogen supply, in terms of fertilizer form used and doses applied (Porto *et al.*, 2008). Manipulation of nitrogen fertilization in terms of rates of application and source type appears as the most applicable means to reduce NO_3^- accumulation in plants (Wang *et al.*, 2008). However, caution should be taken, in order to ensure that a critical minimum level of mineral nitrogen for sustaining plant growth and crop yield is not surpassed and additionally mineral nitrogen manipulation doesn't induce nutritional quality deterioration (Salomez and Hofman, 2009; Stefanelli *et al.*, 2012).

Recent research has shown that the use of organic fertilizers in field grown lettuce can be considered as valid and useful alternative source of nitrogen to the mineral ones (Porto *et al.*, 2008; Montemurro, 2010), but

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information regarding the application of amino acids, another source of organic nitrogen, is scarce. El-Naggar *et al*. (2008) reported that glycine solution sprayed on lettuce plants and soil, served as an effective fertilization strategy as inorganic N fertilizers, but the individual effect of foliar or soil application is not elucidated, neither any other study regarding the application of a mixed amino acids solution on soil of field grown lettuce exist. Although in hydroponic systems, when amino acids were used as alternative for part of NO_3^- supply, plant growth and crop yield of lettuce (Gunes *et al*., 1994) were promoted similar to 100% mineral nitrate fertilization, it is unknown, whether application of amino acids solution is also effective when applied to the soil. Indeed, according to Owen and Jones (2001), organic N applied in soil as amino acids solutions, may not be as effective as inorganic fertilizers, due to the slow movement of amino acids in soil, the rapid turnover of amino acids by soil microorganisms and the poor competitive ability of plant roots to capture amino acids from the soil solution.

Apart from the root supply, amino acids can be successfully applied on plant foliage as foliar sprays. The use of foliar nutrient sprays in agriculture is increasingly widespread, since they are potentially more environment friendly and target-oriented as compared to root treatments (Fernandez and Eichert, 2009; Gonzalez *et al*., 2010; Yasmeen *et al*., 2013). Foliar application of mixed amino acids solution increased fresh and dry weight of shoots in field grown radish plants, while reducing nitrate content (Liu *et al*., 2008a, b). Additionally, foliar application of mixture of amino acids and seaweed extract at different growth stages had a positive effect on vegetative growth, reproductive growth, and berry quality of grapevines (Khan *et al*., 2012). However, in order to be used in vegetables, these should be tested carefully in various crops and cultivars.

Amino16® is a hydrolyzed protein solution containing 11.3% L-amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine), 4% total N and 25% organic matter (according to the manufacturer).

Demand for environment friendly organic materials of plant origin suitably combined with inorganic fertilizers is increasing, in order to preserve soil health, crop yield and improved nutrient use efficiency (Rahman *et al*., 2013), the aim of this study is to investigate the possibility of replacing the supplemental mineral nitrogen fertilization on field grown lettuce by using an organic substitute like Amino16®.

Materials and Methods

Plant Material and Fertilization Treatments

Lettuce plants (*cv*. 'Simpson') were grown in greenhouse condition at Aristotle University of Thessaloniki, Greece from November 2010 to January 2011. Pre-plant incorporated application of synthetic fertilizer (12% N, 12% P_2O_5, 17% K_2O) was made at 1,000 kg/ha. Plants were transplanted at 2-3 true leaf stage at density of 12 plants m^{-2}. During the growing period, the plants were drip-irrigated and weeds were pulled by hands as required. Three weeks after transplanting, supplemental fertilization with ammonium nitrate or Amino16® was applied. Amino16® is patented (EP 2537823 A1) product of EVYP (Greek Industry of Hydrolyzed Protein L.L.P), produced of plant origin raw materials. Ammonium nitrate solution (1.2% w/v) was applied to the soil (100 mL $plant^{-1}$), while Amino16® was applied either to the soil or the foliage (100 ml $plant^{-1}$ of 0.3% v/v or 6.25 mL $plant^{-1}$ of 0.2% v/v, respectively). Two and 4 weeks later, the foliar application of Amino16® was repeated (12.5 mL $plant^{-1}$, 0.2% v/v). Plants without any supplemental fertilization were used as control.

A randomised complete block design was used, comprised from 4 blocks per treatment, each block consisted of three rows with 14 plants in each row. The plants in the outside rows and the end plants of the remaining row were guards. The plants were hand-harvested at once 10 weeks after transplanting and fresh weight of each plant was taken. Plants were graded according to the following weighing scale: 50-99, 100-149, 150-199, 200-249, 250-299, 300-349 and >350 g. Two plants from each block were collected and stored in freezer at -20°C for the determination of the nutritional quality.

Nutritional Quality

Frozen samples were thawed and macerated in a Waring blender and the blended material was used for the determination of dry matter, total soluble solid, nitrate, ascorbic acid and total soluble phenol content, as well antioxidant capacity.

The dry matter was determined after drying the pulp at 70°C for 72 h. The total soluble solids were measured in the juice of the pulp using a portable digital refractometer (Atago Co. Ltd., Tokyo, model PR^{-1}). The nitrates were determined according to Cataldo *et al*. (1975). For the extraction of ascorbic acid, 20 g of the pulp was homogenized with 50 mL 1% oxalic acid solution and filtered through Whatman No 1. The ascorbic acid was measured in the filtrate using RQflex reflectrometer (Merck, Darmstadt, Germany). Total soluble phenols were determined according to Scalbert *et al*. (1989) and expressed as mg gallic acid equivalents (GAE) kg^{-1} FW and antioxidant capacity according to Brand-Williams *et al*. (1995) and expressed as mg ascorbic acid equivalents (AEAC) kg^{-1} FW.

Statistical Analysis

Analysis of variance (ANOVA) was performed in MSTAT and mean separation was done with Duncan's multiple range test ($P < 0.05$). Percentages were statistically processed using arcsin square root transformation and actual percentage values are given in the figures.

Results

Plant Fresh Weight

The fresh weight of harvested plants was not affected by supplemental fertilization (Table 1). The plant fresh weight was not significantly different between the treatments, indicating that pre-plant fertilization was sufficient to sustain plant growth.

However, the distribution of plant fresh weight in the various classes was affected by the treatments (Fig. 1). Particularly, the percentage of 7 weight classes in control treatment did not differ among each other (Fig. 1a), is indicative of low crop uniformity, which is an undesirable trait for the market. On the other hand, inorganic supplemental fertilization resulted in better crop uniformity, compared to the control, as long as 38% of plants were weighing 150-199 g, although not significantly different than classes 200-249 g and 250-299 g, with 20 and 18% of plants, respectively (Fig. 1b). Ten percent of harvested plants were weighing 100-149 g and 15% were weighing >300 g. The application of Amino16® on the foliage was even more effective in improving crop uniformity, as long as 44% and 28% of plants weighing 150-199 g and 200-249 g, respectively, followed by 100-149 g (15%), significantly higher than classes 50-99, 250-299, 300-349 and >350 g, with 2% of plants each (Fig. 1c). However, the best crop uniformity, was obtained when Amino16® was applied to soil, compared to all other treatments (Fig. 1d), as long as plant weight was grouped in 3 distinct weight classes. Particularly, 55% of the harvested plants were weighed 200-249 g, 38% 100-199 g and 5% 250-349 g, without any harvested plant weighing <100 or >350 g (Fig. 1d). In-between the 7 weight classes, significant differences were only observed in 200-249 g class, where the percentage of plants that received Amino16® to the soil was significantly higher (55%), than all other treatments (24-28%).

Although, plant fresh weight and consequently crop yield were not affected by supplemental fertilization (Table 1), crop uniformity was substantially increased when plants received supplemental fertilization with ammonium nitrate and even more when Amino16® was applied and especially to the soil (Fig. 1).

The application of Amino16® to the foliage also resulted in better crop uniformity, compared either to the control or inorganically-fertilized plants, but lower than its soil application.

Nutritional Quality

Dry matter, total soluble solid, ascorbic acid and total phenol contents were not affected by supplemental fertilization (Table 1).

The antioxidant capacity of control plants was 1.88 ascorbic acid equivalents (AEAC) 100 g^{-1} FW, not significantly different than plants treated with inorganic fertilization or Amino16® applied to the soil (Fig. 2a). On the other hand, the application of Amino16® to the foliage resulted in significant increase of antioxidant capacity (3.15 mg AEAC 100 g^{-1} FW), in comparison to the control.

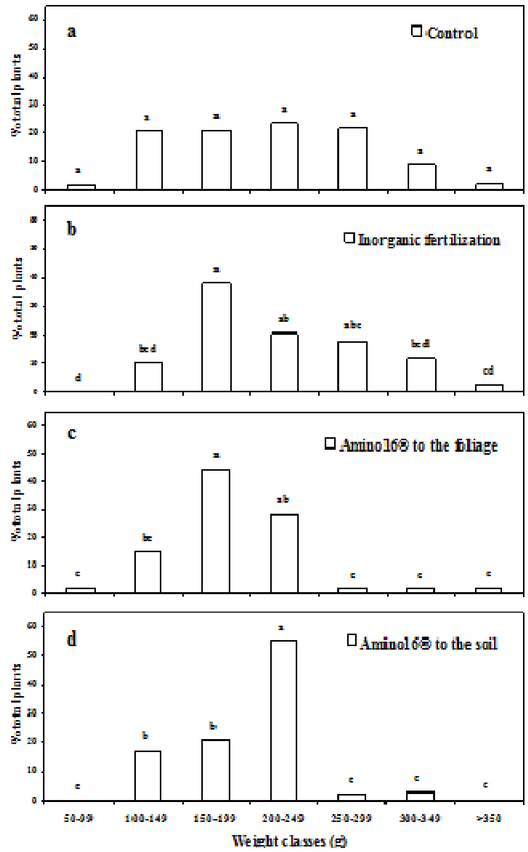

Fig. 1: Lettuce plant fresh weight classification in relation to the supplemental fertilization

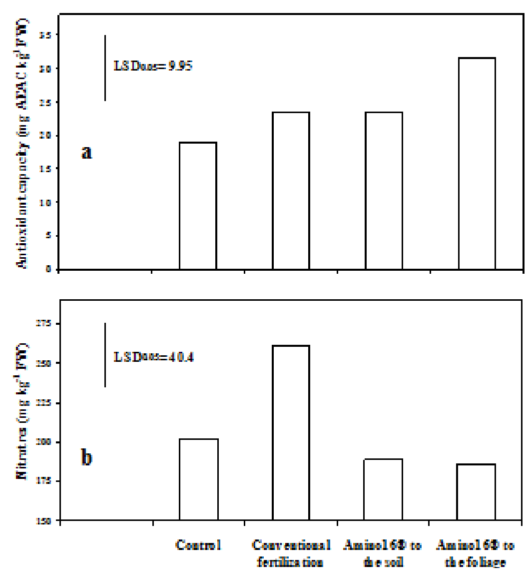

Fig. 2: Total antioxidant capacity and nitrate content of lettuce in relation to the supplemental fertilization

The nitrate content of control plants was 202 mg kg^{-1} FW, not significantly different from plants receiving both Amino16® treatments (186-189 mg kg^{-1} FW) (Fig. 2b). Contrarily, the inorganic fertilization resulted in significantly increased nitrate content, reaching 261 mg kg^{-1} FW.

Correlations

No significant correlations between fresh weight and nutritional quality characteristics were observed (Table 2). Contrarily, dry matter was correlated with soluble solids (r=0.54, *P*=0.031) and total soluble phenols (r=0.57, *P*=0.020), as well as soluble solids with nitrates (r=-0.71, *P*= 0.002).

Discussion

The application of amino acids did not affect lettuce fresh weight in our study (Table 1), in contrast to Liu *et al.* (2008a), the application of mixed amino acids solution on field grown radish resulted in increased fresh weight, when plants were treated frequently. Similar to our results, the replacement of 20% of nitrate in nutrient solutions with mixed amino acids in soilless culture had no effect on fresh weight of lettuce or onion (Gunes *et al.*, 1994, 1996). Likely fresh weight of pak-choi (*Brassica chinensis* L.) was either increased or reduced, depending on the individual amino acids source (Wang *et al.*, 2007).

However, the distribution of lettuce fresh weight in the various classes was affected by the treatments (Fig. 1) and indeed the application of amino acids improved lettuce crop uniformity, especially when Amino16® was applied to soil. Lettuce crop uniformity is of high importance in the produce market and is therefore included as a critical marketing standard in the European Commission Regulation 543/2011 (2011).

Although N concentration in ammonium nitrate or Amino16® solution was equal, the above finding probably implies that amino acids were absorbed by the roots of plants more favourably than ammonium nitrate, either due to higher affinity with the roots or due to higher adsorption of amino acids on soil particles. The form of N uptake is mainly determined by its abundance, accessibility and sets of finely tuned transporters allow the plant to precisely adapt N uptake in response to plant demand and external N supply (Von Wirén *et al.*, 1997). It is well documented that the nitrate adsorption on soil particles is low; leading to nitrate loss through leaching, as well as assimilation of absorbed inorganic N into amino acids is an energy-requiring process (Von Wirén *et al.*, 1997). The use of mixed amino acids solutions as alternative part of nitrate fertilization has successfully sustained lettuce plant growth and yield in soilless growing systems (Gunes *et al.*, 1994), indicating the efficient absorption of amino acids by plants' roots. This is the first report, though, exhibiting the beneficial effect of amino acids application on field grown lettuce crop uniformity.

The reduced effectiveness of foliar application of Amino16® in comparison to soil application, is probably due to the lower amino acids concentration in the plant sap resulting either from a low penetration rate through the foliar cuticle (Fernandez and Eichert, 2009) or from the low solution dispersion on the internal, shielded leaves. Additionally, the total N amount applied through the foliar sprays of amino acids solution was 5-times lower than soil applications of ammonium nitrate or Amino16® solutions, which may explain the present study findings. Indeed, according to Fernandez and Eichert (2009), given the low penetration rates of foliar nutrient solutions, the concentration range applied should be higher as compared to root treatments. Foliar application of amino acids has been shown to increase fresh weight of field grown radish shoots, which is a short-cycle crop, only when application was done intensively i.e., 4 times in a period of 10 days (Liu *et al.*, 2008a). Such practice, though, cannot be adopted in vegetable crops with a longer cycle, such as lettuce, due to the significant increase of production cost. Therefore, the foliar application of amino acids can be properly timed and thus; limited to specific stages of plant growth, when nutrient demand is likely to be high or when soil conditions are known to restrict nutrient uptake (Gonzalez *et al.*, 2010).

The use of mixed amino acids solutions as alternative of part of nitrate fertilization in NFT (nutrient film technique) grown lettuce plants did not affect dry matter content (Gunes *et al.*, 1994), similar to field grown lettuce plants in our study. However, dry weight of Pak-choi has been shown to be negatively affected, when part of nitrate fertilization was replaced with amino acids in the nutrient solution in soilless culture, but this effect was amino acid type-dependent (Wang *et al.*, 2007). Furthermore, our results corresponds with Stefanelli *et al.* (2012) who concluded that reduced nitrate N fertilization can be applied in soilless grown lettuce, without affecting total phenolics and ascorbic acid content, although, N fertilization rates and form have been shown to significantly affect ascorbic acid content in vegetables (Wang *et al.*, 2008).

Interestingly, antioxidant capacity was correlated with dry matter (r= 0.61, P= 0.013) and soluble solids (r=0.63, P= 0.009), but not with total soluble phenols, contrary to other reports (Llorach *et al.*, 2004; Oh *et al.*, 2009; Boo *et al.*, 2011) neither with ascorbic acid with the latter being in consistency with previous results in iceberg or Chinese lettuce (Szeto *et al.*, 2002).

The effect of amino acid application on NO_3^- reduction, found in this study, has also been demonstrated in field grown Pak-choi (Wang *et al.*, 2007), radish (Liu *et al.*, 2008a, b), as well as lettuce (Gunes *et al.*, 1994) and onion (Gunes *et al.*, 1996) grown in NFT. The interpretation of this effect is contradictory among researchers, with other stating that amino acids are preferably absorbed by plants as reduced N source (Gunes *et al.*, 1994, 1996), while others claim that the main role of amino acids on nitrate uptake and assimilation is the regulation of many processes and metabolic pathways of plant N metabolism, such as nitrate and nitrite reductase and glutamine synthetase activities (Liu *et al.*, 2008a, b) or even the inhibition of the expression of HvNRT2 transcript in roots, which induces the synthesis of the mRNA encoding nitrate transporter that is directly related to nitrate uptake (Vidmar, 2000). Alternatively, Aslam *et al.* (2001) suggested that induction of the NO_3^- transporter in the presence of amino acids may be normal, but the turnover of the mRNA encoding the transporter may be increased.

Although the nitrate content of plants receiving inorganic fertilization was much lower than maximum permissible levels established by European Commission Regulation 466/2001 (2001) for lettuce produced in protected areas (4,500 mg/kg FW), the use of amino acids as alternative of mineral supplemental fertilization is of high importance, in order to minimize nitrate content in consumed vegetables. Indeed, European Commission Regulation 1881/2006 (2006) implies that presence of contaminants must be reduced more thoroughly wherever possible by means of good agricultural practices in order to achieve a higher level of health protection, especially for sensitive groups of the population.

In conclusion, crop uniformity was substantially increased when plants received supplemental fertilization with ammonium nitrate and even more when Amino16® was applied especially to the soil. Moreover, the use of Amino16® as alternative of inorganic supplemental fertilization is of high importance, in order to minimize nitrate content in lettuce, without negative effect on other nutritional components or yield.

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