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Responses of Peppermint and Spearmint Crops to Excessive Biostimulant Application and Increased Salinity in a Closed Soilless Production System

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Abstract: A floating system was established in a heated glass greenhouse in order to investigate whether the effect of amino acids (0.25 or 0.50% of a commercial amino acid (AA) solution Amino16[®]) during peppermint and spearmint production on plant developmental and nutritional status may be in part attributed to salinity induced osmotic stress. For this reason, in some nutrient solutions, three levels of salinity were induced by adding 0, 10, or 20 mM NaCl. According to the results, it can be concluded that spearmint is mostly favored by the highest amino acid supplement of the nutrient solution (0.50%) in terms of a substantial improvement of the antioxidant nutritional quality (by up to 130%) at the expense of a reduced biomass production (by <30%). Enzymic antioxidant defense mechanism (APX and POD) was efficiently activated, preventing severe lipid peroxidation and the accumulation of reactive oxygen species such as H₂O₂ and maintaining the proline content at the normal levels. The osmotic stress that was induced by the excessive AA concentration and confirmed by the chlorophyll fluorescence variations was probably related to NH₄⁺ excess supply in the growing media and was not associated with the elevated electrical conductivity in the solution. The absence of any adverse stressful consequences upon the addition of 20 mM NaCl may be attributed to the high salt tolerance of peppermint and spearmint species.

Keywords: medicinal plants; hydroponic production; nutritional quality; APX; CAT; proline; hydrogen peroxide; MDA; chlorophyll fluorescence; antioxidant enzymes



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1. Introduction

Peppermint (*Mentha × piperita* L.) and spearmint (*Mentha spicata* L.) are perennial aromatic herbs that are grown for fresh or dry product or for essential oil extracts to constitute flavor agents in food preparation or even as raw material in herbal medicine production by the pharmaceutical industry [1]. Both mint species are a source of bioactive compounds, rich in antioxidants, phenolics, and other phytonutrients. Due to their health benefits, these species are also classified as medicinal crops [2,3]. However, the active constituents of medicinal plants such as spearmint and peppermint are used in clinical studies and new drug development, but their restricted intensive cultivation sets limitations in the production of medicines [4]. Therefore, the commercial cultivation of medicinal plants in a larger scale by implementing innovative hydroponic techniques is necessary in order to fulfill the demand of the medicine industry.

According to Aktsoylou et al. [5], both peppermint and spearmint crops are suitable for hydroponic cultivation, particularly in a floating raft system. This soilless hydroponic system may be subjected to customized modification in order to enhance the produce quality while employing sustainable agricultural practices [6]. Indeed, the application of biostimulants (plant or animal derived protein hydrolysates) in the soilless production of lettuce and corn also affected both the quality and the crop yield [7].

Protein hydrolysates (PHs) are a mixture of amino acids and peptides deriving from the chemical or enzymatic hydrolysis of animal or plant origin by-products [6]. According to the published literature, it has been confirmed that the biostimulant activity of PHs results in improved quality of leafy vegetables [5,8] and induces protection in plants against several abiotic stressful conditions [9–11]. Moreover, even the application of PHs either as a supplement in nutrient solutions or as a foliar spray solution can serve as alternative nitrogen sources for plant growth and development [12].

However, according to Tsouvaltzis et al. [8], the addition of amino acids (AA) results in a modification of the conditions in the nutrient solution, inducing an increase in electrical conductivity linearly to the concentration of amino acids. Dissolution of ions in water or soil solutions affects the transportation of electrons and therefore a high ion concentration in the nutrient solution (NS) implies, in turn, increased flow of electrons and consequently a greater electrical conductivity (EC) [13], which is an index of the electron transport potential in a solution. Moreover, given that there is a linear regression between the salt concentrations and EC values, high EC levels increase the osmotic pressure of the NS and induce osmotic stress in plants and in turn, adverse growing conditions. Apart from the modification of the EC in the nutrient solutions, the addition of protein hydrolysates has been associated with ammonium toxicity, which is induced by the concentrated nitrogen presence in amino acid solutions [5]. Indeed, this situation may often be encountered in commercial hydroponic production because apart from their biostimulant activity, the amino acids included in the PH solutions are considered as important alternative nitrogen sources for plants [12] and there is always a high risk of excessive application, similar to fertilizer use during crop production on soil.

When plants are exposed to a stressful condition, the homeostasis of their cells may be disturbed due to the generation of reactive oxygen species (ROS) such as H_2O_2 and superoxide radicals, which result in oxidative damage to proteins and DNA. In such conditions, the enzymatic and non-enzymatic antioxidant systems are efficient mechanisms that permit the plants to regulate the oxidative damage by scavenging ROS [14].

Ascorbic peroxidase (APX), catalase (CAT), and peroxidase (POD) are protective enzymes against the oxidative damage induced by ROS and are responsible for controlling the concentration of oxidizing agents in cells such as H_2O_2 . Under various abiotic stresses such as salinity, alkalinity, drought, UV radiation, extreme temperatures, nutrient deficiencies, and air pollution, the accumulation of all the above enzymatic antioxidants in plant cells occurs at high rates [14–16]. These antioxidant enzymes exhibit high specialization in scavenging ROS. In particular, APX has a higher affinity than CAT and POD to H_2O_2 and therefore an important role in the regulation of ROS production, but on the other hand, CAT catalyzes the conversion of H_2O_2 into H_2O and O_2 and is responsible for the removal of ROS [14,17]. Moreover, when the plants suffer from adverse conditions, apart from H_2O_2 accumulation, an increase in malondialdehyde levels (MDA) may be observed. This component is produced during peroxidation of membrane lipids and can be used as an indication of oxidative damage [15].

Phenolics, ascorbic acid, carotenoids, flavonoids, and α -tocopherols are non-enzymatic protective antioxidant compounds that are abundant in plant tissues that also contribute substantially to the nutritional quality characteristics of the horticultural products [17]. In addition, under unfavorable conditions, the plants accumulate solutes such as proline, a reliable stress indicator and an efficient osmolyte for adjusting their osmotic potential. However, according to Chen and Dickman [18], proline can also be regarded as a non-enzymatic antioxidant because it contributes to the maintenance of the membranes' protein stabilization while scavenging OH^- radicals [19,20].

Provided that ROS production takes place in chloroplast, the photosynthetic mechanism of plants is consequently affected. The chlorophyll fluorescence is a small percentage of energy that is emitted when an excited electron returns back to its ground state during photosynthesis [21]. Hence, the chlorophyll fluorescence OJIP transient, which is related to the photosystem II function, may also provide valuable information regarding the photo-

synthetic status of the plant (photochemical and non-photochemical quenching) [22,23]. Indeed, chlorophyll fluorescence has been used as an indicator for detecting damage in the photosynthetic apparatus during plant exposure to various biotic or abiotic stressful conditions such as virus or fungi diseases, nutrient deficiencies, salt, drought, chilling or heat stress, herbicide application, metal pollution, etc. [22,24,25].

As long as high EC levels in the nutrient solution of a soilless production of mint species is related to the concentration of amino acids (AA) in the solution, it is questionable as to whether an abundant supplement of AA may trigger an abrupt rise in EC levels and become harmful in plant growth and produce quality.

The aim of the present study was to investigate whether peppermint and spearmint plants in a soilless production are favored by an excessive amino acid supply in the nutrient solution or if it turns out to be toxic through a salinity induced osmotic stress.

2. Materials and Methods

2.1. Plant Growth

Ten centimeter long rooted shoots were collected from well-established healthy peppermint and spearmint plants and were transplanted on compressed polystyrene trays filled with peat substrate. The grafts were watered daily during the first three weeks until successful rooting.

A floating system was established in a heated glass greenhouse at the farm of Aristotle University of Thessaloniki, Greece and plants were cultivated in a nutrient solution with the following composition: NO₃-N 210 mg/L, NH₄-N 14 mg/L, K 391 mg/L, P 62 mg/L, Mg 49 mg/L, Ca 385 mg/L, S 232 mg/L, Fe 1150 µg/L, Mn 399 µg/L, Zn 150 µg/L, Cu 150 µg/L, B 500 µg/L, and Mo 48 µg/L. Three levels of salinity were induced by adding 0, 10, or 20 mM NaCl, while three levels of a patented commercial amino acid (AA) solution (Amino16[®], EVYP LLP, Thessaloniki, Greece) were prepared by supplementing with 0, 0.25, or 0.50%, and three were left free of either NaCl or AA (control). In total, fifteen plastic basins 70 × 65 × 20 cm³ (L × W × H) were used, three for each amino acid concentration (0.25 or 0.50%) and NaCl level (10 or 20 mM), respectively, as well as three for the untreated control (0% Amino16 and 0 mM NaCl). In each basin, 45 L of the respective nutrient solution was added at the initiation of the experiment as well as 28 days later.

During the production period, the air temperature and relative humidity conditions were recorded with HOBO Onset dataloggers and the electrical conductivity (EC), pH, and temperature levels of the nutrient solutions were monitored by using a portable instrument (C5020, Consort, Turnhout, Belgium). The aeration of the roots was ensured by daily stirring of the nutrient solutions.

The chlorophyll fluorescence of leaves was recorded during the cultivation period, as described below, while the plant height, weight, and root length were measured before being harvested on the 42nd day (6th week) after the initiation of the experiment (placement of the polystyrene trays on the nutrient solutions). All measurements were taken on sixteen plants from each tank, treatment, and specie.

Chlorophyll fluorescence transient was recorded with a Fluorpen FP100-MAX, PAM fluorometer after 30 min of dark adaptation and was determined and analyzed according to the OJIP-test. Several parameters (26 in total) were calculated that describe the energy transport processes at the reaction centers of PSII [26]. Chlorophyll fluorescence was recorded at 0, 1, 2, 4, and 5 weeks after the initiation of the experiment.

The harvested tissue was kept at −20 °C for nutritional composition analysis (phenolics and antioxidant capacity), proline, hydrogen peroxide, malondialdehyde content, and antioxidant enzyme (peroxidase, catalase, and ascorbate peroxidase) activities.

2.2. Antioxidant Capacity and Soluble Phenolic Content

The frozen tissue was partially thawed and homogenized. Five grams of the homogenate was used to measure the antioxidant capacity (DPPH radical scavenging activity) and total soluble phenol content. DPPH radical scavenging activity was determined using

a modified method of Brand-Williams et al. [27]. Ascorbic acid was used as the standard and the DPPH radical-scavenging activity was expressed as mg of ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g fresh weight (mg AEAC 100 g⁻¹ FW). Total soluble phenol content was determined according to the method of Scalbert et al. [28] and was calculated based on gallic acid equivalents (GAE) (mg GAE kg⁻¹ FW).

2.3. Lipid Peroxidation

Lipid peroxidation was determined in terms of malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction as described by [29]. The 1.5 mL tissue extract (the same used in enzyme activities determinations) was heated at 100 °C for 20 min in a boiling-water bath with 2.5 mL 0.5% TBA in 20% trichloroacetic acid (TCA). The cooled mixture was centrifuged at 3000 × g for 5 min. MDA equivalent was calculated from the difference in absorbance at 532 and 600 nm. The concentration of MDA was expressed as nmol MDA g⁻¹ FW.

2.4. Hydrogen Peroxide

Plant tissue (4.0 g) was homogenized in an ice bath with 25 mL of ice cold 0.1% (*w/v*) trichloroacetic acid (TCA). The homogenate was centrifuged at 4 °C and 17,600 × g for 15 min and 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1.0 mL 1 M potassium iodide (KI) at 24 °C. The mixture was vortexed and its absorbance was read at 390 nm. The content of H₂O₂ was calculated from a standard curve [30].

2.5. Proline

Proline was determined according to the method of Troll and Lindsley [31]. In particular, 0.1 g of frozen plant tissue was extracted in 15 mL of 80% ethanol. This extract was incubated at 60 °C in a water bath for 30 min and free proline in the extract was measured with an acid ninhydrin solution.

2.6. Antioxidant Enzyme Activities

Plant tissue (4.0 g) was homogenized in an ice bath with 20 mL of 50 mM phosphate buffer (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 2% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 17,600 × g for 20 min at 4 °C and filtered through Whatman No. 4 filter paper. The filtrate was used for measuring the activities of antioxidant system enzymes.

Peroxidase (POD) and catalase (CAT) enzyme activities were assayed according to Kato and Shimizu [32]. The POD reaction solution (2.1 mL) contained 50 mM phosphate buffer (pH 6.5), 20 mM guaiacol, and 0.2 mL extract. The reaction was initiated at 24 °C by the addition of 40 mM H₂O₂ and the change in the optical density at 470 nm was recorded. The activity of CAT was assayed by measuring the initial rate of disappearance of H₂O₂. The CAT reaction solution (2.1 mL) contained 50 mM phosphate buffer (pH 7.0) and the 0.2 mL enzyme extract. The reaction was initiated at 24 °C by adding 5 mM H₂O₂. The absorbance of the reaction solution at 240 nm was measured every 5 s.

Ascorbate peroxidase (APX) enzyme activity was determined according to Nakano and Asada [33], with the following modification [31]: The reaction solution (2.1 mL) consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, 1 mM H₂O₂, and 0.2 mL enzyme extract. The decrease in ascorbate was followed at 24 °C as a decline in optical density at 290 nm.

All enzyme activities were expressed as units 100 g⁻¹ FW, where one unit of enzyme was defined as an absorbance change of 0.001 units min⁻¹.

2.7. Experimental Design and Statistical Analysis

Data were analyzed by analysis of variance in IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp. using a completely randomized design with three

replications per amino acid and NaCl concentration, with one basin per replication and 16 plants in each specie per replication. Means were separated by the least significant difference (LSD) test at the $p < 0.05$ level.

3. Results and Discussion

3.1. EC, pH, and Temperature of the Nutrient Solution

Electrical conductivity is an indicator of the ionic balance of the nutrient solution (NS). Any change that occurs during plant growth leads to alterations in EC values of the nutrient solution. The water and nutrient absorption by plants during cultivation and the high water and nutrient use efficiency leads to an increase in electrical conductivity in the solutions [5]. Simultaneously, electrical conductivity is also an indication of osmotic pressure in the NS.

In the present study, the electrical conductivity (EC) of the control NS (without any amino acid or NaCl addition) was 3.5 mS/cm at the beginning of the experiment and was gradually increased up to 6.2 mS/cm before the refill of the tanks with fresh nutrient solutions at the 4th week of cultivation when it dropped back to 3.6 mS/cm and never increased higher than 4.6 mS/cm thereafter (Figure 1).

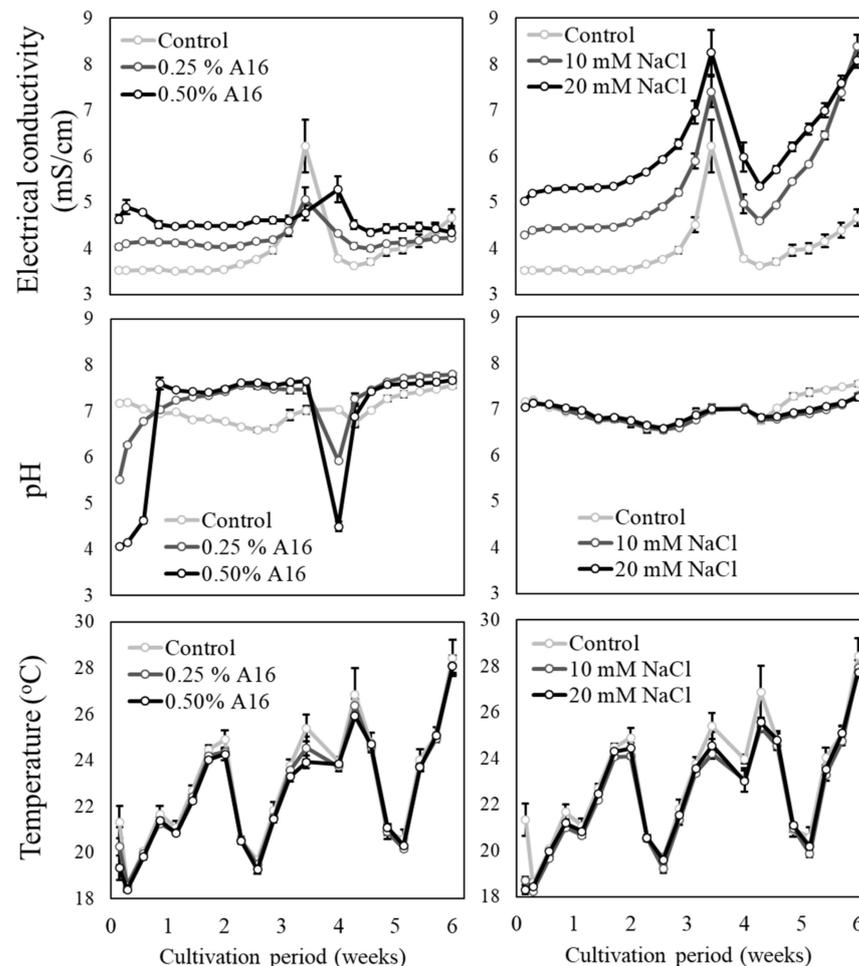


Figure 1. Electrical conductivity, pH, and temperature in the nutrient solution of the hydroponic cultivation of peppermint and spearmint plants grown after supplemented with either 0, 0.25, or 0.50% of a commercial amino acid solution (Amino16[®]) or 0, 10, and 20 mM NaCl. Each line is the mean of 3 replications and vertical bars represent the standard errors. In each basin 45 L of nutrient solution were added at the initiation of the experiment, as well as 4 weeks later.

The EC of the solution at the highest concentration of amino acids (0.50%) was 4.6–5.28 mS/cm during the growing period, while at the low amino acid NS (0.25%) it was 4.0–5.1 mS/cm (Figure 1). When NaCl was added in the nutrient solutions, the electrical conductivity increased significantly compared to the control. In particular, by adding 10 mM NaCl, the EC value was raised from 4.3 to 7.4 mS/cm at the 4th week and at 8.4 mS/cm, two weeks after the refill of the tank (Figure 1). When 20 mM NaCl were added to the solution, the EC value was raised from 5.0 to 8.1–8.3 mS/cm both at the end of the 4th and 6th week of the cultivation season, respectively. In general, the electrical conductivity of the highest salinity level was always higher than the highest amino acid supplemented solution and the same was in the case of low NaCl and amino acid level.

The increase of EC, which was induced upon the addition of salts in the nutrient solution, is related to the presence of concentrated Na^+ and Cl^- ions at the root zone area [34]. Moreover, the increased EC due to amino acid supplement in the nutrient solution was caused by the chemical dissolution of the amino acids, which determines the final concentration of ammonium (NH_4^+) and nitrates (NO_3^-) around the root zone [35] and may in turn significantly affect the osmotic potential [36,37].

Although the pH of the NS was not affected by NaCl during the whole cultivation period (7.1–7.6) (Figure 1), it was observed that upon AA addition, the pH dropped significantly, reverse linearly to the AA concentration reaching 5.5–5.9 in 10 mM NaCl and 4.1–4.6 in 20 mM NaCl at the initiation of the experiment or the refill of the NS, but increased rapidly in less than a week at approximately 7.6–7.7 (Figure 1).

The temperature in the NS of both AA and NaCl solutions was not affected by the treatments but fluctuated during the growing period, while the absolute values were in the range of 18.4–28.1 °C (Figure 1).

3.2. Plant Growth

The protein hydrolysate supplement of the solutions has already been reported to affect both the plant nutritional composition as well as eventually the growth of the plant harvested at the vegetative herbaceous stage [5]. Fresh weight, plant height, and root length in the plants near the flowering stage were significantly affected by the amino acid concentrations, according to the analysis of variance (Table 1). In particular, the fresh weight of both mint species decreased only at 0.50% AA concentration and similar results were also observed in the plant height, where at the highest AA concentration (0.50%), the lowest height of peppermint and spearmint plants was measured. Moreover, the root length of plants was also significantly affected by the inclusion of amino acids in the nutrient solution. In both species, the root length of plants decreased while the AA concentrations increased from 0% (control) to 0.25% and 0.50% (Table 1).

Table 1. Mean weight, height and root length of peppermint and spearmint plants grown hydroponically in a floating system on a nutrient solution supplemented either with 0, 0.25 or 0.50% of a commercial amino acid solution (Amino16[®], EVYP LLP) or 0, 10 and 20 mM NaCl.

Source of Variation	Peppermint						Spearmint					
	Plant Weight (g)		Plant Height (cm)		Root Length (cm)		Plant Weight (g)		Plant Height (cm)		Root Length (cm)	
Amino16 (%)	***		*		***		*		*		***	
0	20.37 ^y	a ^z	34.45	a	12.43	a	16.73	a	37.62	a	11.02	a
0.25	18.35	a	33.77	a	5.45	b	17.65	a	35.76	a	4.47	b
0.50	11.90	b	28.79	b	0.74	c	13.08	b	28.24	b	0.94	c
NaCl (mM)	ns		ns		*		ns		ns		*	
0	20.37		34.45		12.43	b	16.73		37.62		11.02	b
10	25.67		35.97		16.58	a	18.52		39.42		13.03	a
20	24.98		33.37		16.28	a	16.19		33.47		12.22	ab

***, * or ns: Significant effect at $p < 0.001$, $p < 0.05$ or non significant, respectively, according to ANOVA. ^y each value is the mean of 3 replications with 16 plants per replication. ^z means in the same column per treatment (Amino16 or NaCl) followed by different letters are significantly different, according to the least significant differences test (LSD) at $p < 0.05$.

When plants are exposed to abiotic stresses such as low temperature, salinity, heat, or drought, several changes in their physiological status can be observed and plant productivity is restricted [38,39]. In the present study, the highest AA concentration (0.50%) reduced the fresh biomass production probably due to the induction of plant osmotic stress. Kravić et al. [40] reported that a mild osmotic pressure caused a decrease in fresh weight and root length in maize plants, while the crop yield in tomato and the plant height were reduced while increasing the electrical conductivity (EC) of the nutrient solution [41]. Similar results were reported by Ünlükara et al. [42] when the total yield of lettuce was reduced after the plants were irrigated with water at high EC level (6.0 mS/cm). On the other hand, Aktsoglou et al. [5] reported that the plant growth was not affected by the addition of AA in nutrient solution in fresh mint and spearmint production, probably because the plants were harvested at a very early vegetative stage, before any visual symptoms had been exhibited.

In the present study, the plant development was not significantly affected by NaCl addition in the NS at both salinity levels (10 mM and 20 mM), with the exception of root length (Table 1). Neither the plant weight nor plant height in both mint species were affected, while root length was increased at >10 mM NaCl.

3.3. Antioxidant Compounds and Enzymatic Activities

When plants are exposed to abiotic stress conditions, an accumulation of reactive oxygen species occurs, which may lead to cell death upon reaching extreme accumulation rates [16]. Therefore, it is important that the enzymatic and non-enzymatic antioxidant mechanism be rapidly activated, thus preventing any adverse consequences in the plant physiology and development [15].

In the present study, the supplement of NS with 0.50% AA resulted in an increase in the total antioxidant capacity in the peppermint (366 mg AEAC 100 g⁻¹ FW) and spearmint plants (298 mg AEAC 100 g⁻¹ FW) as well as in phenolics (1.31 mg GAE Kg⁻¹ FW and 1.10 mg GAE Kg⁻¹ FW, respectively) (Figures 2 and 3). This finding implies that the non-enzymatic antioxidant mechanism was activated in both mint species during cultivation at high AA concentrations, in order to counteract any stressful factors.

High antioxidant capacity and phenolic content were also observed in peppermint, spearmint, and lettuce plants that were grown in a similar floating system when the concentration of amino acids in the nutrient solution increased [5,8]. Although antioxidants such as ascorbic acid and flavonoids were increased in bean plants grown under saline conditions [43], according to our results, no significant differences were found in the phenolic and the remaining non-enzymatic antioxidant content in both mint species at both levels of salinity (10 and 20 mM NaCl) (Figures 2 and 3 and Table 1). Apparently, the plants did not suffer from the salinity level at these EC values and for this reason, no changes in plant growth at the end of cultivation were observed.

The osmotic stress of peppermint and spearmint plants resulted in the activation of the enzymatic antioxidant mechanism. In particular, the activities of the antioxidant enzymes APX and POD in spearmint plants were significantly higher after AA supplement in the nutrient solution at the highest concentration (Table 2). In peppermint plants grown under 0.50% AA, the APX activity was also significantly higher, although no significant effect was observed in POD activity. Although not significant, there was a trend of increased content of malondialdehyde (MDA) and proline content in both species, linearly to the concentration of the AA concentration (Table 3). Moreover, in both mint species, neither the accumulation of hydrogen peroxide (H₂O₂) was affected by the AA supplement nor the CAT activity (Table 3).

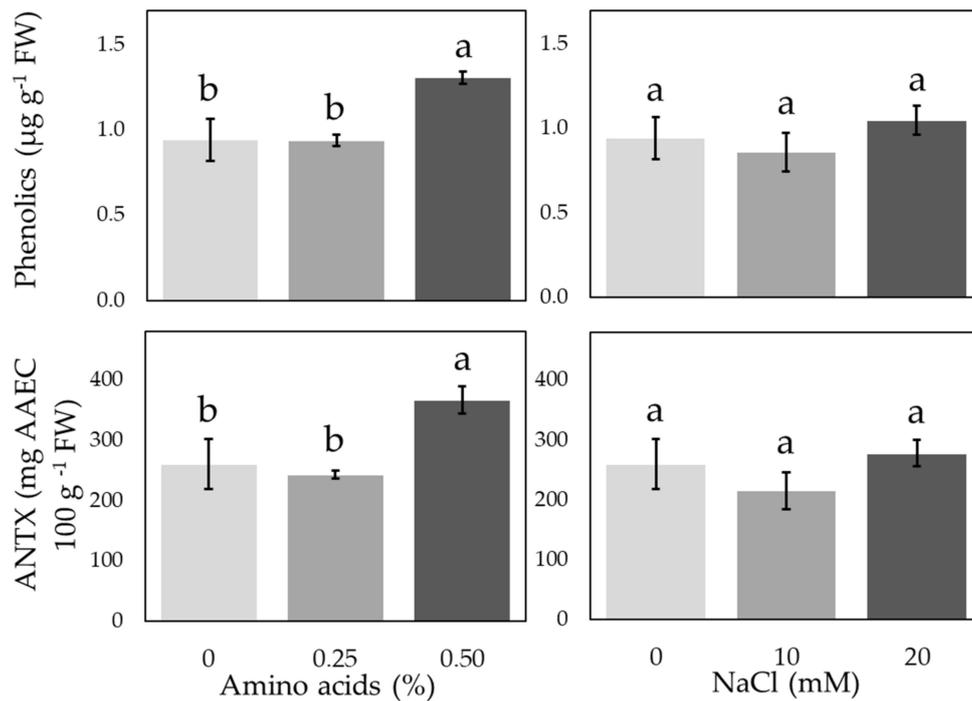


Figure 2. Soluble phenolic content and total antioxidant capacity (ANTX) of peppermint plants grown hydroponically in a floating system on a nutrient solution supplemented either with 0, 0.25, or 0.50% of a commercial amino acid solution (Amino16[®]) or 0, 10, and 20 mM NaCl. Each column is the mean of three replications with 16 plants per replication. Different letters on the columns in each treatment (amino acids or NaCl solution) indicate significant differences between mean, according to the least significant differences test (LSD) at $p < 0.05$.

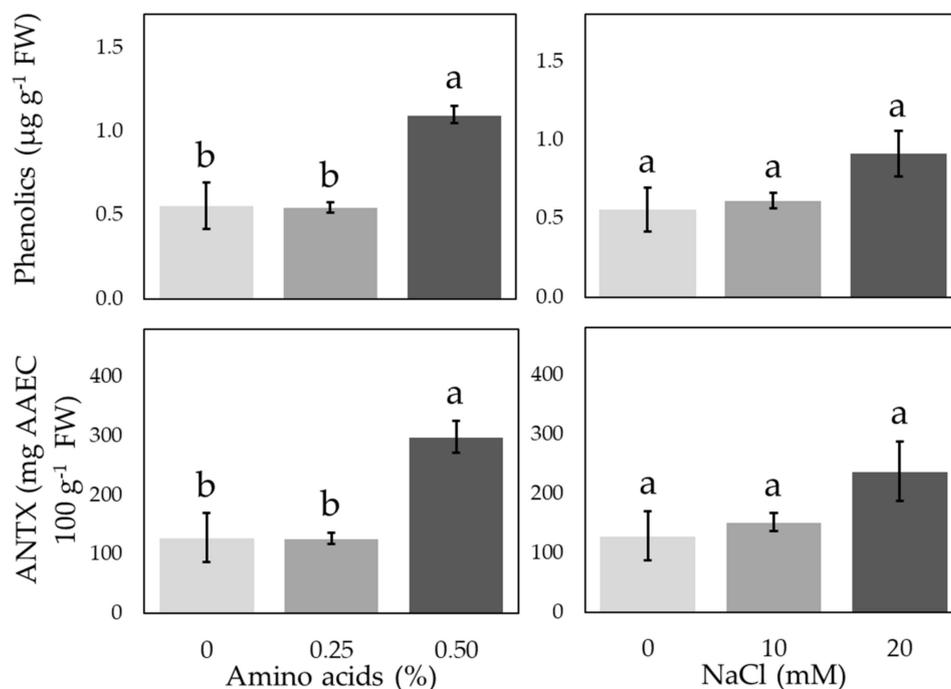


Figure 3. Soluble phenolic content and total antioxidant capacity (ANTX) of spearmint plants grown hydroponically in a floating system on a nutrient solution supplemented either with 0, 0.25 or 0.50% of a commercial amino acid solution (Amino16[®]) or 0, 10 and 20 mM NaCl. Each column is the mean of 3 replications with 16 plants per replication. Different letters on the columns in each treatment (amino acids or NaCl solution) indicate significant differences between mean, according to the least significant differences test (LSD) at $p < 0.05$.

Table 2. Peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) enzyme activities of peppermint and spearmint plants grown hydroponically in a floating system on a nutrient solution supplemented either with 0, 0.25 or 0.50% of a commercial amino acid solution (Amino16[®], EVYP LLP) or 0, 10 and 20 mM NaCl.

Source of Variation	Peppermint			Spearmint		
	POD (U/100 g FW)	CAT (U/100 g FW)	APX (U/100 g FW)	POD (U/100 g FW)	CAT (U/100 g FW)	APX (U/100 g FW)
Amino16 (%)	ns	ns	*	*	ns	*
0	106 ^y	2.33	5.10	111	1.87	5.93
0.25	103	2.73	6.43	125	3.67	8.87
0.50	131	2.47	8.50	132	3.53	12.50
NaCl (mM)	ns	ns	ns	ns	*	ns
0	106	2.33	5.00	111	1.87	5.93
10	106	2.43	5.00	112	3.30	7.10
20	109	2.37	6.00	123	2.33	7.80

* or ns: Significant effect at $p < 0.05$ or non significant, respectively, according to ANOVA. ^y each value is the mean of 3 replications with 16 plants per replication. ^z means in the same column per treatment (Amino16 or NaCl) followed by different letters are significantly different, according to the least significant differences test (LSD) at $p < 0.05$.

Table 3. Malondaldehyde, hydron peroxide and proline content of peppermint and spearmint plants grown hydroponically in a floating system on a nutrient solution supplemented either with 0, 0.25 or 0.50% of a commercial amino acid solution (Amino16[®], EVYP LLP) or 0, 10 and 20 mM NaCl.

Source of Variation	Peppermint			Spearmint		
	MDA (nmol/g FW)	H ₂ O ₂ (μmol/g FW)	Proline (mmol/g FW)	MDA (nmol/g FW)	H ₂ O ₂ (μmol/g FW)	Proline (mmol/g FW)
Amino16 (%)	ns	ns	*	ns	ns	ns
0	0.529 ^y	1.108	0.198	0.442	0.399	0.197
0.25	0.558	1.127	0.259	0.500	0.397	0.280
0.50	0.677	1.111	0.359	0.511	0.406	0.210
NaCl (mM)	ns	ns	ns	ns	ns	ns
0	0.529	1.108	0.200	0.442	0.399	0.197
10	0.400	0.740	0.250	0.433	0.440	0.280
20	0.427	0.913	0.240	0.412	0.440	0.210

* or ns: Significant effect at $p < 0.05$ or non-significant, respectively, according to ANOVA. ^y Each value is the mean of three replications with 16 plants per replication. ^z Means in the same column per treatment (Amino16 or NaCl) followed by different letters are significantly different, according to the least significant differences test (LSD) at $p < 0.05$.

On the other hand, upon the addition of NaCl in the NS, although the electrical conductivity was significantly increased (Figure 1), neither the activity of the antioxidant enzymes (APX, POD, and CAT) affected neither proline, MDA, or H₂O₂ accumulated in both species, in comparison to the control (without AA or NaCl) plants (Tables 2 and 3). According to the literature, the enzymatic activity of the antioxidant mechanism (POD, CAT, APX) in lettuce and cucumber plants was enhanced when plants were grown under salinity conditions (40 and 100 mM NaCl, respectively), which in turn triggered the synthesis of MDA and H₂O₂ in their tissues [44,45].

Obviously, the stressful adverse conditions in plant development upon the inclusion of amino acids in the NS at the highest concentration (0.50%) were not induced by the elevated electrical conductivity in the solution but were most probably due to the increased accumulation of NH₄⁺ cations. This assumption was further confirmed by the fact that while the EC also increased with the NaCl addition, the antioxidant mechanism of plant defense in both species was not activated, and therefore the high value of EC was determined only by the concentration of ions in the nutrient solution. Moreover, our assumption regarding the toxic effect of nutrient accumulation is also supported by the study of Ding et al. [34] in the hydroponic production of pak-choi, where the lower plant growth and leaf size at the highest EC (9.6 mS/cm) was attributed to the concentrated nutrient solution. Apart from the NaCl salt, the addition of KCl or CaCl₂ salts to the nutrient solution led to a large increase in EC up to 8 mS/cm, which in turn induced an osmotic stress in pepper plants that was exhibited with the accumulation of proline [46].

Increased proline content in the roots and leaves of green bean plants was found when nitrate and ammonia ions were supplied in excessive rate [47]. Besides, it was previously reported that nitrogen fertilization can express a dual effect in osmotic stress reaction; when the ammonium form was used for nitrogen supply in maize cultivation, the proline content increased in plants in order to serve as an osmolyte, but when the nitrate form was applied, the antioxidant enzyme activities were accelerated [37].

3.4. Chlorophyll Fluorescence

The Kautsky curves enable major changes that occur during exposure of plants to high irradiance to be observed. The OJIP protocol provides several numerical parameters depicting the status of photosystem II and the operations taking place in that [26]. Each parameter refers to a specific function of the photosystem and describes the fluorescence intensity as it changes over time as well as the energy flow in the molecules of the photosystem complex.

In the present study, two parameters of the OJIP protocol were selected, the Sm parameter calculated by the equation $Sm = Area / (F_m - F_o)$, which represents part of the energy that is required so that all the reaction centers (RC) are closed [48], and the ratio TRo/RC, an index that represents the energy flow at the reaction center through the photosystem II during photosynthesis [49].

In the case of peppermint, the plants near harvesting that were previously grown on the highest (0.50%) AA concentration had the highest Sm value of (0.606), followed by the plants on 0.25% AA concentration (0.584), while the untreated ones (without AA in the NS) had a significantly lower value of Sm parameter (0.550) (Figure 4). The same pattern was observed in the case of spearmint plants where the Sm values were 0.471, 0.475, and 0.502 for the concentrations of the 0, 0.25, and 0.50% amino acid solutions, respectively (Figure 5).

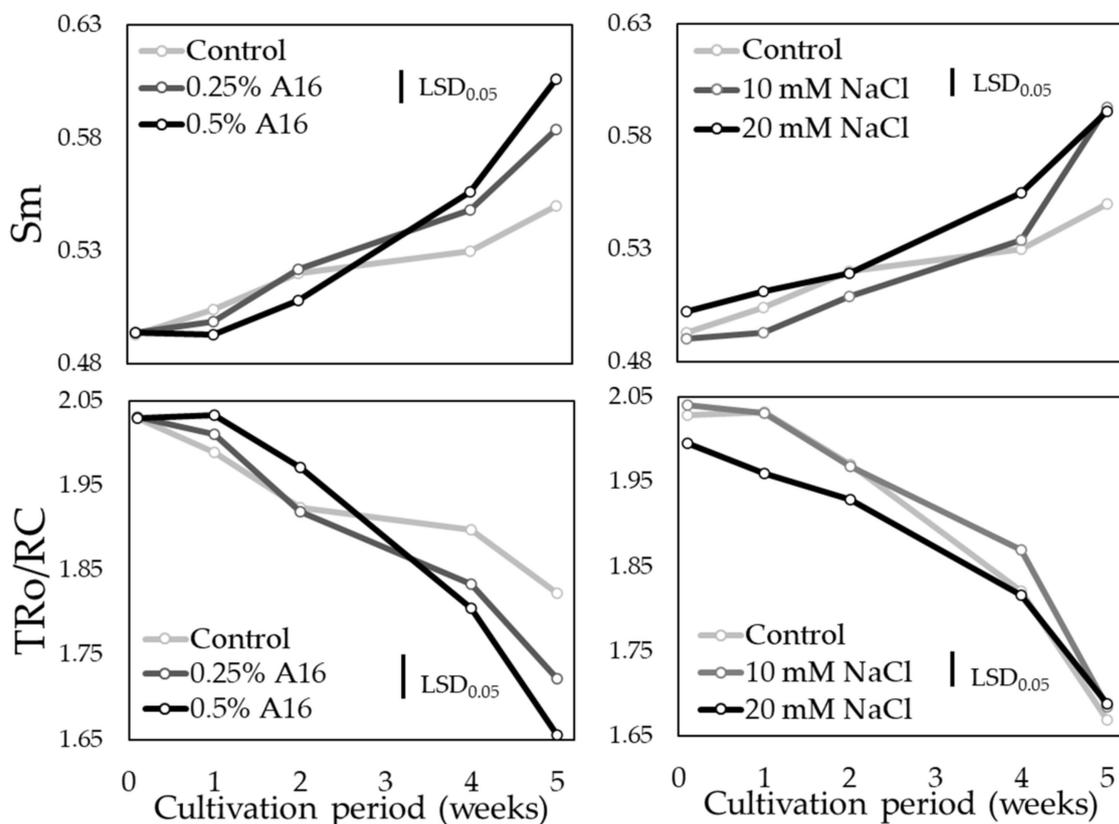


Figure 4. Chlorophyll fluorescence parameters (Sm and TRo/RC) of peppermint plants grown hydroponically in a floating system on a nutrient solution supplemented either with 0, 0.25, or 0.50% of a commercial amino acid solution (Amino16[®]) or 0, 10, and 20 mM NaCl. Each column is the mean of three replications with 16 plants per replication. The vertical lines depict the least significant differences values (LSD) at $p < 0.05$.

When plants perceive any adverse conditions, the leaf tissue reaction centers close, the electron flow to the plastoquinone pool is halted, and consequently, the chlorophyll intensity is restricted [50]. Considering the TRo/RC parameter, which refers to the electron flux in photosystem II (PSII) and provides useful information about cells' photochemistry, the results were definite; the TRo/RC ratio for peppermint plants had the lowest value (1.656) when 0.50% AA was added to the nutrient solution, while the untreated sample exhibited significantly higher value (1.823) at the end of the cultivation period, followed by plants grown with 0.25% AA concentration (1.721) (Figure 4). The changes in the ratio TRo/RC during cultivation exhibited the same pattern in as in the case of the Sm parameter in both species; the lowest TRo/RC value (2.007) in spearmint was recorded when plants were grown under the highest AA concentration (0.50%) in the nutrient solution, while significantly higher values (2.134 and 2.114) were observed for untreated and 0.25% AA supplemented solutions, respectively. This impairment in the electron flux at PSII apparently reduces the available energy for ATP and NADH synthesis in both herb species and was manifested by the suppression in plant growth (weight, height, and root length) at the highest (0.50%) AA concentration.

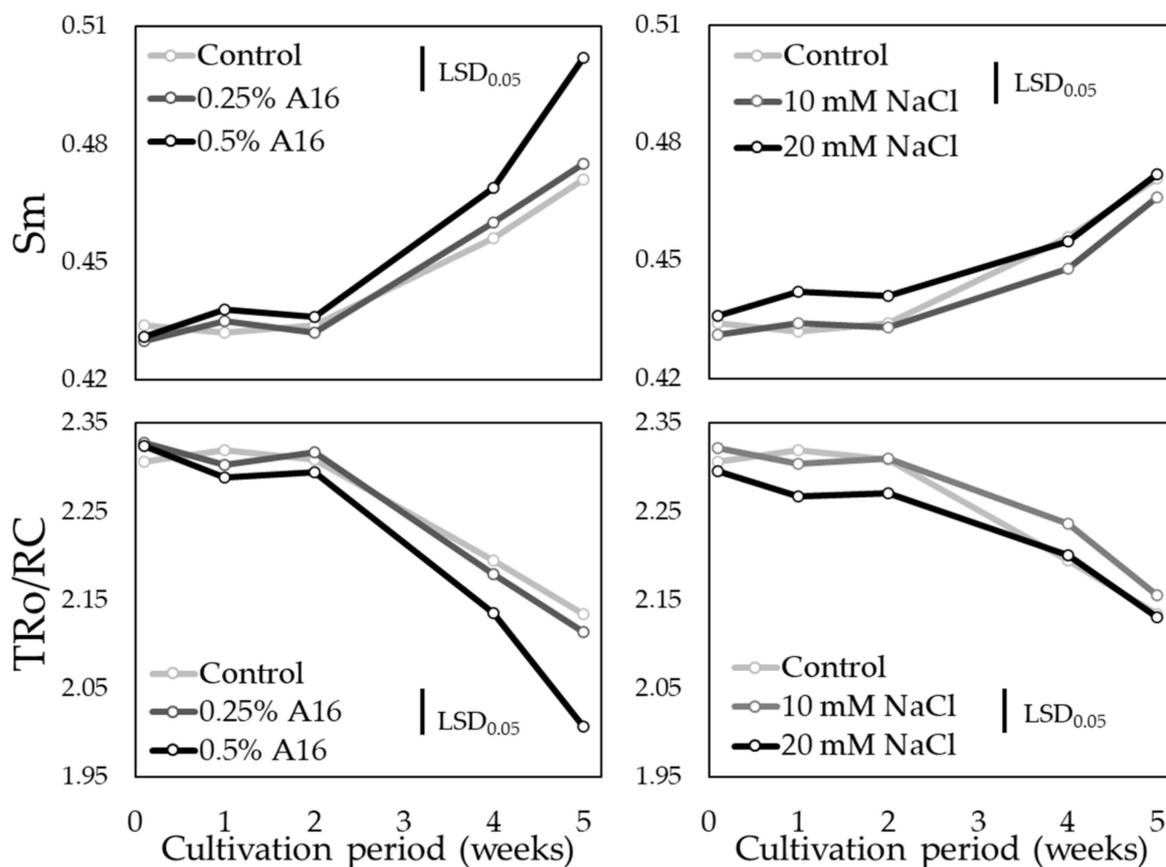


Figure 5. Chlorophyll fluorescence parameters (Sm and TRo/RC) of spearmint plants grown hydroponically in a floating system on a nutrient solution supplemented either with 0, 0.25, or 0.50% of a commercial amino acid solution (Amino16[®]) or 0, 10, and 20 mM NaCl. Each column is the mean of three replications with 16 plants per replication. Different letters in the columns indicate each treatment (amino acids or NaCl solution). The vertical lines indicate significant differences between mean, according to the least significant differences test values (LSD) at $p < 0.05$.

The addition of NaCl in the nutrient solution did not affect the operation of photosynthetic apparatus (Figures 4 and 5). At the end of the growing season, the values of the Sm parameter for both species did not differ between salinity treatments and the control, while similar results were observed for energy flow in photosystem II, with no differences in the TRo/RC parameter between three treatments (Figures 4 and 5). According to Havaux [51],

under salinity stress, a lower energy flux and efficiency of PSII is established and indeed, in wheat and tomato plants, the gradual increase in salt concentration resulted in decreases of TRo/RC in leaves, which could be attributed to the inactivation of PSII reaction centers and the reduced efficiency of electron transfer in PSII [52].

However, in our study, the energy flux in photosystem II was not affected by the high EC in the nutrient solutions. The fact that the chlorophyll fluorescence in both mint species under 10 and 20 mM of NaCl was not significantly affected implies the absence of salt stress, which may partially be attributed to the high salt tolerance of peppermint and spearmint species and that the osmotic stress induced in the amino acid treated plants was related to NH_4^+ excess supply in the growing media.

4. Conclusions

According to the above, it can be concluded that spearmint is mostly favored by the highest amino acid supplement of the nutrient solution (0.50%) in terms of a substantial improvement in the antioxidant nutritional quality (by up to 130%) at the expense of a reduced biomass production (by <30%). Enzymic antioxidant defense mechanism (APX and POD) was efficiently activated, preventing severe lipid peroxidation and the accumulation of reactive oxygen species such as H_2O_2 and maintaining the proline content at the normal levels. The osmotic stress that was induced by the excessive AA concentration and was confirmed by the chlorophyll fluorescence variations was probably related to NH_4^+ excess supply in the growing media and was not associated with the elevated electrical conductivity in the solution. The absence of any adverse stressful consequences upon the addition of 20 mM NaCl may be attributed to the high salt tolerance of peppermint and spearmint species.

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