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Short Communication

The Effect of Aminolevulinic Acid on Physiological and Biochemical Characters of Red-fleshed Apple (*Malus sp. Genotype R_1R_1)* under Salinity Stress

Fatemeh Zahedzadeh, Fariborz Zaare-Nahandi*, Mohammad Reza Dadpour, Alireza Motallebi Azar and Saeide Alizadeh Salteh

Department of Horticultural Sciences, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

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Abstract

The present study was conducted to evaluate the ameliorating effect of 5-aminolevulinic acid (ALA) on physiological and biochemical changes of induced NaCl-salinity stress on in vitro shoot and callus cultured of red-fleshed apple. Shoot and callus segments of the red-flesh apple of Local Iranian genotype were cultured on MS medium containing different levels of NaCl (0, 30, 60 and 90 mM) and ALA (0, 2.5, 5, 10 and 20 µM). Physiological and biochemical variations of treated explants with or without NaCl and ALA treatments were recorded. In both explants, salinity reduced chlorophyll and carotenoids contents, but the activities of antioxidant enzymes and accumulation of total phenol and anthocyanin increased with increasing salinity level (90mM). Exogenous ALA in 5 and particularly in 10 µM was effective in enhancing chlorophyll and carotenoids contents, increasing the activities of superoxide dismutase, ascorbate peroxidase and accumulating of total phenol and anthocyanin. These results indicate that ALA has a powerful salinity-ameliorating potential on in vitro cultured shoot and callus of Iranian red-fleshed apple.

Keywords: Red-fleshed apple, Salinity, 5-aminolevulinic acid, Antioxidant, Anthocyanin

Introduction

Nowadays, it is also evident that exogenous application of plant growth regulators (PGRs) is an accessible and simple way to increase the plant tolerance against abiotic stresses such as salinity. Salinity stress is known to adversely affect plant growth and development that influence a variety of physiological and biochemical processes in plants. The pigmentation of red apple fruits results from the accumulation of anthocyanin in the fruit skin, also in numerous genotypes of apple anthocyanin synthesizes in core and cortex (flesh) tissues [1]. Anthocyanins belongs to the diverse classes of secondary metabolites, known as flavonoids. Anthocyanins biosynthesized from phenylalanine through the phenylpropanoid pathway [2], which is influenced by environmental factors, such as light, temperature, salinity and water deficiency a plant growth regulators (PGR) as internal factors [3]. These compounds improve the growth and productivity of plants by regulating the physiological and biochemical reactions in stressed plants. 5-Aminolevulinic acid (ALA) a precursor for biosynthesis of all porphyrins compounds such as heme, chlorophyll ,phytochrome, heme and vitamin B_{12} [4]. Exogenous ALA in low concentrations increases anthocyanin accumulation in apple fruit [5,6]. Wang *et al*. [5] reported that ALA induces anthocyanin biosynthesis via increasing phenylalanine ammonia-lyase (PAL) activity, the critical enzyme in phenylpropanoid pathway.

Also, ALA is known to play roles in regulating plant tolerance to various abiotic stresses. Recent research has found that ALA exhibits PGR properties, promoting plant growth under standard environments and stressful conditions when applied

∗Corresponding author: Department of Horticultural Sciences, Faculty of Agriculture, University of Tabriz, Tabriz, Iran
Email Address: fzaare@tabrizu.ac.ir

at low concentrations [7]. Improved salinity tolerance with ALA application has been reported mostly in dicot species. Successful recovery from salt damage in cotton, tomato, and wheat seedlings was reported by foliar spraying of ALA (8). Watanabe *et al*. [9] reported that among 12 representatives PGRs examined, ALA was the most effective in promoting plant tolerance to salinity. The positive effects of ALA in mitigating the adverse salt-induced effects associated with the improving reactive oxygen species scavenging compounds and enzymes, reduction in Na^+/K^+ ratio and increasing the photosynthetic assimilation [10-12]. However, until now the mechanism by which ALA promoted anthocyanin biosynthesis is not obvious.

The objectives of this study is evaluation of the physiological and biochemical effects of ALA application under salinity stress in shoot and callus of red-fleshed apple variety in vitro.

Material and Methods

Plant Materials

The annual shoots of the red-fleshed apple of Local Iranian Genotype from Semnan province were chosen for in vitro culture. The shoots segments with 2-3 cm in length were soaked in a detergent solution for 15 min, in 70% ethanol for 5 min and 1% sodium hypochlorite for 20 min then, were rinsed three times with sterile distilled water under aseptic condition. For proliferation the shoots were cultured on the optimized MS medium [13] supplemented with 2 mg l^{-1} BAP (6-benzylaminopurine)+1 mg l^{-1} GA_3 (gibberellic acid) +0.1 mg l^{-1} IBA (indole-3-butyric acid). This medium contained 0.1 mg l^{-1} of iron (FeEDDHA), and 0.05 mg l^{-1} activated charcoal with a pH of 5.7±0.05 and solidified with 0.8% Agar. The treated explants were maintained at a temperature of 25±1 °C under a 16/8 h light regime provided by white fluorescent tubes at an intensity of 40 µmol m^{-2} s^{-1}. Chemical material purchase Sigma Company.

For callus induction, the sterilized leaves with approximately 0.5 cm length, were excised and were cultured on MS media supplemented with 0.5 mg l^{-}NAA and 1 mg l^{-1} 2,4-D. All the cultures were then maintained at 25±1 °C in the dark.

Treatments and Experimental Design

The proliferated shoots and callus explants respectively with 3, and 0.5 cm length were excised and separately cultured on MS medium containing different concentrations of NaCl (0, 30, 60 and 90 mM), with or without ALA (0, 2.5, 5, 10 and 20 µM). Each experiment for shoot and callus was conducted as a factorial on the bases of completely randomized design with four replications.

Tissue samples for all analyses were collected from in vitro plantlets and callus under different treatments after eight weeks of culture.

Quantification of Pigments

Total chlorophyll and carotenoids were extracted from explants using 80% acetone according to Lichtenthaler [14] method. The absorbance of the pigments was measured by UV visible spectrophotometer (Analytik Jena Spekol 1500) at 470, 648, and 664 nm. The concentrations of total chlorophyll and carotenoids after calculation were expressed as mg of each pigment per g fresh weight.

Radical Scavenging Assay

About 1 g of fresh samples was extracted with 10 ml methanol 85% at room temperature, then 50 µl of the methanolic extract was rapidly mixed with 1950 µl of DPPH methanolic solution (40 mg L^{-1}, Sigma). After incubation, the absorbance at 515 nm was measured. The DPPH solution without sample was used as a control.

Radical scavenging activity of plant extracts against stable 2, 2 diphenyl-2-picrylhydrazyl hydrates (DPPH) was determined according to a method of Brand-Williams *et al*. [15]

Antioxidant Enzymes Assay

For assay of antioxidant enzymes, 1 g of fresh explants were homogenized in 10 ml of 100 mM potassium phosphate buffer (pH 6.8) then was centrifuged at 12,000 g for 20 min at 4 °C and the supernatant fraction used as the source of enzymes. The enzyme's activity was expressed based on mg protein of each sample, so the amount of soluble protein in the extract was determined using the Bradford [16] protein assay, with bovine serum albumin as the standard.

The specific activity of catalase (CAT, EC 1.11.1.6) was estimated by UV method of Aebi [17] and reported as mmol decomposed H_2O_2 per min per mg soluble protein (U mg^{-1}), by using the H_2O_2 extinction coefficient of 39.4 mM^{-1} cm^{-1}. The activity of peroxidase (POX, EC 1.11.1.7) was

determined by guaiacol and H_2O_2 substrates as described by Putter [18] and was expressed as mm produced tetraguaiacol (with 26.6 mM^{-1} cm^{-1} extinction coefficient) per min per mg soluble protein (U mg^{-1}). The Ascorbate peroxidase activity (APX, EC 1.11.1.11) was determined according to Nakano and Asada [19] and expressed as mmol ascorbate oxidized per min per mg soluble protein (U mg^{-1}) with 2.8 mM^{-1} cm^{-1} as the extinction coefficient. The activity of superoxide dismutase (SOD, EC 1.15.1.1) was measured by enzyme ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) as described by Beyer and Fridovich [20]. The SOD activity was reported as units per mg soluble protein (U mg^{-1}).

Quantification of Total Phenol and Anthocyanin

Total phenol content was measured by the Folin-Ciocalteu reagent [21]. Briefly, 1 g of fresh samples were extracted with 2 ml 85% methanol then centrifuged at 12,000 g for 10 min. 50 µl of the supernatant was diluted with 450 µl of distilled water, and subsequently, 2.5 ml of freshly prepared 10% Folin-Ciocalteu reagent was added. 2 ml of 7.5% sodium carbonate solution was added, and the absorbance was measured at 760 nm. The phenolic concentration of samples was evaluated from a gallic acid calibration curve, and the total phenol content was expressed as mg of gallic acid equivalent per g fresh weight.

The anthocyanin content of samples was determined by the pH-differential method [22]. Briefly, 1 g samples were extracted with 2 ml 85% methanol. Then 1 ml of extraction separately diluted with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5. The absorbance of each dilution was recorded at the 510 and 700 nm and total anthocyanin of samples calculated as follows:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$$

Total anthocyanin was expressed as mg anthocyanin per kg fresh weight.

Statistical Analysis

Results were subjected to analysis of variance using the statistical analysis program (SPSS ver.22.0). The mean values were compared by Duncan's multiple range test at $p \leq 0.05$.

Results and Discussion

Effects of imposed NaCl-salinity and ALA on most studied parameters in both shoot and callus explants were significant. Salinity and ALA significantly influenced total chlorophyll and carotenoids contents of treated shoots and calli ($p \leq 0.01$). Also, the interaction effects between treatments were significant except for total chlorophyll in shoot explants ($p \leq 0.05$). Increasing salinity stress significantly reduced the total chlorophyll and carotenoids contents respectively down to 48.09 and 50.22% in shoot samples and 30.89 and 55.19% in callus samples. ALA treatment increased photosynthic pigments in unstressed explants. Also, the application of ALA in 5-10 µM efficiently prevented chlorophyll and carotenoids decline in stressed plantlets. However, the high concentration of ALA (20 µM) did not show a positive effect in control of adverse effect of salinity (Table 1, 2). Also, the reduction of chlorophyll and carotenoids contents under salinity stress was reported in many other plant species [23]. Several authors reported that exogenous ALA increased photosynthetic pigments in salinity-stressed plants [12,23,24]. ALA is a crucial precursor of chlorophyll so exogenous ALA application could contribute to the enhancement of chlorophyll biosynthesis [24].

Analysis variance showed that radical scavenging activity of treated shoot and callus explants significantly influenced by salinity and ALA treatment ($p \leq 0.01$). The interaction effects of treatments were non-significant ($p \leq 0.05$) and significant ($p \leq 0.01$) in shoot and callus explants respectively. Radical scavenging activity under severity salinity stress (90 mM) 4.25 and 16.59% was increased in comparison with un-stressed explants in shoot and callus explants, respectively. ALA application in 5-10 µM was increased the radical scavenging activity up to 6.11 and 5.41% in shoot and callus explants, respectively (Table 1, 2).

Plant cells are protected from the adverse effect of salinity stress by an antioxidant defense system that detoxifies reactive oxygen species [25]. The plant antioxidant defense could be enzymatic (SOD, APX, CAT, and POX) or non-enzymatic (glycin between, proline and phenolic compounds).

In both shoot and callus explants, salinity and ALA significantly affected CAT, POX, APX and SOD activities by statistical analysis. The interaction effect of salinity and ALA for antioxidant enzymes activities was significant ($p \leq 0.05$) except APX activity in shoot explants. Measured antioxidant

enzymes (APX and POX) showed a significant increase in response to increasing salinity stress at all levels of ALA treatment in treated shoot and callus explants. CAT and SOD activities of ALA treatment and salinity stress were minimal and CAT and SOD remained relatively unchanged. Antioxidant mechanisms may provide a strategy to enhance salt tolerance in plants, and many changes have been found in the activity of antioxidant enzymes in plants under salinity stress [26]. In the present study, enzymes responded differently to exogenous application of ALA. In both explants, the activities of CAT and POX partial decreased with increasing ALA up to 10 µM under all salinity levels but the activities of APX and SOD increased (Table 1, 2). A considerable increase in the activities of antioxidant enzymes was reported in different plant species in response to ALA treatment [27][28]. However, in pepper seedling [7] and sunflower [29], the activities of antioxidant enzymes did not show increasing trends due to exogenous application of ALA.

The effects of salinity and ALA treatments, as well as their interaction, were significant on phenol content of shoot and callus explants ($p \leq 0.01$). The results indicated that phenol content of treated shoot and callus explants increased with increasing salinity level-up to 13.78 and 16.22% respectively. Treated shoot and callus with 5-10 µM ALA increased phenol content by 19.46 and 9.45% in non-stressed explants, and by 12.30% and 14.90% in explants grown under severe salinity stress, respectively (Table 1, 2). Giorgi *et al*. [30] reported that the amount of phenolic compounds in plants is significantly perturbed depending on the sensitivity of plant species to salinity. Noreen and Ashraf [31] showing no change in phenolic contents in radish under saline stress. Analysis variance showed that salinity, ALA and their interaction for anthocyanin content of shoot and callus were also significant ($p \leq 0.01$). Increasing the anthocyanin content of callus explants in response to salinity stress about 35 times was higher than shoot explants. The application of ALA up to 5 µM (callus explant) and 10 (shoot explant) increased the amount of anthocyanin under all salinity levels up to 2 times in comparison with control explants (Table 1, 2). ALA-induced anthocyanin biosynthesis via increasing phenylalanine ammonia lyase activity [5,6]. Xie *et al*. [6] reported that ALA could up-regulate phenylalanine ammonia lyase expression and increase enzyme activity. According to Wang *et al*. [5] reports, the effect of ALA treatment on anthocyanin biosynthesis was about 16 times as high as that of the light control.

Table 1 The effect of 5-aminolevulinic acid on physiological and biochemical characteristics of plantlet (leaf) explants of red-fleshed apple under salinity stress.

ALA	Salinity	Total Chlorophyll (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	Radical scavenging (%)	CAT (U mg^{-1} protein)	POX (U mg^{-1} protein)	APX (U mg^{-1} protein)	SOD (U mg^{-1} protein)	Total phenol (mg GAE g^{-1} FW)	Total anthocyanin (mg kg^{-1} FW)
0	0	6.35 b	2.191 b*	52.23 i	0.015 jk	0.310 i	0.931 f	0.056 j	290.53 hi	10.18 j
	30	5.52 de	2.046 bc	53.26 hi	0.019 g	0.393 e	1.111 d	0.058 hi	295.46 gh	10.28 j
	60	4.72 fg	1.254 ef	54.30 efghi	0.024 d	0.429 c	1.177 bcd	0.062 ef	304.71 f	10.26 j
	90	2.95 ij	1.086 f	55.79 bcdef	0.029 b	0.465 a	1.301 abc	0.067 c	330.56 de	10.34 j
2.5	0	6.43 b	2.217 b	53.18 hi	0.014 k	0.297 j	0.937 f	0.057 ij	303.06 fg	13.60 hi
	30	5.60 de	2.104 bc	53.24 hi	0.018 h	0.368 g	1.083 de	0.058 hi	328.84 e	13.77 h
	60	4.78 fg	1.459 de	54.74 defgh	0.022 e	0.413 d	1.178 bcd	0.062 ef	332.62 ef	13.72 h
	90	3.21 i	1.177 f	55.64 bcdefg	0.029 b	0.461 a	1.354 a	0.068 bc	346.21 bc	13.45 i
5	0	7.08 a	2.640 a	55.42 defg	0.015 jk	0.309 i	0.918 f	0.057 ij	338.90 cd	14.91 f
	30	6.17 bc	2.216 b	56.33 bcde	0.016 i	0.356 g	1.146 cd	0.058 hi	348.302 b	14.73 g
	60	5.14 ef	2.037 bc	56.79 abc	0.019 fg	0.405 d	1.176 bcd	0.063 e	354.034 b	14.87 fg
	90	4.01 h	1.442 de	57.11abc	0.025 c	0.442 b	1.334 ab	0.068 b	370.330 a	15.02 f
10	0	7.13 a	2.684 a	55.87 bcdef	0.015 jk	0.310 i	0.928 f	0.060 g	347.07 bc	19.50 c
	30	6.35 b	2.274 b	56.75 abcd	0.015 i	0.320 h	1.127 d	0.062 f	352.430 b	19.69 ab
	60	5.30 def	2.000 bc	57.67 ab	0.015 i	0.352 g	1.181 bcd	0.066 d	352.849 b	19.54 bc
	90	4.35 gh	1.571 d	58.40 a	0.019 g	0.365 f	1.330 ab	0.070 a	371.204 a	19.82 a
20	0	5.88 bcd	2.067 bc	52.91 hi	0.015 ij	0.317 hi	0.956 f	0.057 ij	291.740 hi	16.90 e
	30	5.24 ef	1.910 c	53.58 ghi	0.020 f	0.391 e	1.123 d	0.059 h	282.906 i	16.98 de
	60	4.24 gh	1.106 f	53.86 fghi	0.025 c	0.433 c	1.180 bcd	0.062 ef	285.239 i	17.07 de
	90	2.53 j	0.803 g	54.14 fghi	0.033 a	0.458 a	1.310 ab	0.068 bc	312.015 f	17.11 d

* Values followed by the same letter within a column indicate they are not significantly different ($p < 0.05$).

Table 2 The effect of 5-aminolevulinic acid on physiological and biochemical characteristics of callus explants of red-fleshed apple under salinity stress.

ALA	Salinity	Total Chlorophyll (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)	Radical scavenging (%)	CAT (U mg^{-1} protein)	POX (U mg^{-1} protein)	APX (U mg^{-1} protein)	SOD (U mg^{-1} protein)	Total phenol (mg GAE g^{-1} FW)	Total anthocyanin (mg kg^{-1} FW)
0	0	1.46 d*	0.115 c	52.81 t	0.020 h	0.1559 l	0.178 s	0.0043 l	164.29 s	5.12 q
	30	1.37 f	0.088 g	53.54 r	0.022 f	0.177 h	0.315 q	0.0043 l	171.40 q	7.24 o
	60	1.25 h	0.071 j	57.81 r	0.027 c	0.2353 c	0.658 j	0.0045 h	182.25 l	8.64 j
	90	1.00 k	0.052 m	61.57 g	0.030 b	0.2806 a	0.872 d	0.005 d	190.60 h	10.57 g
2.5	0	1.46 d	0.117 c	53.67 q	0.020 h	0.132 m	0.270 r	0.0043 kl	169.34 r	6.46 p
	30	1.39 ef	0.089 g	54.66 o	0.0204 gh	0.162 k	0.380 n	0.0045 i	180.53 m	7.86 m
	60	1.27 h	0.076 i	59.32 i	0.0248 d	0.193 g	0.712 i	0.0046 g	188.66 i	9.64 i
	90	1.06 j	0.061 l	62.53 d	0.03 b	0.254 b	0.911 b	0.00504 c	195.62 f	11.26 f
5	0	1.77 a	0.126 b	54.91 n	0.0175 kl	0.128 n	0.366 o	0.0043 j	179.50 n	8.38 k
	30	1.57 c	0.115 c	57.06 k	0.0185 ij	0.168 j	0.475 k	0.0048 f	188.62 i	10.44 h
	60	1.42 de	0.099 d	61.90 e	0.0217 f	0.175 hi	0.848 f	0.0051 b	201.75 d	13.57 c
	90	1.38 f	0.088 g	64.90 a	0.0262 c	0.204 f	0.937 a	0.0054 a	219.15 a	19.73 a
10	0	1.62 b	0.129 a	54.05 p	0.0171 kl	0.130 nm	0.329 p	0.0043 jk	176.12 o	7.73 n
	30	1.43 de	0.093 f	56.15 l	0.0187 i	0.174 i	0.416 l	0.0048 f	186.80 j	8.36 k
	60	1.33 g	0.082 h	61.74 f	0.0221 f	0.199 g	0.794 g	0.00504 c	199.19 e	12.55 e
	90	1.26 h	0.072 j	64.39 b	0.0264 c	0.208 e	0.895 c	0.0054 a	210.94 c	13.05 d
20	0	1.43 de	0.096 e	53.22 s	0.018 jk	0.153 l	0.324 p	0.0042 m	175.04 p	8.01 l
	30	1.32 g	0.088 g	55.38 m	0.021 g	0.193 g	0.393 m	0.0045 i	182.84 k	8.64 j
	60	1.17 i	0.065 k	60.18 h	0.0235 e	0.205 ef	0.734 h	0.0045 h	195.53 g	12.55 e
	90	1.09 j	0.047 n	62.89 c	0.0308 a	0.215 d	0.854 e	0.0049 e	211.35 b	14.84 b

* Values followed by the same letter within a column indicate they are not significantly different ($p < 0.05$).

Exogenous ALA in low concentrations promoted fruit coloration [6]. ALA induced anthocyanin biosynthesis via increasing phenylalanine ammonialyase (PAL) activity, the key enzyme in phenylpropanoid pathway. However, until now the mechanism by which ALA promoted anthocyanin biosynthesis is not obvious. Analysis variance showed that genotype, salinity and ALA significantly influenced pigments content, the interaction effects between treatments were shown in table 1,2 The total chlorophyll content between callus and plantlet was significantly different and its content in callus 1.77 (mg g^{-1} FW) and plantlet 7.13 (mg g^{-1} FW). Salinity stress significantly reduced the both chlorophyll a and b contents. Results showed that high concentration of ALA (20 µM) did not show positive effect in control of adverse effect of salinity. ALA in concentration of 10 µM slightly enhanced carotenoids content in control and stressed plantlets. Reduction of photosynthesis pigments under salinity stress previously reported by several researchers [12]. The variation of enzymes activity in response to exogenous application of ALA was different. In parallel to our results, a considerable increase in the activities of antioxidant enzymes was reported in different plant species under salinity [12,32]. Our results are somewhat analogous to those results that reported by Korkmaz *et al* [7] Akram *et al* [10] and Ali *et al* [29] in which it was observed that activities of antioxidant enzymes did not show increasing trends due to exogenous application of ALA in stressed plant.

Conclusion

These results indicate that ALA has salinity-ameliorating potential on in vitro cultured shoot and callus of local Iranian red-fleshed apple genotype via increasing antioxidant enzymes activities and enhancing accumulation of phenol and anthocyanin contents. Exogenous application of ALA can help to maintain and to increase photosynthesis pigments via increasing antioxidant enzymes activities and also enhancing phenolic compounds. Also, ALA improves phenylalanine ammonia-lyase activity that leads to increasing flavonoid and anthocyanin accumulation. It is concluded that the enhancement in salinity tolerance by ALA could be associated with increasing the antioxidant enzyme activities and accumulation of defense metabolites such as flavonoid and anthocyanin Combination of salinity stress (90 mM NaCl) and ALA (5-10 µM) could be

used to increase commercial benefits of this plant for in vitro anthocyanin production. these results could be hopefulness given the positive effect of ALA in amelioration of salinity stress under in vivo condition.

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