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Evaluation of exogenous ascorbic acid application as a protective agent against simulated acid rain in Persian maple (Acer velutinum Boiss)

Mehdi Salehi[1]*, Reza Fotouhi-Ghazvini[1], Vahab Jafarian[2]
[1]Department of Horticulture Science, University of Guilan, Rasht, (IRAN)
[2]Department of Biology, University of Zanjan, Zanjan, (IRAN)
E-mail : Mehdisalehi08@gmail.com

ABSTRACT

Air pollution is a problem at recent years which progressively increases. This study was accomplished to determine whether ascorbic acid could be applied in counteracting the adverse effects of acid rain (AR) stress. Accordingly, three-year-old Persian maple seedlings were subjected to the foliar application of ascorbic acid (AsA) at three levels (0, 1 and 2 mM). Afterwards, at each AsA levels, the plants were exposed to four different rain regimes: pH 3, 4, 5 and near neutral (pH 6) as control. At the end of experiment, some visible changes such as necrotic spots and leaf marginal wrinkle were observed in the plant leaves sprayed only with AR of pH 3.0. AsA efficiently impeded of appearance of visible AR-induced injury symptoms. Results also showed that membrane injury, indicated by electrolyte leakage (EL), and lipid peroxidation (LPO) increased following exposure to the acid rain. On the contrary, AsA considerably diminished LPO and EL. It also prevented from leaf chlorophyll degradation caused by AR of pH 3.0. Both AR and AsA treatments did not significantly affect superoxide dismutase (SOD) activity. However, AsA enhanced peroxidase (POD) and ascorbate peroxidase (APX) activities particularly at pH 3.0 AR, thereby plant leaves remained health. A hypothesis can be represented that elevated activity of POD and APX are important in the plant defense against AR stress. © 2012 Trade Science Inc. - INDIA

KEYWORDS

Air pollution;
Acid rain;
Ascorbic acid;
Antioxidant enzymes;
Maple.

INTRODUCTION

Air pollution is a serious problem at recent years. Acid rain (AR) as a pollutant results mainly from dissolution of sulfur dioxide and nitrogen oxides with atmospheric water vapor. These pollutants originate from natural sources and considerably from human activities such as the combustion of burnable waste and fossil fuels within thermal power plants and automobiles[26]. Rain with a low pH, which causes damage to plants, has been registered noticeably near industrial sources and high-traffic cities[35]. Several experiments have been carried out to investigate the effects of AR on plants[6,16,22,41,43,46,50,51,56]. All of the experiments indicate that AR can affect on various growth stages; seed germination to yielding depending on the plant species.

Generally, AR induces changes in cellular biochemistry and physiology of the whole plant[57]. Leaf, however, is the most sensitive organ to pollution which has been the target of many studies. The visible effect of AR on plants is usually the appearance of necrotic lesions on leaves[6,44], so these give rise to deforming of perspective of urban trees and forests. Regardless of the visible AR-injuries, invisible ones such as reduced photosynthesis, nutrient loss from leaves, altered water balance[14] and variations of several enzyme activities devote more importance to themselves which followed by leaf precocious abscissions and even plant death at severe rain acidity[59].

Reactive oxygen species (ROS), e.g. superoxide radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) are produced during numerous processes mainly at stress conditions. The ROS radicals damage membrane lipids, carbohydrates, proteins, and nucleic acids which result in a reduction of plant growth and development[4,34]. The ability of plant to overcome the effects of AR stress and to sustain its productivity may be related to the scavenging of the stress-induced toxic oxygen species[16]. Ascorbic acid (AsA) is regarded as a non-enzymatic antioxidant[42] and it involves in growth and division of plant cells[47,52]. It has been also understood that AsA has anti-stress effects due to its substrate role in activity of some enzymes[2]. Despite its role in scavenging ROS, AsA is also involved in regulating photosynthetic capacity by controlling stomatal movement[8].

Persian maple *(Acer velutinum* Boiss) is a tree species indigenous to the north of Iran (Hyrcanian forests). It is used as a specimen in landscapes and is applied in wood industry as well[58]. Scarce data, however, are available on the variations of antioxidant systems in the plants subjected to AR, as well as, to the best of our knowledge, to date, no data at hand on the involvement of exogenous AsA as a protectant against such type of stress. Therefore, the main objectives of the present paper were to (I) investigate the effect of simulated AR at different pHs on membrane stability, LPO, chlorophyll content, POD, SOD, and APX activities, (II) the possibility of exogenous ascorbic acid to inhibit the injuries of oxidative species in AR-treated maple trees, and finally, (III) the maintenance of urban trees to the acid rain stress through AsA.

EXPERIMENTAL

Plant material and treatments

Three-year-old Persian maple (*Acer velutinum* Boiss) seedlings were transferred from plastic bags to pots (40 Cm diameter and 90 Cm height) containing sand and sterilized manure (2:1). The plants were grown under 28.8/15.5^{-C} average day/night temperatures and relative humidity 79%, then they received full strength of Hoagland nutritive solution[21] every four days throughout the experiment. After one month establishment, they were subjected to the foliar application of AsA at three levels (0, 1 and 2 mM) seven times in two weeks. Afterwards, at each AsA level, the plants were exposed to four different rain regimes: pH 3.0, 4.0, 5.0 and near neutral (pH 6.0) as control about 20 min effective rainfall of 20 mm, during 10 days. The simulated AR was prepared according to Seufert et al.[49] and contained the following components: NH_4NO_3 (1.3 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (3.1 g l^{-1}), Na_2SO_4 (2.5 g l^{-1}), $KHCO_3$ (1.3 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (3.1 g l^{-1}). After dilution of initial solution 1:100, pH value was adjusted to 3, 4 and 5 with 1 N H_3PO_4 and 1 N H_2SO_4. The cocktail used to spray the control plants had the same composition as the simulated AR but the pH value was 6. Tween 80 (0.5%, v/v) was used as surfactant. Six leaves of each plant were collected 24 hours after the last rain application. Leaf material came from the middle region of the expanding leaf at the second, third and fourth nodes from shoot apex.

Analytical methods

(a) Membrane stability

Membrane stability of leaves was measured by EL following the method described by Dionisio-Sese and Tobita[13] with some modification. Leaf pieces were placed in test tubes containing 10 mL distilled deionized water. The tubes were incubated in a water bath at 32^{-C} for 2 h and the initial electrical conductivity of the medium (EC_1) was analyzed. The samples were autoclaved at 121^{-C} for 20 min to release all electrolytes; cooled to 25^{-C}, and then the final electrical conductivity (EC_2) was measured. The EL was calculated using the formula:

$$EL = EC_1/EC_2 \times 100$$

(b) Chlorophyll measurement

To determine the levels of chlorophyll 1 g of fresh leaf material was homogenized in 95% ethanol and filtered. The extract was made up to 25 ml with 95% ethanol. Absorbance was measured at 665 and 649 nm[30].

(c) Determination of the malonyldialdehyde (MDA) content

For the measurement of LPO in leaves, the thiobarbituric acid (TBA) test, which determines MDA as an end product of LPO[19] was used. Leaf material (500 mg) was homogenized in 5 ml 0.1% (w/v) thrichloro acetic acid (TCA) solution. The homogenate was centrifuged at 10 000 rpm for 20 min and 0.5 ml of the supernatant was added to 1 ml 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 10000 rpm for 5 min, and the absorbency of supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

(d) Enzyme analysis

Fresh leaf samples were used for enzyme analysis. Leaves were frozen in liquid nitrogen immediately after harvesting and stored at -20^{-C} until enzyme assays. One gram leaves homogenized in 3 ml of 0.05 M Na-phosphate buffer (pH 7.8) including 1 mM EDTA and 2% (w/v) PVPP. The homogenate were centrifuged at 14 000 rpm for 30 min at 4^{-C}. Supernatant was used for enzyme activity. All assays were done at 4^{-C}. All spectrophotometric analyses were conducted on a Shimadzu (UV-1600) spectrophotometer.

SOD activity assay was based on the method of Beauchamp and Fridovich[3] which measures the inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm. One unit of enzyme activity was defined as the quantity of SOD required to produce a 50% inhibition of reduction of NBT and the specific enzyme activity was expressed as unit mg^{-1} protein g FW.

POD activity was based upon the method as described by Herzog and Fahimi[20] which measures the increase in absorbance at 465 nm, by the rate of formation of 0.15 M Na phosphate citrate buffer the oxidized DAB. One enzyme unit is defined as μmol ml^{-1} destroyed H_2O_2 per min.

APX activity was done according to Nakano and Asada[39]. The assay depends on the decrease in absorbance at 290 nm as ascorbate was oxidized (extinction coefficient of 2.8 mM^{-1} cm^{-1}). One enzyme unit is defined as μmol ml^{-1} oxidized ascorbate per min.

Statistics

The experimental design was entirely randomized, with three repetitions of each factorial combination (4×3), in which four pH values for simulated AR and three concentrations for AsA. The data were subjected to the analysis of variance (ANOVA) and means were compared by LSD test. Only significant results are noted in the text.

RESULTS

Morphological effects

Some visible changes such as necrotic spots and leaf marginal wrinkle were observed in the plant leaves sprayed only with AR of pH 3.0. However, no clear symptoms were appeared by other rain pHs tested. AsA either at 1 and 2 mM concentrations efficiently impeded of appearance of visible AR-induced injury symptoms and maintained leaves health (Figure 1).

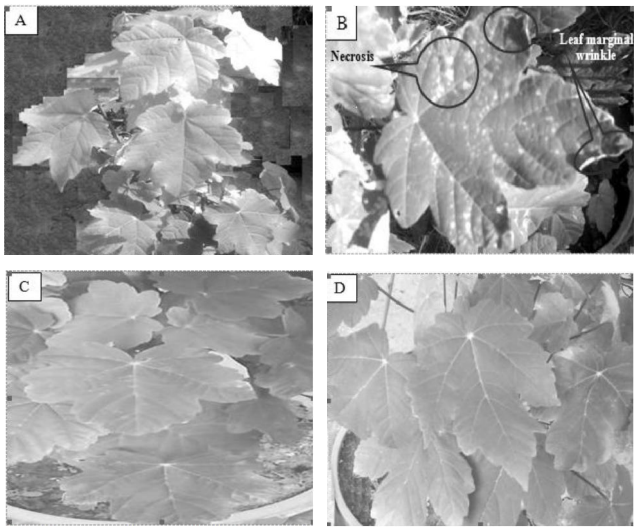

Figure 1 : (A) Persian maple leaves treated with only rain of pH 6.0 as control plant, (B) plant leaves sprayed with simulated AR of pH 3.0, (C and D) plants pretreated with 1 and 2 mM of AsA, respectively, and then sprayed with AR of pH 3.0

Membrane stability

Electrolyte leakage (EL) reflects the damage of cell membrane. As Figure 2 shows, the amount of EL significantly ($P \leq 0.01$) increased with increasing acidity of rain. The increase was more pronounced at pH 3.0, reaching 160% of the control (pH 6.0). Application of AsA at 1 and 2mM concentrations decreased EL by 50 and 56% of that in the plants exposed to pH 3.0, respectively. However, no significant difference was observed between different concentrations of AsA in reduction of EL.

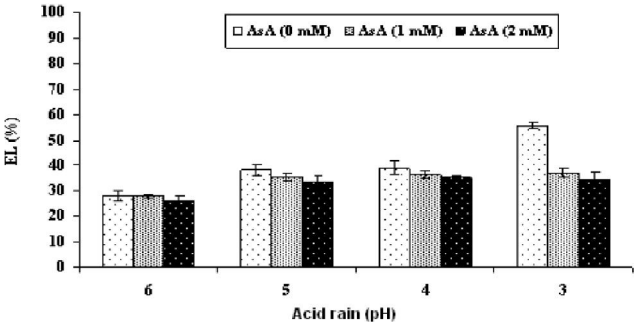

Figure 2 : Changes in electrolyte leakage (EL) as influenced by pretreatment with AsA at different concentrations and treatment with AR at various pHs in leaves of Persian maple trees. Vertical bars represent ± S.E

Lipid peroxidation (LPO)

Variations in LPO were shown by the content of MDA in Figure 3. An increase of MDA following AR application was scored having a maximum at rain of pH 3.0. The response amounted to 156% of the control (pH 6.0). Preliminary supply of AsA at 1 and 2 mM concentrations caused significantly ($P \leq 0.01$) 48 and 51% attenuation in the effect of AR on LPO at pH 3.0, respectively. The level of MDA was not significantly influenced by AsA application at other pHs.

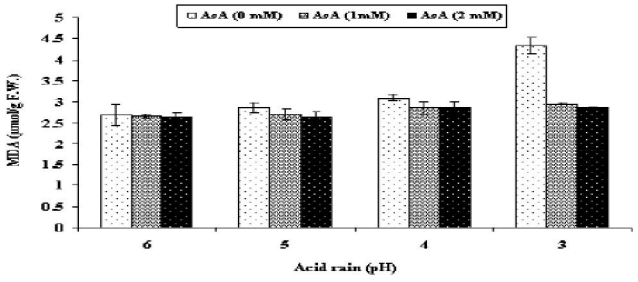

Figure 3 : Changes in malonyldialdehyde (MDA) content as influenced by pretreatment with AsA at different concentrations and treatment with AR at various pHs in leaves of Persian maple trees. Vertical bars represent ± S.E

Chlorophyll content

Changes in total Chlorophyll content are shown in Figure 4. A very slight decrease was observed in leaf chlorophyll content with lowering pH value by 4.0, whereas at pH 3.0, total chlorophyll was considerably reduced to 155% of control. Pretreatment with AsA significantly ($P \leq 0.01$) prevented from chlorophyll degradation at high acidity of rain tested, pH 3.0, and maintained chlorophyll nearly as content as control.

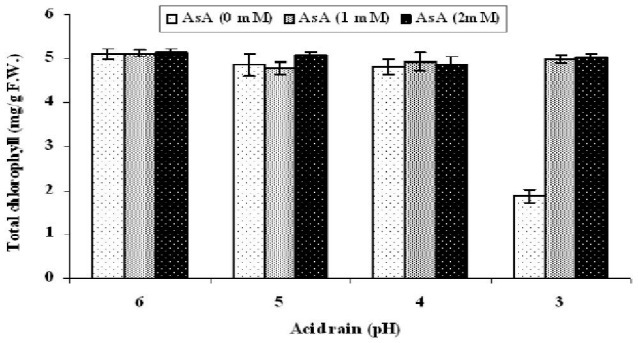

Figure 4 : Changes in total chlorophyll content as influenced by pretreatment with AsA at different concentrations and treatment with AR at various pHs in leaves of Persian maple trees. Vertical bars represent ± S.E

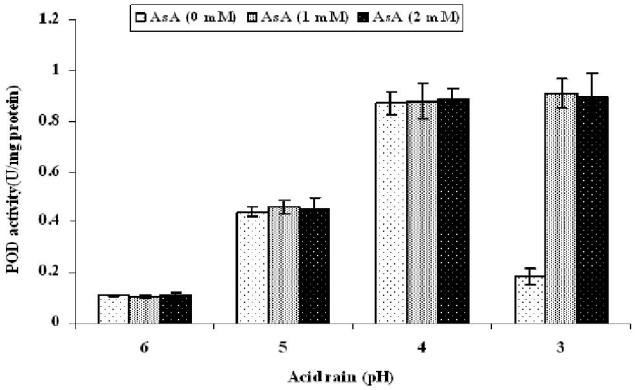

Figure 5 : Changes in activity of peroxidase (POD) as influenced by pretreatment with AsA at different concentrations and treatment with AR at various pHs in leaves of Persian maple trees. Vertical bars represent ± S.E

Enzymes activities

POD and APX activities increased as AR decreased by pH 4.0 values (Figure 5 and 6, respectively), whereas their activities did not significantly ($P \leq 0.01$) increase at pH 3.0 AR as compared with control. However, preliminary application of 1 and 2 mM of AsA led to 710 and 730% increase in POD activity at pH 3.0 in comparison with the control, respectively. These amounts were respectively 850 and 1000% for APX activity.

On the contrary, SOD activity did not significantly ($P \leq 0.05$) affected by both AsA and simulated AR treatments. The supply of AsA to plants at pH 6.0 (control) had no significant impact on APX and POD activities. However, no significant difference was observed between different concentrations of AsA in elevating POD and APX activities. Except at pH 3.0, in which the activity of APX was significantly ($P \leq 0.05$) elevated by 2 mM of AsA more than 1 mM of one.

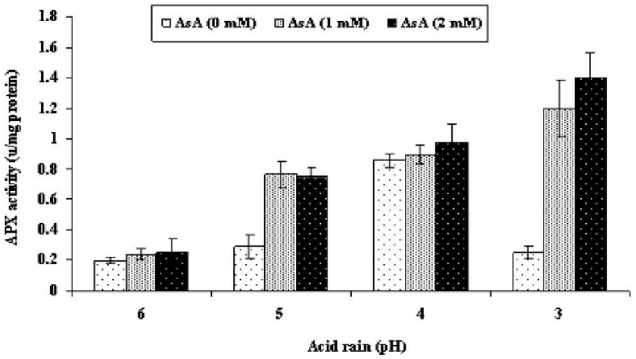

Figure 6 : Changes in activity of ascorbate peroxidase (APX) as influenced by pretreatment with AsA at different concentrations and treatment with AR at various pHs in leaves of Persian maple trees. Vertical bars represent ± S.E

DISCUSSION

In the present study, from a morphological point of view, necrotic spots on the leaf surface and leaf curling were observed as a consequence of the exposure of *A. velutinum* Boiss to the low pH AR (pH 3.0) treatment solely. According to the foliar micromorphological studies of Fransisco et al.[15], *Genipa americana* L. trees submitted to the simulated AR presented deformed stomatal aperture, rupture of stomatal outer ledge and alterations in the cell guard permeability. Percy and Baker[46] showed that cuticular membrane thickness was decreased in *Picea sitchensis* Carr. due to simulated rain pH. They also reported that increase in rain acidity caused changes in wax structure and chemical composition, so these resulted in leaf injuries. The occurrence of visible injuries in our research can be also attributed to the cell disruption resulted from ROS-induced cell wall injuries. In fact, AR as a stress increases level of ROS[16, 27,57]. High ROS levels are very dangerous for cells and damage cell membranes, proteins and DNA in the plants subjected to the severe environmental conditions[12]. As it obtained from our visual results, AsA-pretreated plants showed no visual leaf injuries under any pH of simulated AR tested. It may be due to the protection of cuticular layer by AsA through inhibition wax structure and/or wax chemical composition from changing. It also may be explained by regulatory role of AsA in stomatal closure to stresses[23], which probably resulted in the low penetration of AR to internal tissues. Another reason may be attributed to the scavenging of AR-induced ROS by AsA[42]. As our results showed, increase in acidity value of rain gave rise to increasing EL. On the other word, it enhanced cell wall damaging. The preventing from necrotic spots by AsA can be also explained by reducing both EL and cell wall LPO. Athar et al.[2] stated that AsA caused considerable reinforcement of membrane stability and induced tolerance in wheat exposed to oxidative stress. These are also in agreement with the recent findings reported by Li et al.[31]. They expressed that genes responded to AsA biosynthesis were more expressed under abiotic stresses. In addition, Myung-Min et al.[38] reported that not only AsA itself, but expression of AsA precursors led to the tolerance induction against environmental stresses.

LPO is a reaction in which membrane lipoid compounds being collapse. AsA dramatically prevented from LPO at all pH values of AR tested. LPO in membranes take places when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also increasing the oxidative stress through production of lipid-derived radicals[37]. Base on our results, the Persian maple pretreated with AsA dramatically showed reduced LPO levels particularly under acid rain of pH 3.0. Recently, It has been demonstrated that AsA can provide protection to membranes by directly scavenge the O_2^- and OH^- and by regenerate α-tocopherol from tocopheroxyl radical[17].

The SOD activity of Persian maple leaves was not affected neither by AR nor AsA treatments. It is in conflict with the findings of Athar et al.[2]. They have expressed that SOD activity is greatly enhanced following salt stress in wheat. Unaffecting of SOD after exposure to AR may probably be explained by increasing acidity of leaf tissues resulted from AR. As Gill and Tuteja[17] expressed, O_2^- is dismutated unavoidably at low pH, with one O_2^- giving up its added electron to another O_2^-, and then with protonation resulting in the

generation of H_2O_2. Inversely, POD and APX increased with increasing in acidity of rain applied, but they did not increase in plants treated with only AR of pH 3.0. In the present study, exogenous AsA enhanced both POD and APX activities at all pH values. It can be ascribed to the electron donating characteristic of AsA[9]. Another reason for remaining Persian maple leaves health under rain of pH 3.0 by AsA can be attributed to the high activity of POD in the presence of AsA. The class I POD catalyzed the conversion of H_2O_2 to H_2O and O_2 uses of ascorbate, a reduced form of AsA, as the specific electron donor[1]. The elevated APX in AsA pretreated plants could be another main factor caused leaves to remain health after AR exposure. Increase in APX activity in parallel to AsA application indicates the direct relationship between AsA content and APX activity. Kavitha et al.[25] have reported that oxidative stress induces increase in the transcript of APX in leaves of *Avicennia marina*. They also expressed that APX activity in *A. marina* leaves have a role in reducing the deleterious effect of oxidative stress. Moreover, expression of APX genes by specific factors such as atmospheric pollution was reported by Kubo et al.[29] and Rao et al.[48] as well. The role of high APX activity in reducing injury is also parallel with Bueno and Piqueras[6]. They stated that APX protected tabbaco cells against H_2O_2 under stressful conditions. Role of APX in eliminating ROS from cells in *Euphorbia esula* L. in response to environmental stresses such as salinity, metal toxicity and drought has been reported by Davis and Swanson[11]. Two molecule of ascorbate are required for activation of APX[47]. It has been revealed that ascorbate is one of the most important factors in the scavenging of ozone and ozone-derived ROS in the apoplast[7].

According to the experiment, AsA-pretreated plants showed no pale and discolored leaves when they were exposed to the injurious AR treatment, pH 3.0. It could be explained by the fact that AsA protected chlorophyll from degradation by AR. There are some reports indicate that chlorophyll concentration is strongly dependent on the pH of applied AR, e.g. in cucumber[59] and velvetleaf[36]. However, our results reveal that AsA probably do not have role in chlorophyll biosynthesis because it has not enhanced the chlorophyll contents more than that in the control plants. It only maintained chlorophyll nearly as the same level as the control plants. It is understood from the results that AsA have a role in protection of chlorophyll rather than in biosynthesis of one. Nevertheless, it has been cleared that carotenoid pigments (carotenes and xanthophylls) depend on ascorbate for their regeneration[47].

It has been well established that AR lowers soil pH, and thereby leads to AL toxicity in plants. The Al toxicity constitutes the most important restriction to growth in acidic soils[24]. Conversion of AsA into oxalate has been observed in a number of plant species[32,40]. Oxalate was confirmed to be associated with high Al resistance in taro plant[33] and buckwheat (*Fagopyrum esculentum*)[60]. It is also demonstrated that AsA treatment increased accumulation of oxalate mainly in soluble form in rice seedlings subjected to Al toxicity[18]. Undoubtedly, we could find out from these evidences that AsA have indirectly a protective role in the tolerance induction of plants grown under high soil AL conditions. Therefore, application of exogenous AsA can also certainly ameliorate the detrimental effects of AR-induced Al toxicity, based on the above mentions. Overall, with regard to our results which have revealed the protective characteristics of AsA against simulated AR and according to the its essential role in tolerance to various environmental stresses as well as its indirect role to high soil Al, it is obvious that AsA can be suggested to apply in irrigation water of urban landscapes and crops where located in the areas with the high air pollutant levels. More studies, however, should be done to decipher the precise role (s) of AsA in protecting plants against AR.

CONCLUSION

In conclusion, since acid rain stress is often along with other terrestrial stresses, e.g. cold, flooding stresses, AL toxicity etc., finding a substance with multi-protective role is a most important factor for introducing it as a protective agent against such stress. The present study clears that AsA have an extensive potential for maintaining of plant health under acid rain. It was able to impeded of leaf necrotic spots and marginal wrinkle caused by strong stresses (pH 3.0). Indirect role of AsA in tolerance induction to AL toxicity has been also previously expressed. We believe that

AsA may also have a role in protection of epicuticular layer or its compositions. Overall, these first data provide the basic framework for further researches into the mechanisms of both AsA and AR actions as well as it persuades researchers to investigate other precise role (s) of ascorbic acid in plants under acid rain especially at the subcellular level. In the near future, unless serious investigations are not taken, plants and human beings may face even greater risks resulting from air pollutants. Studies of this kind may help overcome some of the problems and decline the risks.

ABBREVIATIONS

AR; acid rain, AsA; ascorbic acid, EL; electrolyte leakage, LPO; lipid peroxidation, SOD; superoxide dismutase, POD; peroxidase, APX; ascorbate peroxidase, ROS; Reactive oxygen species, MDA; malonyldialdehyde.

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