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Research Article

ASCORBIC ACID AND/OR 24-EPIBRASSINOLIDE TRIGGER PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES FOR THE SALT STRESS MITIGATION IN POTATO (*SOLANUM TUBEROSUM* L.)

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Abstract

In the present study, we examined the role of ascorbic acid (AsA, vitamin C) and/or 24-epibrassinolide (EBL, an active BR) in mitigation of salt-induced stress in potato (*Solanum tuberosum* L.). The 10-d-old plants were exposed to 150 mM NaCl and they were subsequently treated by ASA and/or EBL. The salt stress reduced significantly the plant growth, tuber yield, total chlorophyll and increased proline content and electrolyte leakage in the leaves. Toxic effects induced by salt stress were completely overcome by the combined exogenous application of AsA and EBL. The AsA and/or EBL treatments improved the growth parameters of the salt treated plants, such as shoot length, tuber number and size, fresh and dry mass and other physiological parameters. Our data also indicated that applications of AsA and EBL up-regulated the stress regulating plant hormone such as IAA, IBA and activities of the antioxidant enzymes, such as catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), ascorbate peroxidase (APX) and under salt stress.

Key words: Ascorbic acid, Brassinosteroids; *Solanum tuberosum* L; Antioxidants; Salt stress.

Introduction

Abiotic stress factors including salinity, drought, extreme temperatures, oxidative stress and chemical toxicity from the environment are the key cause of worldwide crop losses leading threats to agricultural products (Mickelbart *et al.*, 2015). Soil salinity is one of the most grave factors restraining the productivity of agricultural crops, with adverse effects on germination, plant vigor and crop yield (Munns and Tester 2008). Salinization affects many irrigated areas mainly due to the use of brackish water. A high salinity in the soil affects the soil porosity, decreases the soil water potential and it also affects the physiology of plants at the cellular as well as the whole plant level (Mahajan and Tuteja 2005). The ion toxicity, ion imbalance and oxidative stress produced by salt stress is serious imposition to plant (Li *et al.*, 2010). Salt stress also severely affects various morphological, physiological, and biochemical processes such as photosynthesis, accumulation of low molecular mass compounds, such as proline and glycine betaine (Koca *et al.*, 2007; Mutlu and Bozcuk 2005), lipid metabolisms and protein biosynthesis change in cell metabolism levels (Parida and Das 2005). While cells are under stress, certain reactive oxygen species (ROS) are produced that may cause membrane peroxidation, protein denaturation, DNA damage, or show toxicity to metabolic functions after conversion to H₂O₂ (

Noctor and Foyer 1998). The accumulation of salinity in soil obviously leads to the hyperosmotic stress in plants that ultimately stimulates the production of reactive oxygen species, dehydration, and turgor loss in cells and tissues (Mittler 2002; Ahmad *et al.*, 2010).

Potato is one of the world's major food crops and production demand is increasing at a greater rate than many other food crops (Chiru *et al.*, 2008). Potato is well propagated predominantly by asexual means (tubers and microtubers). Potato does not thrive well in soils loaded with sodium salts, hence, potato has been classified as moderately salt-sensitive (Farhatullah and Mahmood 2002; Fidalgo *et al.*, 2004) Potato is comparatively sensitive to salinity, particularly in the early growth stages (Nadler and Heuer 1995). Shoot growth of the Andean potato has been reported to be affected by NaCl stress, while some frost resistant genotypes show high salt tolerance which could be related to proline accumulation (Martinez *et al.*, 1996). In vitro micro-tuberization provided an effective experimental model for physiological and metabolic studies of tuberization and the screening of potential potato genotypes for salt tolerance. Adverse effect of the salinity on the potato growth has been reported causing the delayed sprouting and growth, reduced dry weight production especially in tubers, decrease in tuber number per plant and average tuber weight, decrease in total and marketable tuber yield. The

contents of water-soluble carbohydrates and starch of leaves increased and that of total nonstructural carbohydrates also increased by salt stress (Farhatullah and Mahmood 2002; Ghosh *et al.*, 2001; Zheng *et al.*, 2009)

Brassinosteroids (BRs) are structurally similar to animal and insect steroid hormones which belong to a class of polyhydroxy steroids (Goda *et al.*, 2002; Uesusuki *et al.*, 2004). They are considered as sixth group of plant hormones present in very low concentrations in seeds, pollen, young tissues and control a variety of plant responses such as cell division and expansion, xylem differentiation, ethylene biosynthesis, ion uptake, photosynthesis, seed germination, vegetative growth, apical dominance and gene expression (Brosa 1999; Gudesblat and Russinova 2011; Sasse 2003). BRs are reported to regulate several developmental and physiological processes including morphogenesis, cell elongation, and tissue differentiation (Bajguz 2010; Gudesblat and Russinova 2011). These are also involved in conferring both biotic and abiotic stress tolerance in several plants (Anuradha and Rao 2003; Bajguz 2010, 2011; Choudhary *et al.*, 2010, 2011; Fariduddin *et al.*, 2011, 2014; Fariduddin, Khanam, *et al.*, 2009; Hayat *et al.*, 2012; Hayat, Hasan, Hayat, *et al.*, 2010). Signal transduction and molecular mechanism studies have established that the pleiotropic effects of BRs result partially from the interactions with other phytohormones (Choudhary *et al.*, 2010; Divi *et al.*, 2010; Singh and Shono 2003). Epibrassinolide (EBL) improved photosynthetic capacity and decreased oxidative damage in *Vigna radiata* exposed to different levels of heavy metal stress (Yusuf *et al.*, 2012). The BRs are broadly used to support the plant defense system by enhancing the activities and contents of non-enzymatic and enzymatic antioxidants against various abiotic stresses including heavy metals (Anuradha and Rao 2003; Fariduddin, Khanam, *et al.*, 2009), drought (Fariduddin, Yusuf, *et al.*, 2009), and salt stress (Hayat *et al.*, 2012). Application of BRs enhanced seed germination, free proline contents, and activities of antioxidative enzymes (Dhaubhadel *et al.*, 1999; Fariduddin *et al.*, 2014). Epibrassinolide (EBL) has been broadly used to improve the harmful effects of abiotic stress in plants among the known BRs (Ali *et al.*, 2008; Bajguz 2010, 2011).

Ascorbic acid (AsA) is an important antioxidant molecule that acts as a primary substrate in the cyclic pathway for enzymatic detoxification of hydrogen peroxide (H_2O_2) superoxide radicals ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}), and lipid hydro-peroxides (B. P. Yu 1994; Hemavathi *et al.*, 2009, 2010a; Khan and Panda 2007; Smirnov and Wheeler 2000; Upadhyaya *et al.*, 2011). Role of AsA as an ascorbate peroxidase substrate which scavenges hydrogen peroxide in the chloroplast stroma has also been well documented (Gadallah 2000; Nakano and Asada 1981; Shigeoka *et al.*, 2002) which is important metabolic process for abiotic stress tolerance. This AsA is water-soluble with an

additional role on the thylakoid surface in protecting and/or regenerating oxidized carotenes and α -tocopherols which a lipophilic antioxidant molecule (Gallie 2013; Noctor and Foyer 1998; Sadak and Dawood 2014) regulating in the detoxification of oxidants in the cytosol.

The latest advancements in plant hormonal and/or antioxidant interactions have shown a possible involvement in the management of various abiotic stresses (Fariduddin *et al.*, 2011, 2014; Hayat *et al.*, 2012; Hayat, Hasan, Hayat, *et al.*, 2010; Sajid and Aftab 2009a). Although there are reports on the effect of individual AsA or BRs in amelioration of abiotic stress tolerance in several plants, however, no information is available on the interactive effects of AsA and BRs in the mitigation of abiotic stress in potato (*Solanum tuberosum* L). Keeping in view the diverse role of AsA and BRs, the present study was designed with the aim to assess the comparative effect of EBL and AsA against salinity stress on potato.

Materials and Methods

Plant Material and Treatment

Healthy tubers (without any visual symptoms of disease) of the potato cultivars Desiree were grown in sterile sand mixed with agro peat (Versha Agropeat, Bangalore, India) in a plant growth chamber at $23\pm 2^\circ C$ and 75% relative humidity. Nodal cuttings of 1.0 cm long were used to raise the *in vitro* plants. Nodal cuttings were first washed thoroughly with a double distilled water (DDW) and then placed in a 0.7% sodium hypochlorite ($NaClO$) solution containing 0.1% (v/v) Tween-20 for 5–10 min in an Erlenmeyer flask (250 mL, Pyrex) on an orbital shaker at 120 rpm and again washed thrice with autoclaved double distilled water. The nodal cuttings were dried on filter paper and placed on Murashige and Skoog (MS; Murashige & Skoog, 1962) basal medium supplemented with 30 gL^{-1} sucrose, solidified with 0.7% (w/v) agar (Hi-media, India) and adjusted to pH5.8. A single nodal explant was inoculated in each culture vessel and all cultures were incubated in plant growth chamber (Matrix Eco-solution, New Delhi, India) at $23\pm 2^\circ C$ in 16 h continuous light ($200\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) using cool white fluorescent tube lights. These nodes were allowed to grow and the resulting plants were transferred in pots (25cm in size) fill with fixed amount of sand and autoclaved agro-peat mixture and maintained in a plant growth chamber [14-h light period, PAR of $200\text{ }\mu\text{mol (photon) m}^{-2}\text{ s}^{-1}$, $25^\circ C$, 72% relative air humidity) for 45-60 days. To maintain the sufficient nutrients, the supply of nutrient solution was maintained sparingly with regular intervals. After 10 days, the plants were exposed to 150 mM NaCl by watering them with saline water and sprayed simultaneously with 0.5 mM ascorbic acid and/or 24-epibrassinolide. The control plants (without stress) set was sprayed with equal amount of DDW mixed with tween-20 (Hi-Media). A total number of 40 pots were arranged for 8 treatments with 5 replicates each,

having 3 plants per pot in a simple randomized block design. The plants were harvested after 60 d of growth.

Ascorbic Acid and 24-Epibrassinolide Preparation

The stock solution of Ascorbic acid (AsA) was prepared by dissolving the required quantity in 10 ml double distilled water (DDW). The 24-epibrassinolide (EBL) was prepared by dissolving the required quantity in 5 ml of ethanol, in 50 ml volumetric flasks. Tween-20 (2.5 mL) was added and the final volume (of both solutions) was made up to the mark by using DDW. The desired concentrations of AsA or BRs were prepared by the dilution of stock solution with DDW.

Measurement of Growth Parameters

Harvested plants (one plant from each replicate) were removed together with the sand: agropeat and dipped in a beaker filled with tap water. The adhering soil particles were removed while ensuring the integrity of the tubers and roots. The plants were blotted on paper and the number of tubers, weight of tubers, lengths of roots and shoots were measured; then fresh weight (FW) was recorded. The roots and shoots were dried in an oven at 80°C for 72 h and weighed to note their dry mass (DM). Leaf area of randomly selected leaves from each treatment was determined by tracing the outline of the leaf on the graph sheet and counting the number of squares covered by the leaf.

Measurement of Chlorophyll and Quantum Yield of PSII

The chlorophyll (Chl) content was measured by CL-01 Chlorophyll Content System (Hansatech, UK) in the intact leaves of the plants (one plant from each replicate). The Chl fluorescence [*i.e.*, maximum photochemical efficiency of PSII (Fv/Fm)] was measured on the upper surface of the intact leaves of the plants using a Pocket PEA (Plant Efficiency Analyser, Hansatech Ltd., King's Lynn, UK) following the protocol of (Maxwell and Johnson, 2000). The minimal fluorescence level (F₀) was determined by modulated light, which was sufficiently low (< 0.5 μmol m⁻² s⁻¹) not to induce any significant variable fluorescence. The maximal fluorescence (F_m) was determined by a 0.3 sec saturation pulse at 300 μE m⁻² s⁻¹ on dark-adapted potato leaves. The sampled leaf was dark-adapted for 45 min prior to measurement of Fv/Fm. The parameter Fv/Fm [(F_m-F₀)/F_m] reflects the maximum quantum yield of PSII or the potential quantum efficiency if all the PSII centers were open

Electrolyte Leakage (EL)

It has been well established that electrolyte leakage measurements is correlated with numerous biochemical and physiological parameters conditioning the plant responses to environmental stresses. The electrolyte leakage measurements quantify the presence of all charged solutes in the external medium. Total inorganic ions leaked out of the potato leaves were quantified by the method described by (Bajji *et al.*, 2000). Twenty five leaf discs (1.0 cm² size) were kept in a boiling test tube containing 10 ml of DDW,

and electrical conductivity (ECa) was measured by conductivity meter (Thermo Fisher Scientific, UK). The tubes were heated at 50°C and 60°C for 20 min in water bath, and electrical conductivity was measured (ECb) each time. Later, the contents were again boiled at 100°C for 5 min, and electrical conductivity was again recorded (ECc). The electrolyte leakage was calculated using following formula:

$$EL [\%] = [(ECb - ECa)/(ECc)] \times 100$$

Leaf Proline Content

The proline content in fresh leaf samples (one plant per replicate) was estimated by the method described by Bates *et al.* (Bates *et al.*, 1973) with minor modifications. Samples were extracted in sulfosalicylic acid following the standard protocol. An equal volume of ninhydrin solutions and glacial acetic acid were added to the extract. The sample was heated at 100°C, to which 5 ml of toluene was added. Upper layer was taken for analysis. The absorbance was read at 520 nm on a spectrophotometer. Proline concentration was determined from a standard curve and calculated on a fresh weight basis (μmol proline g⁻¹ 230 FW).

Nitrate Reductase Activities

Nitrate reductase (NR) activity was determined following the *in vivo* method (Jaworski 1971). Leaf samples from were taken and cut into small pieces. About 500 mg of leaf pieces from different treatments were incubated separately in a medium containing 1.0 ml of 1.0 M potassium nitrate, 2.0 ml of 0.2 M phosphate buffer (pH 7.5) and 2.0 ml of 0.5% Triton-X-100 for 1 h. The reaction mixture (1.0 ml) was transferred to another test tube containing 1.0 ml of 1% sulphanimide in 2N HCl and 1 ml of 0.2% NEEDA (N-1-naphthyl ethylene diamidedihydro chloride). The absorbance of the mixture was recorded at 540 nm. A standard curve was prepared using different concentrations of potassium nitrite as substrate. The NR activity was expressed in [nmol(NO₂) g⁻¹(FM) s⁻¹].

Antioxidant Enzymes

For the assay of antioxidant enzymes, the leaf tissue (0.5 g) was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone.

The homogenate was centrifuged at 27,600 × g for 10 min at 4°C and the supernatant was used

as source of enzymes.

Catalase (CAT; EC 1.11.1.6) activity was determined by monitoring the decomposition of H₂O₂ at 240 nm. The reaction was initiated by adding 10 mM H₂O₂ to the reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and plant extract, 3 ml in volume. One unit of catalase is defined as the amount of enzyme which liberates half the peroxide oxygen from 10 mM H₂O₂ solution in 100 s at 25°C. Peroxidase (POX; EC 1.11.1.7) activity was

determined by method of (Maral *et al.*, 1977) monitoring the formation of guaiacol dehydrogenation product (extinction coefficient 6.39 mM cm^{-1}) at 436 nm, following the method used by Putter (1974). The reaction mixture (3.1 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol and plant extract. To initiate the reaction, 0.1 mM H_2O_2 was added to the reaction mixture. One unit of peroxidase is defined as the oxidation of guaiacol from 0.3 mM guaiacol and 0.1 mM H_2O_2 per min at 25°C at pH 7.0. To determine the superoxide dismutase (SOD; EC 1.15.1.1) activity, a method described by (Beyer *et al.*, 1991) was followed. The reaction mixture (30.25 mL) contained 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 57 mM nitroblue tetrazolium (NBT) and an appropriate volume of plant extract. The reaction was initiated by light illumination. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined using spectrophotometer at absorbance 290 nm as described by (Chen and Asada 1989). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.2 mM H_2O_2 and a suitable volume of plant protein extract.

Estimation of Endogenous Plant Hormones

Plant hormones, in particular, indole-3-acetic acid (IAA) and abscisic acid (ABA) is the major hormone reported to participate in abiotic stress tolerance. These hormones were determined in potato growing under conditions via HPLC method as described by Kelen *et al.*, (2004). 1 g fresh leaf tissue was homogenized in 70% (v/v) methanol and stirred overnight at 4°C . Leaf extracts were filtered and methanol was vacuum evaporated. The ether phase was evaporated and the pellet was dissolved in 2 ml of absolute methanol. The chromogenic analysis was performed on a HPLC (Shimadzu, Japan). The mobile phase used was acetonitrile: water (26:74; v/v) at $25 \pm 1^\circ\text{C}$. The separation was carried by isocratic elution with a flow rate of 0.8 ml min^{-1} .

Statistical Analysis

The experiment was conducted according to simple randomized block design. Each treatment was replicated five times. Data were statistically analyzed for analysis of variance (ANOVA) using SPSS software and least significant difference (LSD) was calculated to separate the means.

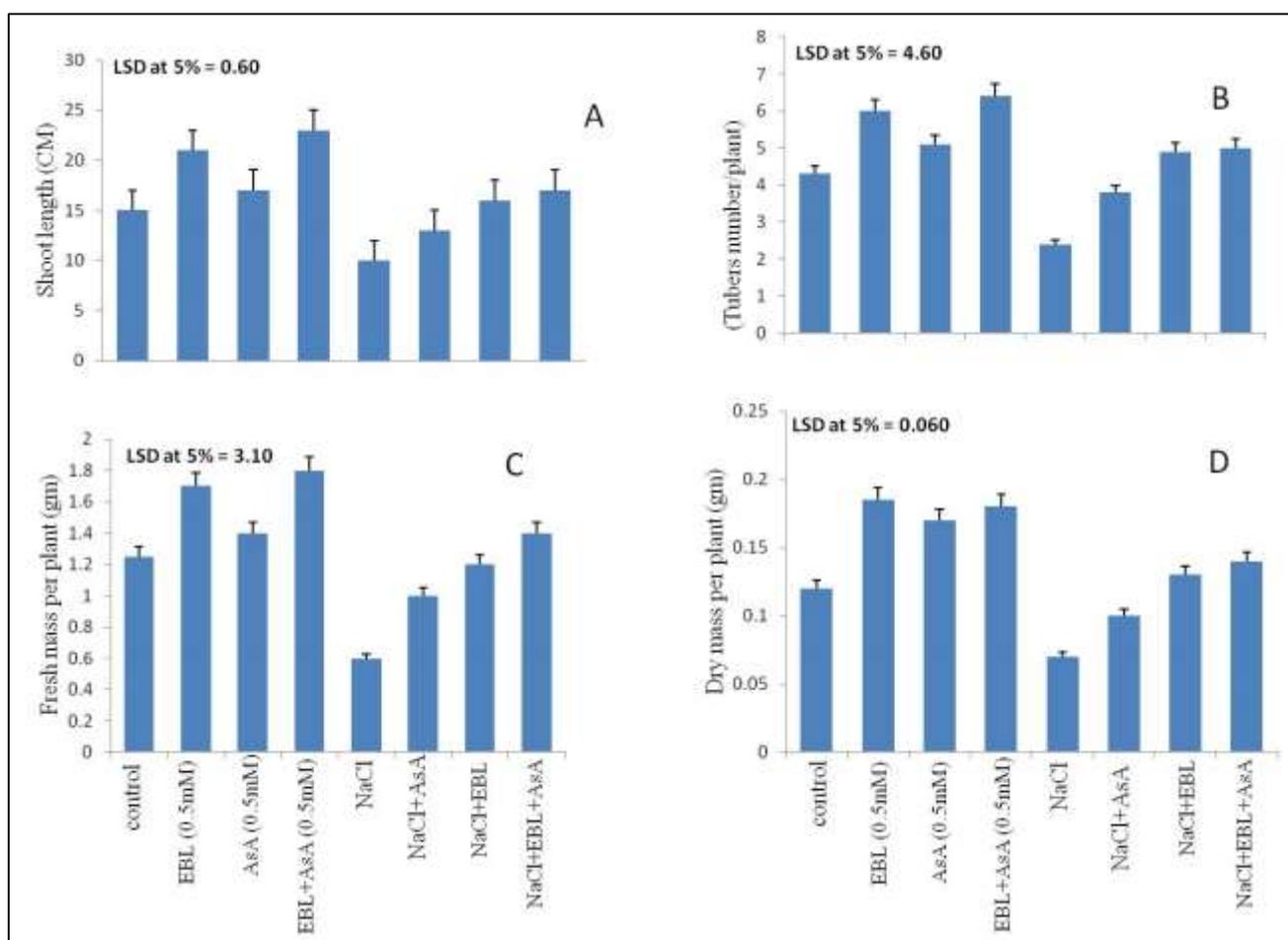


Fig. 1: Effect of Ascorbic acid (AsA; 10.5mM) and/or 24-epibrassinolide (EBL; 0.5mM) on the NaCl (150 mM) induced changes in the (A) shoot length, (B) tuber numbers, (C) fresh mass per plant, (D) dry mass per plant.

Results

Growth parameter analysis:

A significant reduction in all the growth parameters was observed in plants exposed to salt stress @150mM (Fig. 1). The shoot length, number of tubers, FW, DW, and decreased by 38.5, 51.6, 48.3, 46.1 respectively, compared with their respective controls (Fig. 1). Plants treated with ASA or AsA+ EBL overcame completely the inhibitory effects generated by NaCl stress. The plants treated with AsA+ EBL showed enhancement of all growth parameters, such as shoot length, number of tubers, FW, and DW, and leaf area by 42, 44.2, 36.9, 47.5, and 35.2 %, respectively, when compared with their respective controls. It is very important to mention that the AsA and/or EBR treated plants showed increased tuber size, weight and root length (data not shown) as compared to their respective controls. The tuber size and weight also increased in the AsA or EBA treated plants growing without salinity stress.

Chlorophyll Content and Photosynthetic Performance

The stress conditions generated by NaCl decreased the leaf area, total Chl content and Fv/Fm value by 42.4, 28.8 and 19.2%, respectively, compared to the respective controls (Fig. 2). Treatment with AsA and/or EBL improved significantly both Chl content and Fv/Fm. Total leaf area increased in the plants treated with AsA+EBL, AsA or EBL by 35.2, 31.5 and 30.5%. Total chlorophyll content increased by 43.2, 31.4, and 19.3%, respectively, whereas Fv/Fm increased by 33.3, 22.1, and 19.2% compared with the respective control. The treatment with AsA or EBL + AsA helped the plant to overcome the negative effect exerted by the NaCl stress.

Electrolytic Leakage:

Plants exposed to salt stress induced greater EL (30.8%) as compared with the controls. However, the exogenous application of AsA alone or together with EBL in stressed plants reduced the EBL by 20.4 and 23.7%, respectively, as compared with the controls (Fig. 2D). Moreover, the application of AsA alone to the plants exposed to NaCl (150 mM) completely restored the leakage of ions.

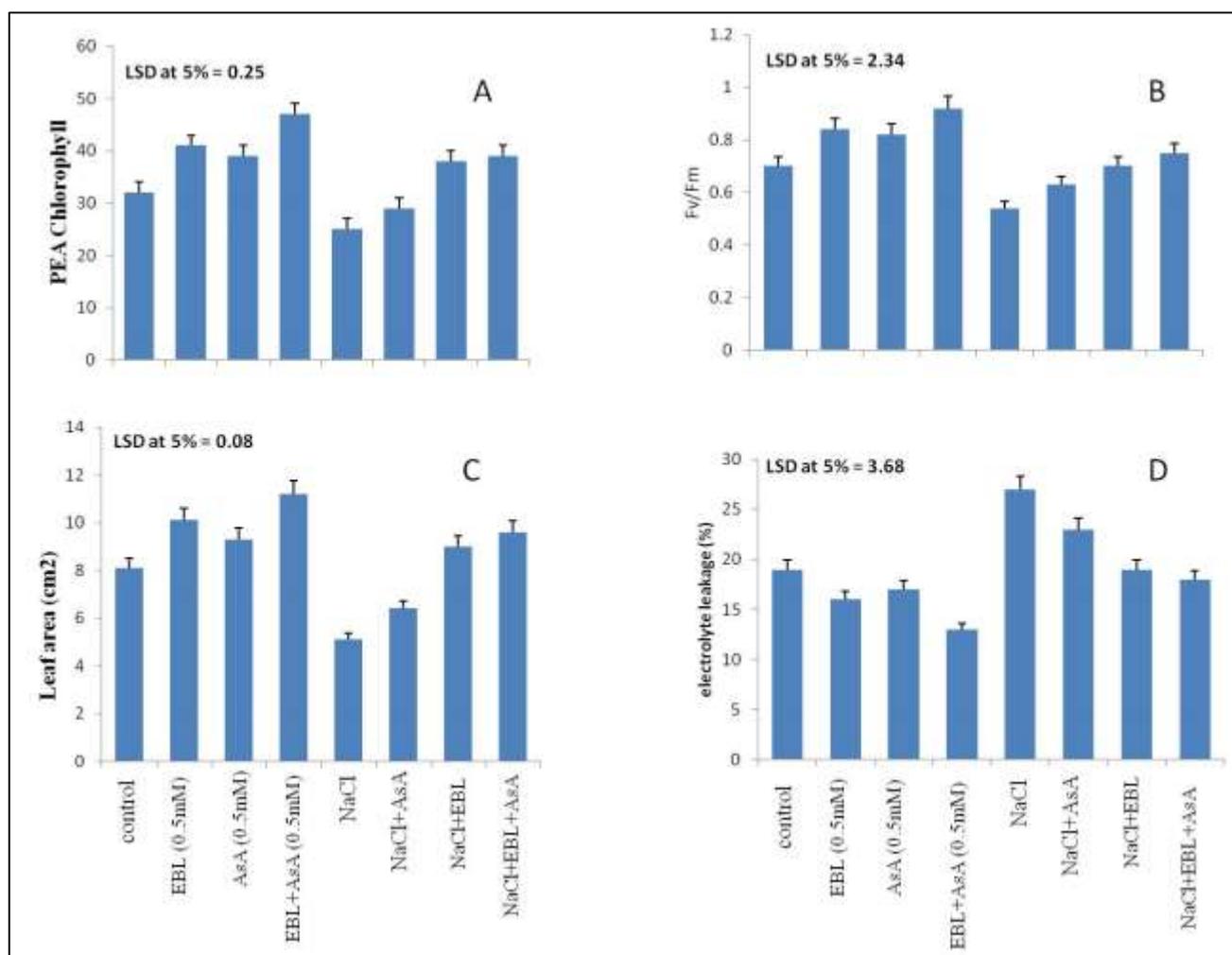


Fig. 2: Effect of Ascorbic acid (AsA; 10.5mM) and/or 24-epibrassinolide (EBL; 0.5mM) on the NaCl (150 mM) induced changes in the (A) PEA chlorophyll, (B) Fv/Fm, (C) Leaf area, (D) electrolytic leakage

Proline Content and NR Activity

Potato plants exposed to salt stress were found to have higher proline content than the control plants (Fig. 3 A). Proline content in response to NaCl was 36.8% higher. However, EBL and/or AsA treatment of the salt stressed plants had an additive effect on the proline content. Application of EBL, AsA or both increased the proline content by 30.2, 20.0, and 44.5%, respectively, over the

control. The plants exposed to salt stress and subsequently treated with EBL+AsA were found to have the highest content of proline. The plants treated with either EBL or AsA showed higher NR activity than the respective control (Fig. 3 B). The NR activity increased by 40.2 and 25.0% when treated with EBL or AsA alone compared with the respective control. However, salt stressed plants treated by both improved significantly the NR activity by 20.9 compared with the unstressed controls.

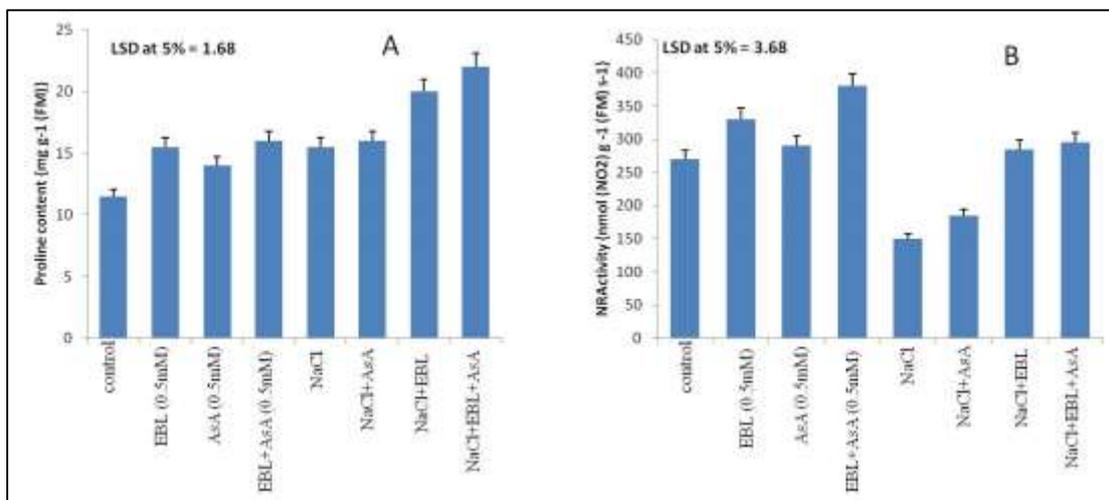


Fig. 3: Effect of Ascorbic acid (AsA; 10.5mM) and/or 24-epibrassinolide (EBL; 0.5mM) on the NaCl (150 mM) induced changes in the (A) Proline content (B) Electrolytic content

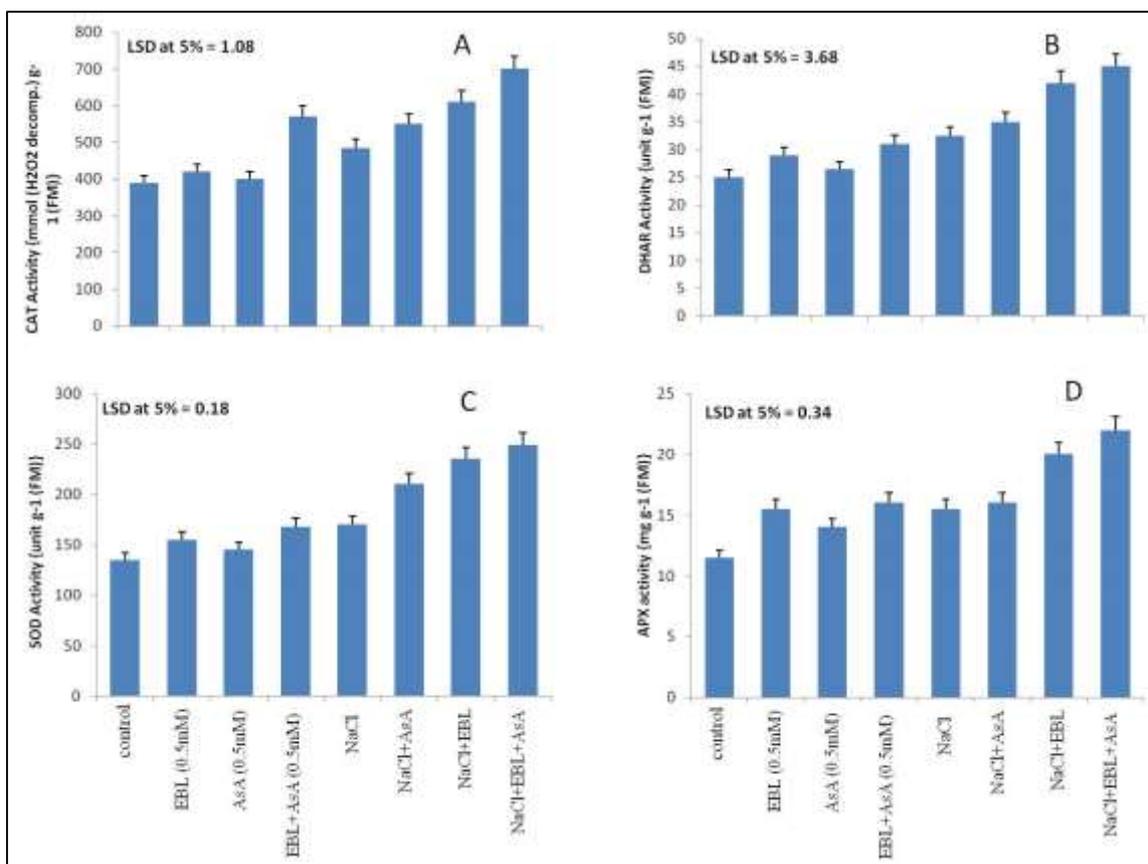


Fig. 4: Effect of Ascorbic acid (AsA; 10.5mM) and/or 24-epibrassinolide (EBL; 0.5mM) on the NaCl (150 mM) induced changes in the antioxidant enzymes (A) CAT activity (B) POX (C) SOD and (D) APX activity

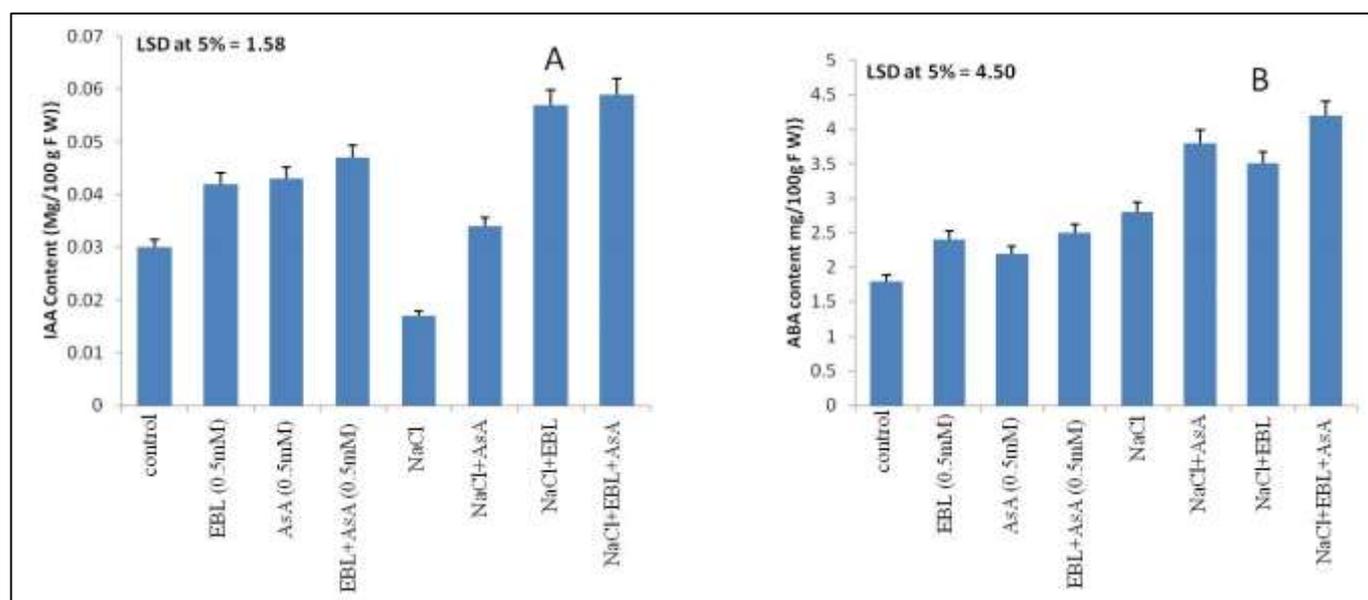


Fig. 5: Effect of 24 Ascorbic acid (AsA; 10.5mM) and/or 24-epibrassinolide (EBL; 0.5mM) on the NaCl (150 mM) induced changes in the endogenous hormone level (A) IAA(B) ABA

Antioxidant Enzymes

The activities of CAT, POX, SOD and APX were enhanced significantly by 30.1, 40.3, 35.2 and 42.2% in the plants exposed to salt stress (Fig 4). The application of EBL alone showed higher activities of CAT, POX, SOD and by 15.8, 15.8, 27 and 22%, respectively; whereas, AsA alone stimulated the activities of these enzymes by 8.8, 9.1, 11.2 and 13%, respectively, as compared with the respective controls. Furthermore, the maximal activities of

CAT, POX, SOD and APX were found in the plants exposed to stress and consequently applied with both EBL and AsA in combination.

Discussion

The salinity limits crop production in semiarid and arid regions, where saline soil is naturally high and its precipitation is insufficient to drain away salt from soil. The present study highlights the role of ascorbic acid and epibrassinolide to enhance salinity tolerance in potato. Potatoes are relatively sensitive to salinity, particularly in the early growth stages (Ghosh *et al.*, 2001; Nadler and Heuer 1995; Patell *et al.*, 2001). Several studies have indicated possible role of individual ascorbic acid and epibrassinolide in abiotic stress tolerance. Results of the present study indicated that all the morphological parameters in potato (shoot length, number of tubers, size & weight of tubers, FW, DW) were significantly inhibited by the salinity. Our results were in agreement with that of (Potiuri and Devi Prasad 1993) reported reduced in vitro growth in potato under 0.4–0.6% NaCl stress. Ghoulam *et al.*, (2002), Ekanayake and Dodds (1993) also showed marked reduction in growth parameters of sugar beet and *Ipomoea batatas* subjected to NaCl stress. Furthermore, reports on salt stress influencing the metabolic processes occurring in the chloroplast and mitochondria (Cheeseman

1988) suggested the prevention of important physiological phenomena such as osmosis and diffusion, disturbance in water and osmotic potential (Azooz *et al.*, 2004), toxicity of excessive Na^+ and Cl^- , disturbance in the accumulation of nutrients, disruption in the structure and the activity of the enzymes, (Feng *et al.*, 2002) damage in cell organelles and plasma membrane, disturbances in photosynthesis, respiration, and protein synthesis, (Munns 2005). Therefore, the reduced growth and yield of potato plants under salt stress in this study might be due to one or a combination of the aforementioned factors. In our experiments, the subsequent application of AsA and/or EBL to the plant growing under salt stress was favorable to withstand the growth and to reduce toxic effects of salt stress in comparison to respective controls. The stressed plants treated with AsA or EBL increased their FM and DM, shoot length and leaf area as well as tuber number and tuber size per plant, compared with those grown without AsA or EBL. A similar increase by AsA pretreatment in potato plant has been reported by (Sajid and Aftab 2009a). Similarly, Shalata and Neumann (2001) and Al-Hakimi and Hamada (2001) also observed that additional supply of ascorbic acid (0.5 mM) to the growth medium, before salt treatment considerably helped the recovery and long-term survival of wilted tomato seedlings and wheat plant respectively. Likewise, the EBL treatment also enhanced several morphological parameters as reported (Ali *et al.*, 2008; Hayat *et al.*, 2012; Fariduddin *et al.*, 2009). In our experiments, the BR- treated plants exhibited the improved growth parameters which are known to improve the growth of root and/or shoot in various plant groups (Anuradha and Rao 2003). The increase in the growth parameters caused by BR is due to involvement of BR-regulated genes in plant development, such as cytoskeleton formation, cell wall modification, growth hormone biosynthesis (Vert *et al.*,

2005) activated cell division and cellular enlargement (Hu *et al.*, 2000) up-regulation of xyloglucan transferase/hydrolase (cell wall loosening enzyme) and its enhanced gene expression or by activating the H⁺-ATPase activity (Sun *et al.*, 2005). Interestingly, the combined application of AsA and EBL showed more pronounced effects on the improvement of growth parameters under salt stress, perhaps due to their synergistic or additive effects. Although there are no reports on synergistic effect of AsA and BR on amelioration of salt stress in plant, however, the synergistic effects of Polyamines (PAs) and BRs were shown by (Choudhary *et al.*, 2011) and (Fariduddin *et al.*, 2014).

In addition to the primary effects, salinity stress leads to oxidative stress through an increase in reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen (Zheng *et al.*, 2009). It is now established that these cytotoxic ROS are accountable for various stress-induced damages to macromolecules and ultimately to cellular structure (Amor *et al.*, 2006; Sekmen *et al.*, 2012; Ulrich Heber, Chikahiro Miyake, Junichi Mano 1996). ROS detoxification by enzymatic antioxidants (SOD, CAT, POX, APX and other) is the effective defense mechanism against oxidative damage in plants (Hemavathi *et al.*, 2010a; Khan and Panda 2007; Verslues *et al.*, 2006) However, increasing evidence suggests that ascorbate peroxidase provides resistance to various environmental stresses in plants (Kim *et al.*, 1999; Mano *et al.*, 2001, Hemavathi et al 2010, Upadhyaya et al 2011) In this study, we observed the enhanced activities of antioxidative enzymes such as CAT, POX, SOD and APX, as well as that of proline content in the plants exposed to NaCl stress with or without AsA and /or EBL treatments. The increase in activities of antioxidative enzymes by application of AsA or BRs is a gene- regulated phenomenon. Ascorbic acid is considered an important antioxidant, protecting plants from oxidative stress by eliminating several reactive oxygen (Smirnoff and Wheeler 2000). Exogenous application of ascorbic acid was suggested to be utilized in cell metabolism to enhance the cell division efficacy of growing cells (Citterio *et al.*, 1994). Moreover, ascorbate has also been reported to play a role in elimination of H₂O₂ indirectly via activity of APX, CAT, glutathione peroxidase, glutathione reductase, and SOD (Asada 1992). Further, (Goda *et al.*, 2002) reported the expression of POX-encoding genes, ATP2 and ATP24a, to be regulated by BRs in Arabidopsis. Since the activity of POX is activated by various environmental stresses, it is possible that BRs might mediate the detoxification of ROS (Andre *et al.*, 2010). Our results validated the synergistic interactions of AsA and EBL for amelioration of the oxidative stress generated by salinity stress.

Salt stress also are also reported to cause damages to photosynthetic machinery at multiple levels, such as

structure and function of thylakoids, pigment content, electron transport and enzymes activity, stomata functioning, and gas exchange (Geissler *et al.*, 2009). Salt stress also causes a decrease in Chlorophyll content via the acceleration of Chlorophyll degradation or inhibition of its biosynthesis (Gururani *et al.*, 2012). In our experiment, a decrease in value of Chlorophyll was found which may be the result of effect of these processes. Similar decrease in Chlorophyll content was observed in plants by (Bhomkar *et al.*, 2008; Gururani *et al.*, 2013; Hayat *et al.*, 2012; Sajid and Aftab 2009a). However, the subsequent treatment by AsA and/or EBL improved the Chl content in our experiment. Cellular enhancement in ascorbic acid content via AsA application or transgenic approaches also results in the protection of chloroplast membrane integrity (Gadallah 2000; Hemavathi *et al.*, 2010b; Venkatesh *et al.*, 2012). It is very important to note that AsA or EBL application to unstressed potato also increased the Chl content; which was also it was also found by others in plants (J. Q. Yu *et al.*, 2004; Ali *et al.*, 2008; Hayat *et al.*, 2012; Fariduddin *et al.*, 2014). One of the possible reasons may be AsA/EBL-induced transcription and/or translation involves the expression of specific genes responsible for synthesis of enzymes determining Chl synthesis. In this study, decrease in Fv/Fm value was also observed which may be due to decrease the turnover of MSP protein (D2 protein) of PSII under salinity stress. Such a decrease was also observed in *Vigna radiata* ((Hayat, Hasan, Yusuf, *et al.*, 2010), and in *Triticum aestivum* (Shahbaz *et al.*, 2008) exposed to salinity. Gururani *et al.*, (2012) reported a very interesting result where untransformed control potato showed decrease in Fv/FM under growing salinity, however, the transgenic potato overexpressing MSP2 protein resisted the salinity stress and the Fv/Fm level was restored even under higher salinity stress without any yield penalty. In our experiment, application of AsA and /or EBL on potato improved the values of Fv/Fm growing under salinity condition indicating that AsA and EBL helped in the protection of PSII under stress that is often associated with decrease in photosynthetic electron transport activities due to inhibition of PSII activity (Kao *et al.*, 2003). Cellular enhancement of BRs improved the quantum yield of PSII under salt stress in wheat is reported by (Shahbaz *et al.*, 2008) and in potato (Venkatesh *et al.*, 2012). All these results suggested that AsA and BR application reduced the sensitivity of potato to NaCl stress similarly as in our study.

The activity of nitrate reductase (NR) is a measure of the habitat-dependent nitrate utilization of plants (Larcher W 1995). Potato exposed to salt stress showed significantly decrease in NR activity. This decrease might be subsequent consequence of the inhibition and/or metabolic dysfunction of NR or inhibited nitrate transport to the shoot interference with nitrate uptake and xylem loading (Cramer MD et al 1995). Moreover, stress factors affect the structure and fluidity of the membrane ((Mohanty and Pardha Saradhi

1992), (Karim *et al.*, 1999) as also obvious from the increase in electrolytic leakage after the exposure to salt stress in our experiments. This might limit the uptake of nitrate, the inducer and substrate for the NR (Campbell 1999) resulting in the reduced NR activity. However, treatment of AsA and /or EBL to both stressed and non-stressed plants enhanced the activity of NR. This may be due to impact of BR on membrane anion channels to facilitate the uptake of nitrate and increased transcription and/or translation of the metabolic enzymes (Z. Zhang *et al.*, 2005). Moreover, increased NR activity by steroidal and other polyamine like compounds is attributed to the fact that these compounds are involved in increasing the transcript levels of NR and its cofactor- binding domain genes, thereby stimulating the activities of NR and nitrate reduction (Shi *et al.*, 2008).

A significant decrease in free IAA was recorded in salt when compared with controls. Further, EBR treatment restored the contents of free IAA levels up to 65% of their control values. The improved may be due to enhanced biosynthesis of IAA similar to earlier reports in radish seedlings grown under stress (Choudhary *et al.*, 2010). Auxins have been reported to ameliorate the inhibitory effects of salinity (Farooq *et al.*, 2009), and heavy metals (Dimkpa *et al.*, 2008) in plants. It was found that interactions between ABA and BR in *Arabidopsis* occur at a platform called ARF2 (auxin response factor - an integration point for auxin and BR promoters), a transcription factor that responds to auxin, could be regulated by BR insensitive (BIN2), a negative regulator of BR signaling (Zhang *et al.*, 2009). Moreover, ascorbate has also been reported to be an important cofactor for a number of enzymes involved in hormone synthesis, e. g., gibberellins ((Prescott and John 1996). This opens yet another possible explanation for better growth parameters of potato cultures as observed in our study. These reports and the findings from the present study could advance our understanding about the stress mitigation role of BRs via auxins.

Conclusion

The role of AsA and BRs in restoring growth has been shown independently in several studies. It is apparent that both the AsA and BR crosstalk to induce defensive genes countering stress conditions. Salt stress decreased the growth, physiological, and biochemical parameters in potato. The synergistic effect of AsA and EBL was more effective in alleviation of salinity stress than their individual impacts. Our study confirmed further that the amelioration of salinity tolerance in potato (*Solanum tuberosum* L.) may be achieved by exogenous application of AsA and BRs.

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Conflict of Interest

The authors declare no conflict of interest for present investigation.

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