Salt stress relief potency of whortleberry extract biopriming in maize

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Abstract

Berries have gained public attention for their presumed positive effects on cancer patients. In contrast, the potential of berries to mitigate damage caused by abiotic stress in plants has not received significant attention. This is the first quantitative analysis of the efficacy of *Vaccinium arctostaphylos* L. (Ericaceae) fruit extract (VAFE) used to bioprime maize to limit damage caused by salt stress. Salt stressed maize seedlings exhibit lower quantum efficiency of photosystem II (Fv/Fm) and photosynthetic pigment content relative to untreated controls however, Fv/Fm increase caused by VAFE was found marginal. VAFE biopriming limited pigment loss and increased levels of antioxidant enzymes. It improved the growth of salt stressed seedlings by reducing salt-induced biomass loss, damage to roots and shoots, lipid oxidation, proline synthesis and endogenous hydrogen peroxide concentrations. In sum, VAFE biopriming may provide a new approach to improve yields in soils containing high salt levels as an alternative to traditional agricultural practice.

Keywords Berry extract · Antioxidant · Salinity · Priming · *Zea mays*

Introduction

With roles in nutrient availability and pH balance salts are cornerstones for healthy soil and optimal crop yields. However, high soil salt concentrations have long been one of the main threats to agriculture. Global warming in current era, rising 0.13 °C each decade since 1950 (Bhau et al. 2016), poses an additional threat by increasing the damage caused by salt stress on crops up to catastrophic levels. In addition, population increase combined with the shrinking availability of arable land is raising public concern about the number of undernourished individuals. Unfortunately, however, the crops feeding the world population are glycophytes and thus not be able to evolve fast enough to tolerate high salt concentrations (Zhang and Shi 2013). Maize is one of those crops as it is moderately salt-sensitive and globally important plant for human daily needs, taking third place after rice and wheat among cereals (FAO 2016). Even though some salinity-tolerant varieties of maize may exist because of cross pollination or high polymorphism that occurred through the evolution (Hasegawa et al. 2000), the capacity

of breeding alone to increase yield, germination and/or plant survival under salt stress remains in doubt. Plant growth management procedures that can be used in conjunction with breeding offer the hope of a sustainable strategy for coping with salt stress and improving food security.

Ionic toxicity due to the sodium and/or chloride accumulation in the cytoplasm can dramatically inhibit the growth of sensitive plants. The main cause is not chloride but sodium toxicity, which is the major problem in salt stressed maize and other members of the Poaceae (Paterniani 1990). Sodium has detrimental effects on cell metabolism because NaCl may replace by K⁺ which is important in the enzymatic reactions occurring in the cytoplasm. Therefore, it is not prominently the Na⁺, but the high concentrations of Na⁺ to K⁺ ratio in the cytoplasm causes toxicity (Yue et al. 2012).

Low yields due to salt stress are a global problem that is not yet under adequate control despite an array of tolerance strategies that have been employed. The complex nature of the NaCl stress, including a role in inducing oxidative stress, makes achieving salt tolerance through genetic engineering a difficult goal (Jha et al. 2013). In addition, public concern about genetically transformed crops further complicates their introduction. To this end, organic plant growth promoting preparates may provide an acceptable alternative to both gene modification and to chemicals (chemical priming) which may be harmful to human health, the integrity of

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the microbial community in rhizosphere and environmental quality.

Intake of crude extracts of plant organs rich in phenol compounds (leaves, roots, seeds and fruits (Sowndhararajan et al. 2013) is an increasing social trend in the field of herbal medication for human health. Their redox balancing capability as reducing agents, hydrogen donors and singlet oxygen quenchers (Kähkönen et al. 1999) are considered to protect against chronic diseases such as diabetes, cancer, muscular dystrophy and neurodegeneration (Pascual-Teresa and Sanchez-Ballesta 2008). In contrast, synthetic antioxidants are implicated in toxicity and carcinogenicity (Sowndhararajan et al. 2013). Since these naturally occurring antioxidant molecules prevent free radicals from damaging basic biomolecules such as DNA, proteins and lipids (Sowndhararajan et al. 2013) in animal cells, they may also activate similar defense mechanisms in plant cells. Hence, this phenomenon might be used to increase salt stress tolerance in plants as well. Furthermore, formulated bioavailable anthocyanin rich plant organ extracts could be more cost-effective than currently available mineral fertilizers.

Plant phenols are phytochemicals that can be divided into three major groups of secondary metabolites: (i) flavonoids, (ii) terpenoids and (iii) alkaloids (Crozier et al. 2006). The flavonoid family includes proanthocyanins, flavonols, catechins and anthocyanins (Kähkönen et al. 1999). Anthocyanidins, aglycon forms of anthocyanins, are structurally based on the flavilium ion or 2-phenylbenzopyrylium and have hydroxyl and methoxyl groups in different positions. Anthocyanidins present in nature are mainly the linked forms of one or more glycosidic units (Pascual-Teresa and Sanchez-Ballesta 2008). The most commonly found are pelargonidin, cyanidin, delphinidin, petunidin, peonidin and malvidin (Pascual-Teresa and Sanchez-Ballesta 2008).

Grapes and berry varieties (whortleberry, strawberry, blackberry, blueberry, raspberry, bilberry, cherry), with the help of their wide range of flavonoids and phenolic acids, play a bottleneck role regarding stabilizing molecules acts as free radicals in the cell in many human health related studies (Suzuki et al. 2011). When free radicals exceed the level that a plant can tolerate through its own defense mechanisms, exogenously applied antioxidants might be useful in the neutralization or elimination of radicals that may overwhelm the plant's endogenous defenses. For example, fresh strawberry extract has been shown to inhibit lipoprotein oxidation with 15 times higher antioxidant capacity than a water soluble antioxidant, vitamin E (Wang et al. 1997). Berries, an anthocyanin-rich fruit, have been widely known in traditional herbal therapies for the treatment of an array of disorders since ancient times (Milbury et al. 2007). In a study, phenolic extracts of berries were shown to be controlling the decrease of oxidation by their extraordinarily high scavenging potential against chemically generated

superoxide radicals (Constantino et al. 1992). Extracts of *Vaccinium arctostaphylos* fruits, with their high anthocyanin levels (Soltani et al. 2014), was chosen here for further study. This species is native to northern forests of Turkey as well as Azerbaijan, Georgia, Russia, Iran and Armenia.

While there are many studies of the antioxidant effects of berry extracts in animal cells, especially rodent models (Joseph et al. 1999; Milbury et al. 2007; Tang et al. 2015), little has been reported of potential uses of berry extracts to alleviate damage caused by abiotic stresses in plants. Of note, the role of organic extracts in salt stress tolerance remains unclear. This report is an evidence of the antioxidant potential of natural *Vaccinium arctostaphylos* fruits against salt stress. This is the first study showing modulation of salt stress-related oxidative stress defense by application of fresh *Vaccinium arctostaphylos* fruit extracts (VAFE).

Materials and methods

Preparation of whortleberry extract

Fresh fruits of whortleberry (*Vaccinium arctostaphylos*) were harvested from the Eastern Black Sea Coast of Turkey, Rize during spring season in May 2015. After the plants were authenticated by a plant taxonomist the fresh fruits of whortleberry were stored in the Recep Tayyip Erdogan University Berry Research and Application Center. The fruits were washed thoroughly in tap water, shade dried at room temperature (25 °C), pulverized and used for solvent extraction. Powdered fruit samples were sequentially extracted with ethanol and dimethyl sulfoxide (DMSO). The extraction was performed according to Lim et al. (2014). Briefly, fresh fruit equivalent to 30 g dry weight was digested with the help of a mechanical stirrer (Heidolph RZR 2020, Germany); a little amount of ethanol added, then it was further mixed with 1:3 volume of ethanol (w/v) at 40 °C for 30 min in an ultrasonic water bath (Elmasonic S 30 H, Germany). The extract was then centrifuged (Hettich® Universal 320R Zentrifugen, Germany) at 3500 rpm for 10 min. Two further re-extractions were performed in the same manner by using the same extraction solvent to obtain the supernatant. Combined supernatant was filtered through Whatman filter paper (Whatman International, England) and evaporation was conducted with a rotary evaporator (Genevac EZ-2 Elite personal, USA) – 40 °C to evaporate the solvent. Obtained extracts were further extracted in DMSO and stored at – 20 °C for the analyses. The freeze-dried extract (in DMSO) was diluted with the water. After pilot experiments the final concentration of 0.5 mg/ml was decided to be used for the assessment of antioxidant capacity through various bio-chemical assays.

Plant materials and growth conditions

Maize seeds (*Zea mays* L. Samada 07) were surface sterilized in 70% (v/v) ethanol for 5 min, then 15% (v/v) sodium hypochlorite for 8 min and finally rinsed with sterile H₂O for several times. Sterilized seeds were stratified in water at 4 °C for approximately 1 h and then were germinated in petri dishes in the dark for 4 days. Each pot (16 cm × 14 cm × 11 cm) was sown with 6 seeds, selected for uniformity in their radicle emergence rate (radicle length) and grown for another 10 days in soil under constant conditions (23 °C ± 2, temperature, 60% ± 5 relative humidity and 400 μmol m⁻² s⁻¹ light intensity) in a growth chamber. The leaves of the plants were then collected at 30 days of growth and stored at - 80 °C until use for the measurements of relevant parameters. Values in all experiments represent the mean of at least three biological and three technical replicates.

Salt tolerance test and measurements of growth and biomass

Priming with VAFE (0.5 mg/ml) was performed right after the germination stage by giving VAFE/water solution to the maize seedlings, and salt (NaCl) stress was initiated after 14 days of growth at 4-day intervals. A 50-mM NaCl solution was used to irrigate the plants on day 14, with subsequent NaCl solutions used for watering in which the concentration raised to 200 mM in 50-mM increments. Plants treated with neither NaCl nor VAFE served as one type of control. Plants treated with DMSO alone only (DMSO is the polar solvent of the extract) were used as another control group (although DMSO has a very low toxicity towards plant cells, a DMSO treated group was included to eliminate uncertainty in this respect). The experiment was stopped after 30 days of growth. After pictures were taken on the last day of experimental period, root and shoot lengths were measured with the help of Image J (Rueden et al. 2016). Root and shoot fresh weight (FW) was measured by weighing the above or below-ground portion of plants. Subsequently, these plant portions were dried in an oven at 65 °C for 48 h and dry weights (DW) were measured.

Analyses of physiological characteristics

Chlorophyll (Chl) measurement was performed by the method of methanol extraction (Porra et al. 1989). Hundred mg of fresh leaf samples was excised and incubated in methanol absolute for 24 h at 4 °C. An aliquot of 1 ml of the extraction solution was used per plant to calculate the concentrations of total Chl based on absorption at A₆₆₅ and A₆₅₂ with a BioTek Synergy HT microplate reader (BioTek Instruments, Inc. Vermont, USA). Absorption values were

then converted to the relative amounts of Chl a + b for each sample using the specific molar extinction coefficient (Porra et al. 1989).

Chl fluorescence ratio values (Fv/Fm) were determined using a portable fluorometer (OS5p, OptiScience Corporation, USA) after 30 min dark adaptation of leaves and maximum photochemical efficiency of Photosystem II (PSII) was determined.

Enzyme activities

The extraction of enzymes was conducted at 0–4 °C. Leaves (0.5 g) were pulverized under liquid nitrogen. The resulting powder was transferred to a centrifuge tube in 5 ml of 50 mM phosphate buffer (pH 7.0), 1% polyvinylpyrrolidone and 1 mM EDTA. This mixture was centrifuged at 20,000 g for 20 min at 4 °C and the aqueous supernatant was stored at - 20 °C for all further enzyme analyses. For all enzyme assays, blanks contained only buffer and no extract.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined by a modified method by Dhindsa and Matowe (1981) in 1 ml volumes containing 75 μM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 2 μM riboflavin, 13 mM methionine, 50 mM phosphate buffer (pH 7.8) and 50 μl of supernatant. The reaction was illuminated with two 20 W (watt) lamps for 10 min and absorbance was read at 560 nm. A unit (U) of SOD activity was given as the amount of enzyme needed to inhibit the rate of the NBT photoreduction by 50%.

Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was analyzed by the method of Urbanek et al. (1991). The increase in tetraguaiacol absorbance was measured at 470 nm for 3 min. The extinction coefficient of tetraguaiacol is 26.6 mM⁻¹ cm⁻¹ at a wavelength of 470 nm.

Catalase (CAT, EC 1.11.1.6) activity was assayed by H₂O₂ depletion (25 °C, $\epsilon = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$) at 240 nm (Aebi 1983). 50 mM potassium phosphate buffer (pH 7.0), H₂O₂ and stored extract for 1 ml of total volume were used and reaction was initiated by adding 10 mM H₂O₂ into the mixture.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined based on the decrease in absorbance at 340 nm (25 °C, $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) according to Foyer and Halliwell (1976). One ml of reaction mixture containing 150 μM NADPH (the reaction initiator), 500 μM oxidized glutathione (GSSG), 50 mM Tris-HCl (pH 7.8) and 50 μl of extract was used for the experiment.

Total protein content was measured for each group of plants according to Bradford (1976) with bovine serum albumin as standard.

Analyses of chemical compounds

Four-week-old maize plants were used to determine the lipid oxidation rate by using the analysis of thiobarbituric acid-Malondialdehyde (MDA) complex (Heath and Packer 1968). 100 mg of leaf samples were used for absorbance readings collected by a spectrophotometer (Biochrom) at A_{532} and A_{600} . The results are represented as nmol of MDA per gram of plant FW.

Endogenous hydrogen peroxide accumulation as a stress marker was detected at 390 nm following a modified method of Velikova et al. (2000). 0.25 g leaf samples was pulverized in 3 ml of 5% trichloroacetic acid in combination with 0.1 g of activated charcoal at 0 °C. 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 0.75 ml of 1 M KI were transferred to a 0.5-ml aliquot of the supernatant. H_2O_2 concentration is expressed as $\mu\text{mol g}^{-1}$ FW.

For proline measurements, 0.25 g leaf samples were homogenized in 5 mL of 3% sulfosalicylic acid and homogenate was centrifuged at 8000×g for 15 min as described by Bates et al. (1973). The proline concentration was determined by a standard curve.

Data and statistics analysis

All data were analyzed by normality (Shapiro–Wilk) and equal variance test (Levene). One-Way ANOVA was used to compare the parameters in different groups. Whenever significant differences were detected among different treatments, Tukey's tests were performed. Statistical significance was 5% ($p < 0.05$). Statistical analyses were performed using the SigmaPlot 12.3 statistical software package (Systat Software INC.).

Results

The efficacy of VAFE biopriming on the activities of antioxidant enzymes in maize during salt stress

The effect of VAFE biopriming on antioxidant enzymes was found to be significant. The activity of all antioxidant enzymes increased during salt stress in maize seedlings and the activity rates were higher in the VAFE bioprimed seedlings. The SOD, GPX, CAT and GR activities increased by 90, 55, 20 and 37% in salt only treated seedlings as compared to the untreated seedlings. Furthermore, VAFE biopriming alone (absence of salt stress) leads to a remarkable increase in SOD, GPX, CAT and GR activities as compared to the DMSO only treated seedlings. Additionally, VAFE biopriming further upregulated the

activities of SOD, GPX, CAT and GR as compared to salt only treated seedlings (Fig. 1).

Total chlorophyll content and Chl fluorescence (Fv/Fm ratio)

The results pertaining to the outcome of VAFE biopriming before induction of salt stress on total Chl content in maize is shown in Fig. 2a. Percent reduction in total Chl content was significantly increased under salt stress. Significant decrease was recorded by 23% in total Chl in salt only treated seedlings. However, Chl content in VAFE bioprimed only seedlings (in the absence of salt stress) did not change significantly as compared to the DMSO only treated seedlings. When the VAFE bioprimed and salt stressed seedlings (SE) were compared with the salt only treated seedlings (S), total Chl content in VAFE bioprimed and salt-stressed seedlings were higher than that of salt only treated seedlings. These data indicate that VAFE biopriming relieves the injurious effects of salt stress on total chlorophyll. Salt stress inhibited maximum quantum yield of PSII photochemistry (Fv/Fm) as compared to the DMSO and water only treated seedlings. On the other hand, the Fv/Fm increase caused by VAFE was found marginal. The VAFE and DMSO group showed slightly less Fv/Fm comparing to well-watered seedlings (Fig. 2b).

The effects of VAFE biopriming on lipid peroxidation and H_2O_2 content under salt stress

While salinity caused a severe H_2O_2 accumulation in the seedlings, VAFE biopriming markedly diminished the H_2O_2 concentration of the seedlings under stress (Fig. 3a). The results regarding the outcome of VAFE biopriming on lipid peroxidation in maize seedlings are shown in Fig. 3b. The VAFE alone did not alter the MDA content of the non-stressed plants as compared to the DMSO only treated seedlings. Yet salt stress immensely increased the lipid peroxidation in maize leaves. Furthermore, VAFE biopriming significantly mitigated the lipid peroxidation under NaCl stress (Fig. 3b).

Proline content

Salt stress caused a remarkable increase in proline content. VAFE biopriming alone induced proline accumulation as compared to the water and DMSO only treated seedlings. When VAFE bioprimed seedlings were compared with the salt only treated seedlings, proline content in VAFE bioprimed and salt stressed seedlings was slightly lower than that of salt only treated seedlings (Fig. 3c).

Fig. 1 The activities of CAT (a), GPX (b), GR (c) and SOD (d) enzymes in maize seedlings with/without dose-dependent NaCl and/or whortleberry extract. 200 mM NaCl solution applied in 50 mM increments every 4 days starting on day 14. Whortleberry extract (0.5 mg/ml in DMSO) was applied since germination till the end of the experiment. W, water only; D, DMSO only; E, whortleberry extract only; S + E, 200 mM NaCl plus whortleberry extract; S, 200 mM salt only treated seedlings on day 30. Error bars show standard deviation and different letters (a, b, c, d, e, f) indicate significant difference (ANOVA, $p < 0.05$) among the different treatments

Fig. 2 Total chlorophyll (a + b) contents (a) and maximum quantum efficiency of PS II (Fv/Fm) (b) in the absence or presence of dose-dependent NaCl and/or whortleberry extract. 200 mM NaCl solution was applied in 50 mM increments every 4 days starting on day 14. Whortleberry extract (0.5 mg/ml in DMSO) was started to be applied right after the germination till the end of the treatment. W, water

only; D, DMSO only; E, whortleberry extract only; S + E, 200 mM NaCl plus whortleberry extract; S, 200 mM salt only treated seedlings on day 30. Error bars show standard deviation and different letters (a, b, c, d, e, f) indicate significant difference (ANOVA, $p < 0.05$) among the different treatments

The effects of VAFE on the physiological parameters of maize during salt stress

First, the effects of VAFE bioprimer were examined on the physiological parameters in salt stressed maize. Salt stress significantly lowered the shoot and root dry weight as well

as the length of each organ. Maximal decline in the length of shoot and root was 20 and 23% under salt stress compared to the water only treated seedlings (control), respectively (Fig. 3d). VAFE bioprimer alone caused an increase in root length and root/shoot dry weight while no change was observed in shoot length of maize seedlings as compared to

Fig. 3 Biochemical and growth response of maize seedlings with/without dose-dependent NaCl and/or whortleberry extract. H_2O_2 (a), MDA (b), proline (c) contents and root and shoot growth (d) of maize seedlings. 200 mM NaCl solution was applied in 50 mM increments every 4 days starting on day 14. Whortleberry extract (0.5 mg/ml in DMSO) was started to be applied right after the germination till the end of the treatment. W, water only; D, DMSO only; E, whortleberry extract only; S + E, 200 mM NaCl plus whortleberry extract; S, 200 mM salt only treated seedlings on day 30. Error bars show standard deviation and different letters (a, b, c, d, e, f) indicate significant difference (ANOVA, $p < 0.05$) among the different treatments

the DMSO only treated or well-watered seedlings (Fig. 3d). On the other hand, there were significant increases in fresh and dry weight of shoots of salt-stressed plants treated with VAFE (Fig. 4). While VAFE bioprimer in combination with salt stress (SE) was measured as 18.31 ± 1 g and 1.86 ± 1 g in shoots for FW and DW, the mean of salt only group (S) was 13.18 ± 1 g and 1.4 ± 1 g for the respective parameters. Accordingly, root FW and DW data were comparable (6.85 ± 1 g and 0.6 ± 1 g for SE and 6.46 ± 1 g and 0.60 ± 1 g for S for the respective parameters (FW and

DW). In the light of these results, VAFE bioprimer clearly enhances length and both dry and fresh weights of maize roots and shoots compared to the salt only treated seedlings and alleviates the detrimental effects of stress.

Discussion

The population explosion, urbanization and industrialization and other anthropogenic factors are inevitably changing our ecological balance and climate. In addition to these

Fig. 4 The fresh (a) and dry (b) weight of maize shoots and roots with/without dose-dependent NaCl and/or whortleberry extract. 200 mM NaCl solution applied in 50 mM increments every 4 days starting on day 14. Whortleberry extract (0.5 mg/ml in DMSO) was applied since germination till the end of the experiment. W, water

only; D, DMSO only; E, whortleberry extract only; S + E, 200 mM NaCl plus whortleberry extract; S, 200 mM salt only treated seedlings on day 30. Error bars show standard deviation and different letters (a, b, c) indicate significant difference (ANOVA, $p < 0.05$) among the different treatments

factors, abiotic stresses (particularly table salt, NaCl) can arise at any time and threaten our agricultural production at a global scale. It is important to limit and reverse negative environmental impacts of human activity and find sustainable solutions for stress tolerance. Even though using natural products, especially those derived from the *Vaccinium* genus, is becoming popular in the eye of public as a radical scavenger and balancing agent, no reports on the potential utility of *Vaccinium arctostaphylos* fruit in reducing the effects of salt stress on plants have been found. As maize is an agro-economically vital cereal (FAO 2016) which is generally salt sensitive (except for some resistant varieties as stated in Hajibagheri et al. 1987), our goal was to analyze the efficacy of VAFE biopriming of maize seedlings. Since the duration of the biopriming application and difference of responsiveness between different plant varieties that have genetically different backgrounds might have different tolerance responses, our study might be important in this particular cultivar (Samada 07).

Even though results might not be the same for other stages of maize growth such as flowering stage, it is clear that VAFE biopriming reduced the adverse effects of salt stress in maize seedlings during the first 30 days of growth. These include considerable reduction in root and shoot fresh/dry biomass as well as the length of each organ. Thus, VAFE biopriming might be effective in promoting plant growth under both saline and non-saline conditions. In parallel, Yasmeen et al. (2013) reported that naturally occurring plant growth stimulants reduced root and shoot biomass failure in wheat seedlings under salt stress. It was also reported that moringa leaf extract in tomato enhanced canopy biomass, lateral vegetative shoot number and plant height compared to untreated control plants (Basra and Lovatt 2016). Considering *Vaccinium* genus is a potential source of anthocyanin which has very strong antioxidant properties, mitigation of the negative effects of salt stress may be mediated by the high antioxidant properties of anthocyanin in the VAFE. This extract may also improve growth by effects on nutrient status and upregulation of phytohormones coding genes.

Numerous studies demonstrated that salinity results in a loss of balance between generation and degradation of reactive oxygen species (ROS) (Chawla et al. 2013; Abdelgawad et al. 2016). There are several pathways to convert toxic ROS to less toxic forms in plants (Mittler 2002). Given the fact that the upregulation of antioxidant enzymes is a protective process for plants against the harmful effects of ROS, data presented here support this notion, specifically that an increase in SOD, CAT, APX and GR activities occurs in VAFE bioprimed maize seedlings in the absence and presence of salinity stress (Fig. 1). Yasmeen et al. (2013) also reported that the exogenous application of growth stimulators positively modified antioxidant enzyme activities under salt stress. Our results demonstrated that VAFE bioprimed

maize seedlings have higher activities of antioxidant enzymes than untreated controls, consequently resulting in increased tolerance to excessive salt.

Another response to salinity is changes in photosynthetic pigment contents. It is well known that salt stress suppresses the pigments in the photosynthetic apparatus in an array of plant species. In this respect, the values presented in Fig. 2a show that VAFE biopriming relieved the toxic effects of salinity on Chl content. Rehman and Basra (2010) reported that foliar application of moringa leaf extracts prevents premature leaf senescence and results in more leaf area with higher photosynthetic pigment contents. In addition to these, increases were reported to be found in photosynthetic pigment content of tomato seedlings treated with *Ascophyllum nodosum* (Whapham et al. 1993) and seaweed extract application in maize and bean (Lingakumar et al. 2004). The improved photosynthetic pigment contents in plants exposed to *Vaccinium* fruits extract might be associated with the decreased lipid peroxidation and ROS production such as H_2O_2 . In this regard, exogenous application of VAFE reduced MDA and H_2O_2 concentrations remarkably in salt-stressed maize seedlings (Fig. 3a, b). These data strongly suggest that VAFE alleviates salt-induced oxidative damage in maize cells by virtue of its antioxidant properties. Additionally, given its positive effect on Chl content, VAFE biopriming might be attributed to the improvement in Chl pigment intactness and subsequently in the rate of photosynthesis (Fig. 2.). In accordance with that, *Fv/Fm* is also a favorable parameter which allows detection of damage to and possible photo-inhibition of PSII (Ahmed et al. 2002). Our results show that salt stress caused a remarkable decrease in *Fv/Fm* ratio and that exogenous application of VAFE under stress significantly moderates this effect (Fig. 2). Low *Fv/Fm* values in plants under stress generally indicate damage to the PSII reaction center. In this case, the effect of VAFE on the *Fv/Fm* ratio is the one possible way of VAFE which presumably minimizes damage to the cell membrane and thereby limits injury caused by ROS (Hashem et al. 2014).

Free proline is widely known as a compatible solute, scavenger of free radicals, stabilizer of macromolecules, metal chelator and also a source of reduced nitrogen and carbon. It is thus thought to alleviate negative effects of stressful conditions (Ben Rejeb et al. 2014) by accumulating in a variety of plants in response to environmental stresses, including salt. Free proline may also act directly as a scavenger of reactive oxygen species as well as an inducer of gene expression of several antioxidative enzymes and a protectant of PSII (Hossain and Fujita 2010; Szabados and Savaouré 2010; De Carvalho et al. 2013; Filippou et al. 2014). The positive impact of VAFE biopriming on the photosynthetic apparatus might thus be explained by the accumulation of anthocyanins and proline, leading to osmotic regulation under saline conditions (Carillo et al. 2011; Hayat et al. 2012).

Both proline and anthocyanins may protect plant cells from salt stress-induced oxidative injuries (Winkel-Shirley 2002; Verslues and Sharma 2010; Hayat et al. 2012). Our findings indicate an important role of proline in the protection of the photosynthetic apparatus, which is evident by reduction in maximal quantum yield of PSII under salinity. Interestingly, however, proline level of VAFE treated seedlings was significantly lower than that of salt stressed only seedlings (Fig. 3c). Since proline accumulation is very dynamic in the cell and depends on the sensing of stress signals, there might be a stress relief induced by VAFE biopriming and upon this relief the level of accumulated proline might decrease dramatically to a basal level as the synthesis process of this particular amino acid is completely reversible when stress conditions are changed. VAFE biopriming may exert a repressive role on proline synthesis, meaning seedlings have not been forced enough into accumulating proline to protect structure and function of cellular macromolecules. In our case, rather than proline, anthocyanins might be the prominent actors that bind to the toxic ions and protect cytoplasmic structures and chloroplasts from the adverse effects of NaCl on photosynthesis as stated by Wahid and Ghazanfar (2006) as well.

Accumulation of MDA as a degradation product of lipid peroxidation indicates an increase in membrane injury under abiotic stress conditions. In our study, VAFE exhibited highly beneficial results by markedly lowering lipid oxidation (Fig. 3b). Likewise, H_2O_2 amounts were actively reduced by VAFE in maize seedlings while salt stress triggered the accumulation of H_2O_2 (Fig. 3a). These results support our hypothesis that VAFE biopriming might act as a plant growth enhancer under stress due to the presence of anthocyanin, antioxidants and essential plant nutrients in the fruit. The decrease found in H_2O_2 and MDA suggests a role for anthocyanin in the alleviation of the oxidative injuries occurring in maize seedlings. In accordance with this notion, in a field study, Agati et al. (2007) found that anthocyanin-deficient mutants of *Arabidopsis* grown under strong light showed more lipid peroxidation than wild-type. Hatier and Gould (2008) also experimentally proved that anthocyanins alleviate stress-induced damage by scavenging free radicals and restricting H_2O_2 movement in plant cells.

Taken together, maize is a valuable crop and hardening it off by natural products under stress conditions is important not to harm environment with synthetic growth stimulators. This study assigns protective roles to VAFE in salt-stressed maize via modulating H_2O_2 accumulation, protecting photosynthetic apparatus and organizing antioxidant machinery. Although biopriming is still a method under active development, it offers several key advantages that can be used in conjunction with the existing agronomy practice. Since most of the priming agents have favorable outcomes only in a very narrow concentration

range (Savvides et al. 2016) our dose-dependent outcomes might be helpful in designing approaches for creating cost-effective and natural salt stress tolerance agents. Unless we have cost-effective and standardized procedures which allow for transfer of prototype priming studies to the field, we will not be able to use this method efficiently to fight against salt and other abiotic stresses. Moreover, length of effects after priming and difference of responsiveness between different genetic backgrounds of maize have not yet been addressed but should be in the future.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest between third parties.

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