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ORIGINAL PAPER

Modulation in growth, photosynthetic efficiency, activity of antioxidants and mineral ions by foliar application of glycinebetaine on pea (*Pisum sativum* L.) under salt stress

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Abstract A pot experiment was carried out to explore the role of glycinebetaine (GB) as foliar spray foliar on two pea (*Pisum sativum* L.) varieties (Pea 09 and Meteor Fsd) under saline and non-saline conditions. Thirty-two-day-old plants were subjected to two levels 0 and 150 mM of NaCl stress. Salt treatment was applied in full strength Hoagland's nutrient solution. Three levels 0, 5 and 10 mM of GB were applied as foliar treatment on 34-day-old pea plants. After 2 weeks of foliar treatment with GB data for various growth and physiochemical attributes were recorded. Rooting-medium applied salt (150 mM NaCl) stress decreased growth, photosynthesis, chlorophyll, chlorophyll fluorescence and soluble protein contents, while increasing the activities of enzymatic (POD and CAT) and non-enzymatic (ascorbic acid and total phenolics) antioxidant enzymes. Foliar application of GB decreased root and shoot Na^+ under saline conditions, while increasing shoot dry matter, root length, root fresh weight, stomatal conductance (g_s), contents of seed ascorbic acid, leaf phenolics, and root and shoot Ca^{2+} contents. Of three GB (0, 5, 10 mM) levels, 10 mM proved to be more effective in mitigating the adverse effects of salinity stress. Overall, variety Pea 09 showed better performance in comparison to those of var. Meteor Fsd under both normal and salinity stress conditions. GB-induced modulation of seed ascorbic acid, leaf phenolics, g_s, and root Ca^{2+} values might have contributed to the increased plant biomass, reduction of oxidative stress, increased osmotic adjustment and better photosynthetic performance of pea plants under salt stress.

Keywords Pea (*Pisum sativum* L.) · Salinity · Glycinebetaine · Ascorbic acid · Antioxidants

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Introduction

Abiotic stresses (e.g., drought, salinity, cold and heat) adversely affect growth and yield of economically important crops and more than 50 % yield losses are direct result of these environmental factors (Rodriguez et al. 2005; Ahmad et al. 2012). Among these abiotic stresses soil salinity is a major issue which acts as a limiting factor for the productivity of crops and is expected to destroy about 50 % cultivable land up to the middle of twenty-first century (Mahajan and Tuteja 2005). High salt level in the irrigation water or soil causes hyperionic and hyperosmotic stress effects which leads to various metabolic disorders and ultimately plant death (Mahajan and Tuteja 2005).

Various growth, physiological and biochemical attributes, e.g., fresh and dry biomass (Shahbaz et al. 2011, 2012; Shaheen et al. 2012; Kausar et al. 2013), photosynthesis (Kanwal et al. 2011; Kausar and Shahbaz 2013; Perveen et al. 2010, 2013; Shahbaz et al. 2013; Kanwal et al. 2013), chlorophyll fluorescence (Kanwal et al. 2011; Habib et al. 2013; Perveen et al. 2013), water relations (Perveen et al. 2012a, 2014; Odjegba 2013), mineral nutrients (Perveen et al. 2012b) and activities of antioxidant enzymes (Perveen et al. 2011; Ashraf et al. 2012; Odjegba 2013) are severely inhibited under saline conditions (Shahbaz and Ashraf 2013). However, plants protect

themselves from negative effects of salt stress by synthesis or accumulation of low-molecular-weight compatible organic compounds such as glycinebetaine (GB), proline (Pro) and raffinose (Wakeel et al. 2011; Chen and Murata 2002, 2011; Sakr et al. 2012), etc.

Glycinebetaine (*N*,*N*,*N*-trimethyl glycine) is a quaternary ammonium compound that is ubiquitous in occurrence (Rhodes and Hanson 1993) and known as an effective compatible solute that accumulates in the chloroplasts of certain plants under abiotic stress conditions like drought and salinity stresses (Robinson and Jones 1986; Giridarakumar et al. 2003). GB plays protective role against salt stress primarily by osmotic adjustment (Gadallah 1999), protection of photosynthetic machinery (Cha-Um and Kirdmanee 2010), stabilization of proteins structure (Makela et al. 2000) and scavenging of reactive oxygen species (Ashraf and Foolad 2007). However, GB accumulation varies not only under different environmental conditions, e.g., extreme temperatures (Karabudak et al. 2014), drought (Abbas et al. 2014), salinity (Girija et al. 2002) and alkaline stress (Cui et al. 2008), but also on type of plant species (Moghaieb et al. 2004), plant varieties (Hassine et al. 2008) and plant organelles (Zhu et al. 2003).

McCue and Hanson (1990) reported that activity of betaine aldehydehydrogenase, a GB synthesizing enzyme, is increased under salinity stress. Varshney et al. (1988), however, were of the view that choline and betaine accumulation was higher in salt sensitive *Trifolium alexandrinum* lines. Similarly, Wyn Jones et al. (1984) did not find positive correlation in accumulation of GB and salt tolerance in Elymus, Agropyron and Triticum genera. Accumulation of GB is a widespread and sporadic and varies in different plant species (Ashraf and Harris 2004). For example, the concentration of GB is tenfold more in sorghum than that found in maize (Grieve and Maas 1984; Rhodes et al. 1987). Yildiztugay et al. (2014) reported that osmolytes such as GB, proline and choline accumulate under mild level of salt stress in *Sphaerophsa kotschyana* plants. However, some other crops did not/little possess natural ability of GB-accumulation under abiotic stresses (Subbarao et al. 2001).

In recent decades, exogenous application of osmoprotectant such as proline, glycinebetaine and trehalose has helped reduce the negative effects of salinity stress (Hoque et al. 2008; Zeid 2009; Kausar et al. 2014).GB enables plant species to tolerate wide range of environmental stresses (Yang and Lu 2005) by helping plant cells with poor or no solute accumulation in osmotic adjustment (Ashraf and Foolad 2007). For example, under abiotic stresses foliar application of GB has been reported to increase growth of wheat (Shahbaz et al. 2011), rice (Chaum et al. 2013) and maize (Reddy et al. 2013). Exogenous application of GB has been reported to stimulate growth of tomato (Chen et al. 2009), rice (Shahbaz and Zia 2011), canola (Sakr et al. 2012), soybean (Ali et al. 2012) and maize (Nawaz and Ashraf 2010; Kaya et al. 2013) although adverse effects of higher doses of GB have also been reported in grapevines (under low temperature) (Wilson 2001), kidney bean (under salt stress) (Lopez et al. 2002) and cotton (under normal conditions) (Makhdum and Shababuddin 2006). However, complete mechanism of GB-mediated salt-stress tolerance is not well-known in crop plants.

Pea (*Pisum sativum* L.) is an important leguminous crop that ranked on 4th position globally and occupies 2nd position in Pakistan on productivity basis (Shahid et al. 2011, 2012). It is an excellent source of proteins, carbohydrates, vitamins, minerals, salts and antioxidants (Hussein et al. 2006; Noreen and Ashraf 2009). Pea is cultivated in tropical and sub-tropical regions of the world (Javaid and Ghafoor 2002). Pea is cultivated on an area of 528.71 thousand hectares with total production of 441.53 thousand tons globally (Ashraf et al. 2011). In Pakistan pea is cultivated as winter crop and used as food and fodder throughout the country. It is cultivated on an area of 10.00 thousand hectares with total annual production of 82.00 thousand tons (Khan et al. 2013). Punjab is the leading province and contributes 70–80 % of total pea production (Zaidi et al. 2013). However, production level of pea could not meet the domestic needs as its annual consumption goes up to 160.00 thousand tons. Salt stress adversely affects germination rate, fresh and dry biomass, plant height, photosynthetic efficiency and mineral nutrients of pea crop (Shahid et al. 2011, 2012). No information is yet available on the effect of foliar application of GB on pea plants under salt stress conditions. So, keeping in view the protective role of GB and economic importance of pea crop a hypothesis was made whether or not foliar application of GB could be effective in ameliorating the deleterious effects of salt stress on pea plants. So the objectives of current study were to assess the effect of foliar spray of glycinebetaine on various growth (root and shoot fresh and dry weights and root and shoot length), physiological (gas exchange characteristics and chlorophyll fluorescence) and biochemical (chlorophyll pigments, activities of enzymatic and non-enzymatic antioxidants, soluble proteins and mineral ions) attributes of pea plants under saline and non-saline regimes.

Materials and methods

Plant material and experimental design

To explore the role of foliarly applied glycinebetaine (GB) on various growth and physiochemical parameters of two

pea (*Pisum sativum* L.) varieties (Pea 09 and Meteor Fsd) under saline (150 mM) and non-saline conditions, an experiment was carried out in the wire-house of Botanical Garden, University of Agriculture, Faisalabad, under natural climatic conditions with average day and night temperatures 31.83 ± 4 °C and 10.66 ± 3 °C, respectively, 9.33 and 14.67 light and dark period at PPFD 800–1,100 μmol m^{-2} s^{-1}, respectively, and relative humidity from 68.83 to 50 %. Seeds of two pea varieties (Pea 09 and Meteor Fsd) were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad. Each plastic pot was filled with thoroughly washed river sand (10 kg per pot) and ten seeds per pot were sown. The design of the experiment was completely randomized with four replicates. When seedlings were 1 week old thinning was performed to reduce the number of plants to 6 in each pot. Plants were supplemented with full-strength Hoagland's nutrient solution at the rate of 2 l per pot every week. Salt stress of 150 mM NaCl was applied after 36 days of sowing. To avoid osmotic shock to plants salt solution (NaCl + Hoagland's nutrient solution) was applied by increasing the salt level gradually in aliquots of 50 mM NaCl every day up to day 3 until final volume 150 mM was attained. Salt treatment was applied at the rate of 2 l per pot every week until the end of experiment. Moisture content of sand were maintained by adding 150 ml water per pot every day. Glycinebetaine (mol. wt. 117.15 of Sigma Aldrich, Germany) solution was prepared by dissolving solid GB in distilled water and 0.1 % tween-20 to ensure effective penetration in leaf cells. Three levels of glycinebetaine, i.e., 0, 5 and 10 mM were applied at the rate of 25 ml per pot as foliar spray in the evening to avoid evaporation after 38 days of sowing. Data for various growth and physiochemical attributes were recorded after 2 weeks of foliar treatment with GB. Plastic zipper bags were used for the collection of fresh leaf samples during early morning to avoid desiccation and stored at −20 °C for determination of various biochemical attributes. Two plants were carefully up-rooted from each replication, measured shoot and root fresh weights (g $plant^{-1}$), and shoot and root lengths (cm). Then the same plant material was oven-dried at 65 °C, and shoot and root dry weights (g $plant^{-1}$) were recorded.

Physiological attributes

Determination of gas exchange characteristics

Net CO_2 assimilation rate (A), transpiration rate (E), stomatal conductance (g_s) and sub-stomatal CO_2 (C_i) were determined by using a portable infrared gas analyzer (Analytical Development Company, Hoddesdon, LCA-4 ADC). The gas exchange measurements were performed in situ from 10.30 a.m. to 12.30 p.m. with some specific adjustments/specifications as mentioned in Perveen et al. (2010). PAR (Qleaf) at the surface of leaf was 941 μmol m^{-2} s^{-1}.

Chlorophyll fluorescence determination

A Multi-Mode Chlorophyll Fluorometer of model OS5P-Sciences, Inc. Winn Avenue Hudson, USA, was used for the determination of maximum quantum photosynthetic yield (F_v/F_m) according to the method of Strasser et al. (1995). The oscillations were produced by light intensity of 2,800 μmol m^{-2} s^{-1}. All the leaves were dark adapted for 30 min before recording the data for maximum fluorescence with all PSII reaction centers open (F_m), minimum fluorescence with all PSII reaction centers open (F_o), variable fluorescence (F_v) and efficiency of quantum yield of PSII (F_v/F_m). Measuring beam frequency was 6 and 20 kHz for all measurements of F_o and F_m, respectively, during saturation flash. F_o was recorded by weak red light of <0.1 μmol m^{-2} s^{-1} intensity. F_m were recorded at 8,000 μmol m^{-2} s^{-1} saturation pulse of 0.8 s duration, while F_v/F_m, electron transport rate (ETR) and coefficient of nonphotochemical quenching (qN) were determined by 1,500 μmol m^{-2} s^{-1} actinic light intensity.

Following formulae were used for ETR and qN calculation:

$$\mathrm{ETR} = Y \times \mathrm{PAR} \times 0.84 \times 0.5$$

$$\mathrm{qN} = F_m - F'_m / F_m - F_o,$$

where 0.84 is absorption coefficient of leaf and 0.5 is absorbed light fraction of PSII antennae.

Biochemical attributes

Determination of chlorophyll pigments

Arnon (1949) method was used for the determination of chlorophyll (chl. *a* and *b*) contents. Fresh leaf samples (0.1 g) were extracted in 5 ml acetone (80 %). The optical densities of the supernatant were recorded at wavelengths 645 and 663 nm using a UV–visible spectrophotometer (Model IRMECO U2020, Germany).

Chlorophyll contents were calculated by using the following formulae:

$$\mathrm{Chl}a = [12.7\,(\mathrm{OD}\ 663) - 2.69\,(\mathrm{OD}\ 645)] \times v/1{,}000 \times w$$

$$\mathrm{Chl}b = [22.9\,(\mathrm{OD}\ 645) - 4.68(\mathrm{OD}\ 663)] \times v/1{,}000 \times w$$

where v is the volume of the extract (ml), and w is the fresh leaf mass (g).

Determination of total phenolics

Total phenolic contents were determined by following the method of Julkenen-Titto (1985) using Folin and Ciocalteau's phenol reagent (MP Biomedicals, USA). Fresh plant leaf (0.5 g) material was extracted in 80 % acetone. Then centrifuged extract at 10,000×*g* for 10 min. The aliquot (0.1 ml) was diluted with 2 ml of distilled H_2O plus 1 ml of Folin and Ciocalteau's phenol reagent. After shaking the mixture, 5 ml sodium carbonate (Na_2CO_3) (20 %) was added. Then to the above mixture was added distilled water and volume maintained to 10 ml in test tubes. After vortex read the OD at 750 nm on a spectrophotometer (US-Visible, IRMECO, GmbH, Germany).

Seed ascorbic acid (Asc) content determination

Mukherjee and Choudhuri (1983) method was used for the determination of ascorbic acid contents. Fresh seeds 250 mg were homogenized with trichloroacetic acid (6 %) and volume raised to 10 ml in test tubes. Then 4 ml extract was mixed with 2 ml of 2 % dinitrophenyl hydrazine and then one drop of thiourea (10 % dissolved in 70 % ethanol) was added. The mixture was boiled for 15 min. in a water bath at 95 °C. Then cooled down and added 5 ml of sulphuric acid (80 %) to the sample mixture. The absorbance was read at 530 nm and Asc values were calculated with the help of standard curve.

Antioxidant enzymes activities

Extraction of enzyme Fresh leaf 0.5 g was homogenized in 10 ml of 50 mM cooled phosphate buffer (pH 7.8), placed on an ice bath and then centrifuged at 15,000×*g* for 20 min at 4 °C.

Determination of superoxide dismutase (SOD) Giannopolitis and Ries (1977) method was used for SOD determination. For the appraisal of SOD enzyme activity, criteria of nitroblue tetrazolium (NBT) photoreduction inhibition was used as one unit of SOD enzyme activity will be equal to the amount of enzyme that inhibit 50 % NBT photoreduction. SOD reaction mixture (1 ml) in plastic cuvettes consists of 500 µl phosphate buffer (pH 7.8), 0.5 ml distilled H_2O, 100 µl methionine, 50 µl NBT and 50 µl sample extract, which were kept under light for 20 min. After that optical density of irradiated aliquots was read at 560 nm using a spectrophotometer.

Catalase and peroxidase determination CAT and POD activities were determined by following Chance and Maehly (1955) with slight modifications. The CAT reaction solution (3 ml) consisted of 1.9 ml phosphate buffer (50 mM; pH 7.0), 1 ml H_2O_2 (5.9 mM) and 0.1 ml enzyme extract. Reaction was initiated by adding an aliquot (100 µl) of the enzyme extract. Changes in enzyme activity were recorded by spectrophotometer at 240 nm after every 20 s for 2 min. For determination of POD activity reaction mixture consists of 750 µl phosphate buffer (50 mM; pH 5.0), 100 µl guaiacol (20 mM), 100 µl H_2O_2 (40 mM) and 100 µl enzyme extract. Changes in absorbance values of reaction mixture were read at 470 nm after every 20 s for 3 min by spectrophotometer.

Determination of total soluble proteins Bradford (1976) method was used for the determination of total soluble protein contents. Fresh leaf (500 mg) was triturated with 10 ml of 50 mM potassium phosphate buffer (pH 7.8) in an ice bath. The aliquot was centrifuged at 10,000×*g* for 15 min at 4 °C.

Determination of mineral ions Dried root or shoot material (0.1 g) was finely ground in digestion flasks with digestion mixture (2 ml) by following Allen et al. (1985) method. The digestion flasks were left for 24 h at room temperature. The flasks containing samples were heated by gradually increasing the temperature of hot plate up to 250 °C and kept at this temperature for 40 min. until fumes are produced, after which, 35 % H_2O_2 (0.5 ml) was poured down along the sides of flasks. When sample mixture became colorless, heating was stopped and the samples cooled down. Then volume of samples was maintained up to 50 ml with distilled water and the samples filtered and used for the determination of Ca^{2+}, K^+ and Na^+ by flame photometer (Jenway PFP 7).

Application of state on data

Data were analyzed statistically using analysis of variance (ANOVA) for all parameters and calculated using computer software (Co-STAT). Snedecore and Cohran (1980) method was used for comparing mean values via least significant difference.

Results

Effect of salt (NaCl) and glycinebetaine (GB) on growth attributes

Root and shoot biomass and root and shoot lengths

Rooting-medium applied salt stress of 150 mM NaCl significantly decreased shoot and root fresh weights (Fig. 1a, b), shoot and root dry weights (Fig. 1c, d), and shoot and root lengths (Fig. 1e, f) of two pea varieties, pea 09 and meteor Fsd (Table 1). Of both varieties, Pea 09 was

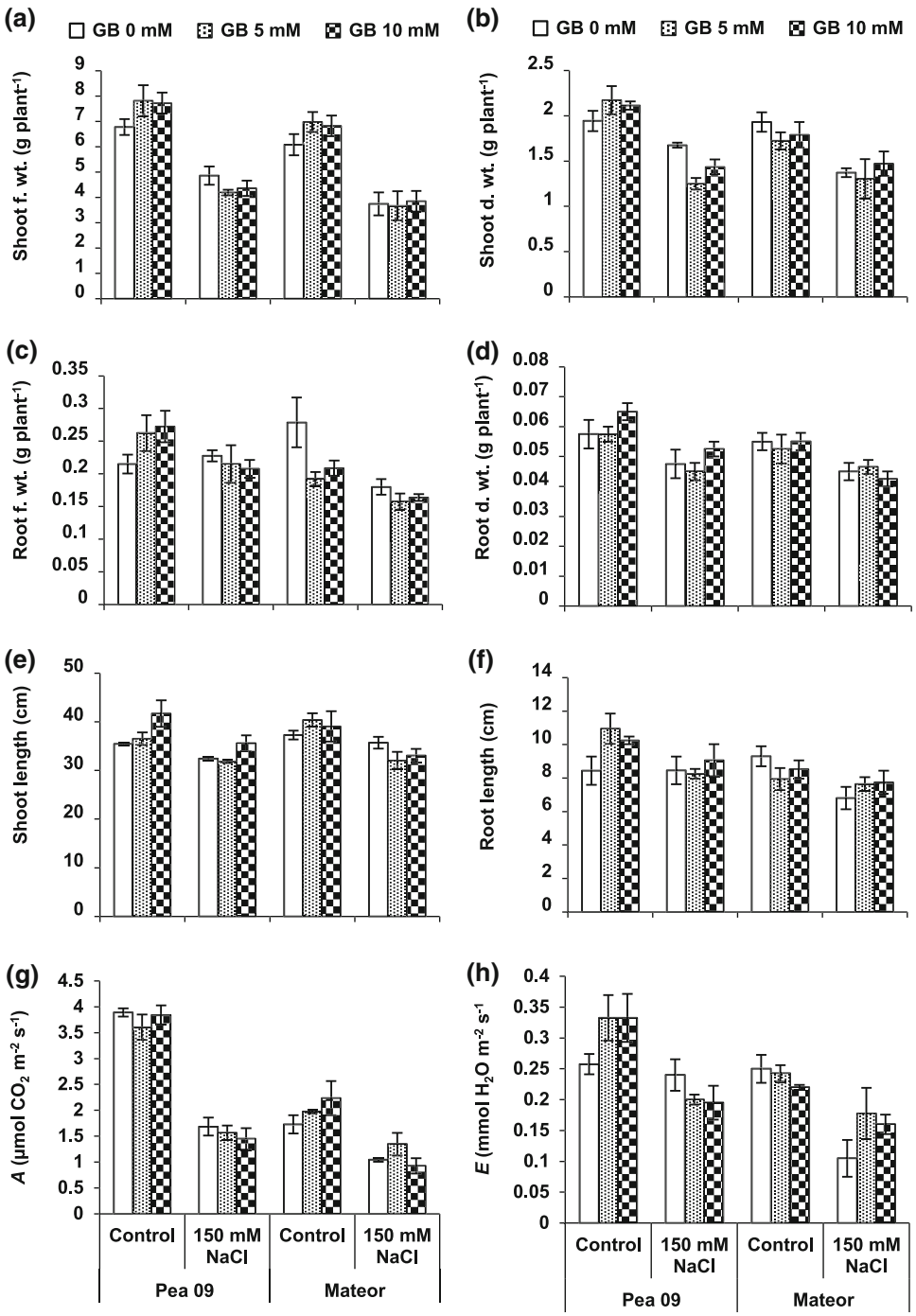

Fig. 1 Shoot and root fresh and dry weights, shoot and root lengths and photosynthetic attributes of pea (*Pisum sativum* L.) plants foliarly sprayed with glycinebetaine under saline and non-saline conditions

significantly higher in root and shoot fresh and dry biomass, and root length than Meteor Fsd under both control and salt stress conditions. Glycinebetaine (GB) applied as foliar spray did not alter these growth attributes significantly under non-stress and salt stress regimes. However, foliar application of GB increased shoot dry weight (Fig. 1b) and root fresh weight (Fig. 1c) in variety Pea 09, while decreased root fresh weight (Fig. 1c) and root length (Fig. 1f) in var. Meteor Fsd under non-saline conditions.

Effect of salt (NaCl) and glycinebetaine (GB) on physiological attributes

Effect on gas exchange characteristics

Gas exchange characteristics like net CO_2 assimilation rate (A) (Fig. 1g), transpiration rate (E) (Fig. 1h) and stomatal conductance (g_s) (Fig. 2a) significantly decreased in both pea varieties under salt stress of

Table 1 Mean squares from analysis of variance of the data for various growth, gas exchange, chlorophyll fluorescence, chlorophyll and key metabolites of pea (*Pisum sativum* L.) plants subjected to different concentrations of foliar-applied glycinebetaine under saline and non-saline conditions

Source of variation	*df*	Shoot f. wt.	Shoot dry wt.	Root f. wt.	Root dry wt.	Shoot length
Varieties (var.)	1	6.984***	0.330*	0.015**	2.707*	5.528 ns
Salinity (S)	1	102.2**	3.339***	0.025***	0.001***	293.7***
Glycinebetaine (GB)	2	0.516 ns	0.059 ns	0.001 ns	4.908 ns	24.50 ns
Var × S	1	0.028 ns	0.108 ns	0.002 ns	1.408 ns	1.244 ns
Var × GB	2	0.058 ns	0.003 ns	0.006*	8.325 ns	32.07 ns
S × GB	2	1.968 ns	0.069 ns	2.224 ns	1.158 ns	21.20 ns
Var × S × GB	2	1.968 ns	0.178*	0.001*	1.158 ns	6.950 ns
Error	36	0.692	0.052	0.001	4.491	10.28

Source of variation	*df*	Root length	Chl. *a*	Chl. *b*	Chl. *a*/*b* ratio	Total chl.
Varieties (var.)	1	18.58**	0.013 ns	0.049 ns	0.559 ns	0.112 ns
Salinity (S)	1	18.46**	0.558***	0.840***	2.617***	2.777***
Glycinebetaine (GB)	2	1.758 ns	0.037 ns	0.050 ns	0.123 ns	0.167 ns
Var × S	1	0.026 ns	0.008 ns	9.100 ns	0.034 ns	0.007 ns
Var × GB	2	2.213 ns	0.032 ns	0.001 ns	0.208 ns	0.037 ns
S × GB	2	0.269 ns	0.032 ns	0.031 ns	0.593 ns	0.0623 ns
Var × S × GB	2	6.152*	0.084 ns	0.029 ns	0.378 ns	0.194 ns
Error	36	1.825	0.0387	0.016	0.183	0.0752

Source of variation	*df*	*A*	*E*	g_s	C_i	F_v/F_m
Varieties (var.)	1	15.35***	0.054***	602.1**	6,244***	0.058*
Salinity (S)	1	28.59***	0.10***	2,799***	6,986***	0.017 ns
Glycinebetaine (GB)	2	0.005 ns	0.003 ns	413.3**	226.2 ns	0.028 ns
Var × S	1	5.380***	1.02 ns	133.3 ns	1,643*	0.004 ns
Var × GB	2	0.239 ns	3.583 ns	45.07 ns	71.19 ns	5.233 ns
S × GB	2	0.298 ns	4.083 ns	508.1**	154.1 ns	0.031 ns
Var × S × GB	2	0.051 ns	0.013*	160.7 ns	855.9 ns	0.029 ns
Error	36	0.135	0.002	65.85	286.8	0.012

Source of variation	*df*	*ETR*	*qN*	*NPQ*	Total phenolics	Ascobic acid
Varieties (var.)	1	3.685 ns	0.007 ns	8.333 ns	3.866***	2.855 ns
Salinity (S)	1	0.010 ns	6.302 ns	0.012 ns	2.253***	14.28***
Glycinebetaine (GB)	2	1.751 ns	0.007 ns	0.142***	0.552***	7.810***
Var × S	1	70.32 ns	3.502 ns	0.053 ns	0.423***	0.004 ns
Var × GB	2	8.776 ns	7.170 ns	0.016 ns	0.244**	1.248 ns
S × GB	2	7.926 ns	0.006 ns	0.002 ns	0.012 ns	0.953 ns
Var × S × GB	2	50.25 ns	0.026*	0.066*	0.011 ns	0.321 ns
Error	36	24.46	0.007	0.013	0.030	0.790

df degrees of freedom, *Chl. a, b and a/b ratio* chlorophyll *a*, *b* and chlorophyll *a*/*b* ratio, respectively, *Ci* sub-stomatal CO_2 conc, g_s stomatal conductance, *E* transpiration rate, *A* net photosynthetic rate, F_v/F_m efficiency of photosystem II, *ETR* electron transport rate, *NPQ* non-photochemical quenching, *qN* co-efficient of non-photochemical quenching

***, **, and * significant at 0.001, 0.01 and 0.05 levels, respectively; ns, non-significant

150 mM NaCl (Table 1). Of two varieties, Pea 09 exhibited greater decline as compared to Meteor Fsd to net CO_2 assimilation rate (*A*) under saline stress, while in var. Meteor Fsd the reduction in transpiration rate (*E*) was more prominent in comparison to that of Pea 09 under saline and non-saline regimes. Overall, var. Pea 09 was higher in g_s value as compared to var. Meteor Fsd under both NaCl stress and non-stress conditions. Foliar application of GB significantly improved stomatal conductance of var. Pea 09 under non-stress condition only (Table 1).

Considerable ($P \leq 0.001$) improvement was observed in sub-stomatal CO_2 (C_i) (Fig. 2b) of both pea varieties under salt prevailing regimes. GB had non-significant effect on C_i of both varieties under salt stress as well as non-stress condition. Meteor Fsd showed better performance in maintaining the C_i under salt-stressed conditions (Table 1).

Effect on chlorophyll fluorescence

Maximum quantum yield of photosystem-II (F_v/F_m) (Fig. 2c), electron transport rate (ETR) (Fig. 2d), non-

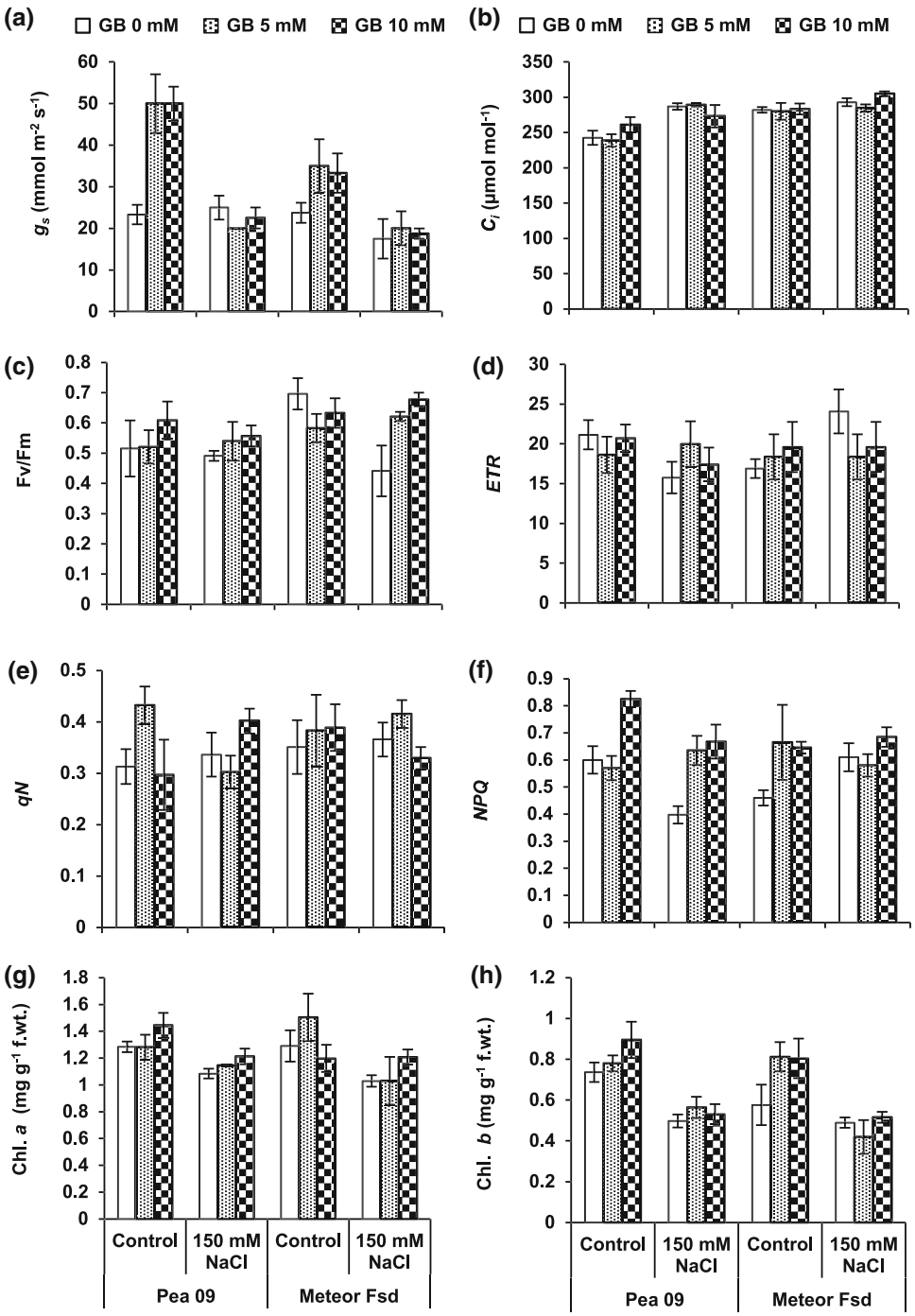

Fig. 2 Photosynthetic and chlorophyll fluorescence attributes and chlorophyll *a* and *b* contents of pea (*Pisum sativum* L.) plants foliarly sprayed with glycinebetaine under saline and non-saline conditions

photochemical quenching coefficient (qN) (Fig. 2e) and non-photochemical quenching (NPQ) (Fig. 2f) remained uniform under saline regimes (Table 1). Foliar-applied GB did not alter these chlorophyll fluorescence attributes under both saline and non-saline condition except qN and NPQ. Foliar-applied GB prominently increased ($P \leq 0.001$) the NPQ value of both pea varieties both under control and 150 mM NaCl level (Fig. 2f). However, var. Pea 09 showed high qN and NPQ values by foliar-applied GB under non-saline conditions. Of both varieties, Meteor Fsd was superior ($P \leq 0.05$) in F_v/F_m ratio as compared to that of variety Pea 09 under both control and saline regimes. Among various concentration of GB, 10 mM was proved to be more effective as compared to others.

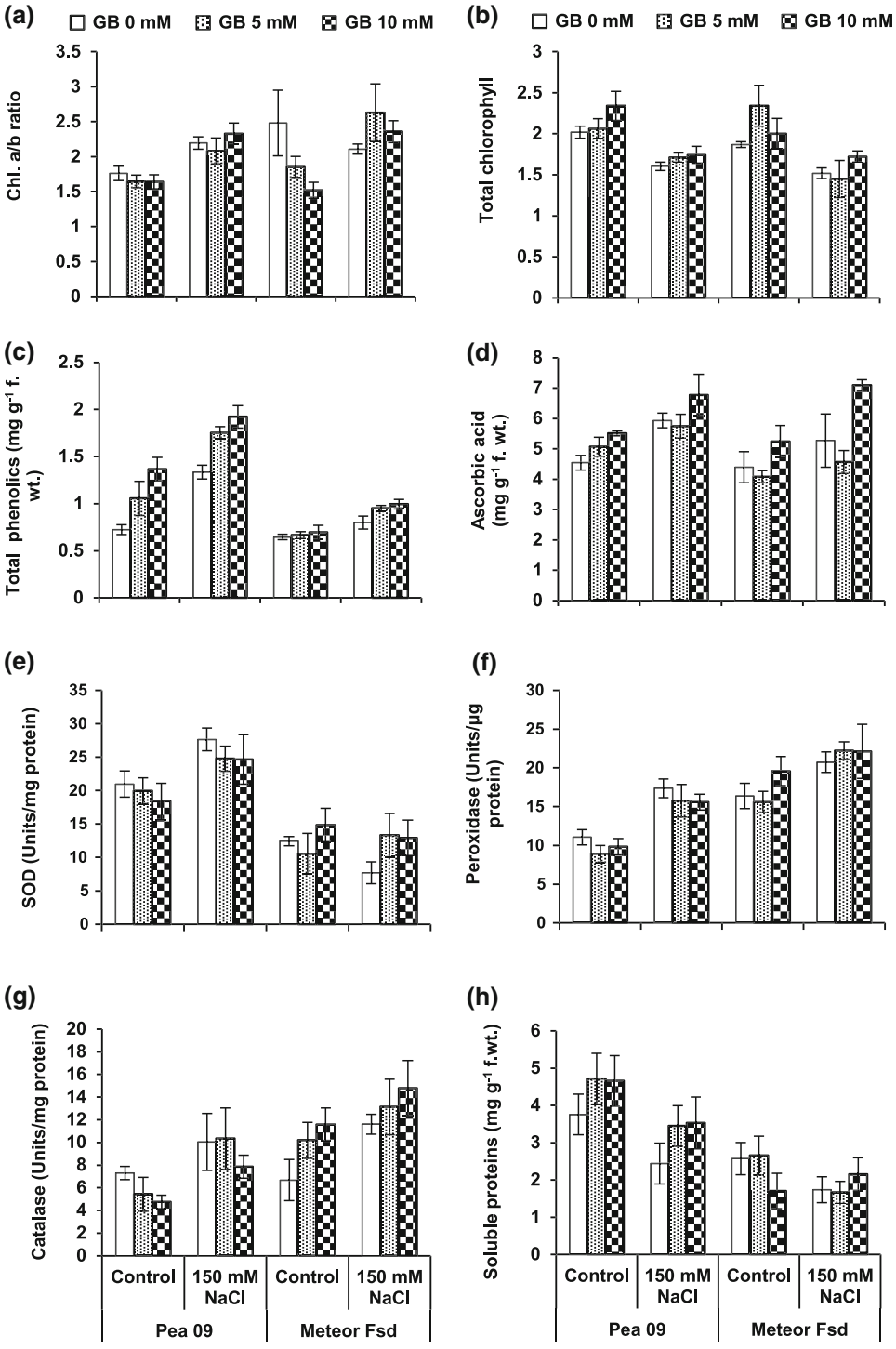

Fig. 3 Chlorophyll *a/b* ratio, total chlorophyll, total phenolics, ascorbic acid, activity of antioxidant enzymes and total soluble proteins of pea (*Pisum sativum* L.) plants foliarly sprayed with glycinebetaine under saline and non-saline conditions

Effect of salt (NaCl) and glycinebetaine (GB) on biochemical attributes

Effect on chlorophyll pigments

Chlorophyll *a*, *b* (Fig. 2g, h) contents significantly ($P \leq 0.001$) decreased, while chl. *a/b* ratio (Fig. 3a) increased ($P \leq 0.001$) in both varieties of pea under salt stress. Contents of total chlorophyll (Fig. 3b) also decreased under salt stress in both pea varieties. Exogenously applied various levels of GB had no pronounced effect on chlorophyll contents of both varieties under both control (0 mM NaCl) and salt-stressed (150 mM NaCl) conditions (Table 1).

Table 2 Mean squares from analysis of variance of the data for activities of leaf antioxidant enzymes and shoot and root mineral contents of pea (*Pisum sativum* L.) plants subjected to different concentrations of foliar-applied glycinebetaine under saline and non-saline conditions

Source of variation	*df*	SOD	POD	CAT	Soluble proteins	Shoot K^+
Varieties (var.)	1	1,393***	484.9***	164.8***	33.75***	30.88 ns
Salinity (S)	1	64.28 ns	350.0***	158.9***	8.643**	338.6***
Glycinebetaine (GB)	2	1.477 ns	5.51 ns	3.837 ns	1.073 ns	1.598 ns
Var × S	1	155.6*	9.674 ns	0.034 ns	1.808 ns	9.360 ns
Var × GB	2	44.02 ns	14.61 ns	41.17 ns	1.678 ns	8.317 ns
S × GB	2	8.073 ns	6.715 ns	0.710 ns	0.779 ns	31.14 ns
Var × S × GB	2	22.82 ns	2.269 ns	4.301 ns	0.481 ns	26.81 ns
Error	36	23.72	11.17	12.76	1.130	13.64
Source of variation	*df*	Root K^+	Shoot Na^+	Root Na^+	Shoot Ca^{2+}	Root Ca^{2+}
Varieties (var.)	1	48*	18.75 ns	147**	12.50 ns	16.92*
Salinity (S)	1	705.3***	1,800***	833.3***	94.92***	34.17**
Glycinebetaine (GB)	2	13.77 ns	27.08 ns	103.5**	8.973 ns	36.85***
Var × S	1	35.02 ns	1.333 ns	90.75*	30.88*	8.755 ns
Var × GB	2	14.06 ns	0.25 ns	6.812 ns	25.25*	11.73 ns
S × GB	2	26.27 ns	42.25*	105.1**	16.51 ns	14.39*
Var × S × GB	2	0.145 ns	1.583 ns	0.437 ns	2.880 ns	13.16*
Error	36	10.65	12.04	16.58	6.605	3.671

df degrees of freedom

***, **, and * significant at 0.001, 0.01 and 0.05 levels, respectively; ns, non-significant

Effect on total phenolics

Total phenolic (Fig. 3c) contents of both pea varieties significantly ($P \leq 0.001$) increased under saline condition (Table 2). Both pea varieties significantly ($P \leq 0.001$) differed in total phenolics. Variety Pea 09 was superior to that of var. Meteor Fsd in total phenolic contents under saline and non-saline regimes (Fig. 3c). GB application as foliar spray significantly increased total phenolic contents of both pea varieties under both control and salt-stressed conditions (Table 2). However, var. Pea 09 accumulated more total phenolics than var. Meteor Fsd under non-saline conditions.

Effect on seed ascorbic acid

Root-medium applied salt stress markedly enhanced ascorbic acid (Fig. 3d) contents in pea seeds. Both pea varieties did not differ significantly in seed ascorbic acid contents. GB application as foliar spray had prominent effect ($P \leq 0.001$) on the level of seed ascorbic acid contents in both varieties (Table 1). Of various GB levels, 10 mM GB significantly ($P \leq 0.001$) enhanced seed ascorbic acid contents of both pea varieties under both control and 150 mM NaCl level (Fig. 3d).

Effect on activities of antioxidant enzymes

Salt stress did not alter activity of superoxide dismutase (SOD) (Fig. 3e) in both pea varieties. Variety Pea 09 was higher in SOD enzymatic activity than that of Meteor Fsd under both control and 150 mM NaCl conditions. GB applied as foliar spray did not improve activity of SOD in both pea varieties under non-saline or saline regimes (Table 2).

Peroxidase (Fig. 3f) and catalase (Fig. 3g) activities were appreciably ($P \leq 0.001$) elevated in both pea varieties subjected to saline regimes (Table 2). Variety Meteor Fsd showed high activity of CAT and POD as compared to variety Pea 09 under both control and 150 mM NaCl level. Foliar application of different GB levels did not alter the activities of POD and CAT in both varieties under control and saline conditions.

Effect on total soluble proteins

Salt stress significantly decreased total soluble protein (Fig. 3h) contents in both pea varieties (Table 2). This reduction ($P \leq 0.001$) was higher in variety Meteor Fsd than that of variety Pea 09 under both control and 150 mM salt level (Fig. 3h). GB application as foliar spray did not alter the level of soluble proteins in both varieties of pea crop (Table 2).

Effect on mineral ions

Root and shoot K^+ contents (Fig. 4a, b) significantly decreased in both pea varieties under saline stress. Both pea varieties showed uniform behavior in accumulation of shoot K^+ (Fig. 4a) under both salt-stressed and non-stressed conditions; however, Meteor Fsd was prominently higher in accumulation of root K^+ (Fig. 4b) as compared to

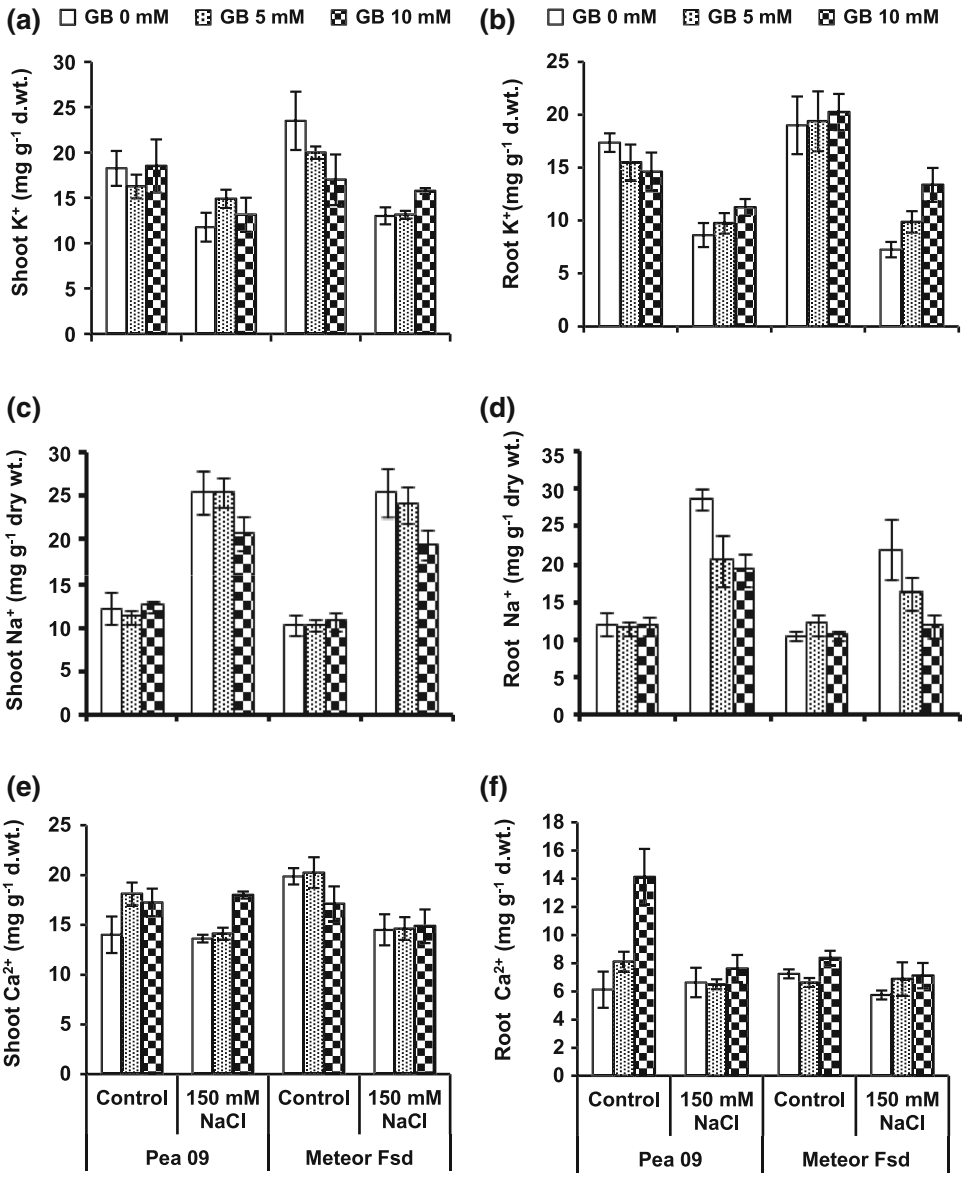

Fig. 4 Shoot and root potassium, sodium and calcium contents of pea (*Pisum sativum* L.) plants foliarly sprayed with glycinebetaine under saline and non-saline conditions

that of variety Pea 09. Foliar-application of GB did not alter root and shoot K^+ contents in both pea varieties (Table 2).

Root and shoot sodium ions significantly increased (Fig. 4c, d) in both pea varieties under salt stress. Variety Pea 09 was higher in root Na^+ (Fig. 4d) contents to that of var. Meteor Fsd under salt stress condition. Foliar application of GB significantly decreased root and shoot Na^+ contents in both pea varieties under 150 mM NaCl level (Table 2).

A marked reduction ($P \leq 0.001$) in root and shoot Ca^{2+} (Fig. 4e, f) contents was observed in both pea varieties under saline regimes. Varietal behavior was uniform in shoot Ca^{2+} (Fig. 4e), while variety Pea 09 had high root Ca^{2+} (Fig. 4f) than that of Meteor Fsd under control and salt-stressed condition. Foliar-applied GB significantly increased shoot Ca^{2+} in var. Pea 09 (Fig. 4e) and root Ca^{2+} (Fig. 4f) in both pea varieties under both non-stressed and salt-stressed conditions (Table 2).

Discussion

Glycinebetaine regulates a plethora of physiological and biochemical phenomena under saline environment (Kaya et al. 2013). Glycinebetaine when applied externally to the plants ameliorates damaging effects of salinity stress by regulating various stomatal and non-stomatal factors under salt stress (Kausar et al. 2014). Photosynthetic machinery components such as ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) (Nomura et al. 1998) and oxygen evolving complex of photosystem II (Murata et al. 1992)

are protected by GB via enhancing antioxidant enzymes activities (Khan et al. 2014) and inhibiting peroxidation of membrane lipids (Demiral and Turkan 2004). GB accumulation led to improved photosynthesis through reduction in oxidative stress under salt stress (Khan et al. 2014). The current study was conducted to assess whether foliar spray of different concentrations of glycinebetaine can reduce negative effects of salinity stress on pea (*Pisum sativum* L.) plants or not.

In the current study, 150 mM NaCl stress drastically reduced plant growth of both pea varieties. High amount of salt in the rooting medium has been reported to decrease plant biomass of various crop species like canola (Shahbaz et al. 2013), pepper (De Pascale et al. 2003a), celery (De Pascale et al. 2003b), pea (Shahid et al. 2011, 2012) and wheat (Perveen et al., 2014). Although foliar application of 5 and 10 mM GB could not improve growth significantly under saline conditions, however, under control environment (0 mM NaCl) foliar-applied GB enhanced shoot dry weight, root fresh weight and root length of pea variety Pea 09. There are some reports which show non-significance of GB when applied exogenously in different crop species like sunflower (Ibrahim et al. 2006), cotton (Meek et al. 2003), kidney bean (Lopez et al. 2002) and tomato (Heuer 2003).

In the current study, under salinity stress of 150 mM NaCl chlorophyll contents (chl. *a* and *b*) and total chlorophyll decreased in both pea varieties. Similar results were reported by Shahid et al. (2012) in seven pea genotypes under 75 mM of NaCl. Foliar application of GB has no considerable effect on salinity-induced reduction in the chlorophyll content. Similarly, Akhter et al. (2007) was of the view that seed treatment with GB did not significantly change chl. *b* contents in wheat.

Long time exposure to NaCl stress leads to premature senescence of leaves and ultimately decreases photosynthetic rate (Cramer and Nowak 1992). Sodium and chloride ions' accumulation in the leaf tissues decreases turgor potential within cell due to which stomata closed, resulting in reduced stomatal conductance (g_s). In this study, 150 mM NaCl stress decreased all gas exchange parameters such as g_s, transpiration rate (E) and principally net photosynthetic rate (A) of both varieties. Shahid et al. (2012) reported similar results in seven pea genotypes. GB plays a role in maintaining ionic balance and osmotic adjustment in various plant species under salinity stress (Sakamoto and Murata 2000; Yildiztugay et al. 2014). In this study, foliar-applied GB did not alter gas exchange characteristics. These results can be correlated with the findings of Meek and Oosterhuis (1999) in which GB did not improve photosynthetic rate of cotton. Contrarily, Raza et al. (2006) reported that photosynthetic rate (A) improved when glycinebetaine applied exogenously under NaCl stress in wheat. GB has been reported to enhance salt stress tolerance due to increased stomatal conductance and consequently high photosynthetic rate of field-grown tomato plants (Makela et al. 1999). In another report exogenous application of GB improved all gas exchange characteristics such as water use efficiency (*WUE*), sub-stomatal CO_2 (C_i), stomatal conductance (g_s) and net CO_2 assimilation rate (A) of maize under NaCl stress (Kausar et al. 2014). Timasheff (1992) reported that GB increases stomatal conductance due to increasing the amount of bound water in plant cells.

Kalaji et al. (2010) reported that photochemical efficiency of PSII (F_v/F_m) can be used as criteria for evaluating plant performance under stressful conditions. Lower F_v/F_m values indicate reduced electron transport rate and damaged PSII reaction centers under NaCl stress (Basu et al. 1998). In this study, all chlorophyll fluorescence attributes like F_v/F_m, ETR, qN and NPQ remained unaffected under salt stress. Similar to these results, salinity stress did not alter PSII activity in cotton and barley (Morales et al. 1992; Brugnoli and Bjorkman 1992). Under mild salt (150 mM NaCl) stress F_v/F_m did not get affected, while under high level of salt (300 mM NaCl) F_v/F_m significantly decreased in *Sphaerophsa kotschyana* plants (Yildiztugay et al. 2014). In this study, foliar applied GB did not alter F_v/F_m and ETR significantly, however, increased NPQ value under saline conditions in var. Pea 09 and qN and NPQ values in both pea varieties under non-saline conditions. Raza et al. (2006) reported that GB did not exert any significant effect on efficiency of photosystem II (F_v/F_m) in wheat under salt stress.

Plants tolerance criteria are related with low Na^+ uptake and higher accumulation of beneficial K^+ and Ca^{2+} to maintain optimal K^+/Na^+ ratio (Munns and Tester 2008). Our results can be related with earlier findings explained by Heuer (2003) that exogenously applied GB and proline did not reduced the uptake of sodium and chloride ions in the leaves and roots of tomato. GB as seeds' treatment did not alter status of mineral nutrients in wheat plants both under non-stress or salt stress conditions (Akhter et al. 2007).

In this study, total phenolic and ascorbic acid contents increased under saline condition in both pea varieties. Contrary to our results, Navarro et al. (2006) reported that NaCl stress reduces the ascorbic acid and total phenolic contents in pepper. In previous studies it has been reported that GB can ameliorate unfavorable effects of salinity by up regulating enzymatic antioxidant activity (Raza et al. 2007; Nawaz and Ashraf 2010). In our study, CAT and POD enzymes' activities increased under NaCl stress in both pea varieties; however, GB did not modulate antioxidant enzyme activities under either salt stress or non-stress conditions. GB is not always a compatible organic compound for all plant species (Ibrahim et al. 2006); it could be

phytotoxic because various physiological and biochemical processes such as photosynthetic rate and activity of metabolic enzymes are negatively affected at higher concentrations of GB. It has been reported that the effect of GB varies according to environmental conditions, plant species, cultivars, concentration and number of application (Ashraf and Foolad 2007; Reddy et al. 2013).

In conclusion, 150 mM of NaCl markedly reduced root and shoot fresh and dry matter, lengths of root and shoot, photosynthetic pigments such as chl. *a*, *b* and total chlorophyll, *A*, *E*, g_s and protein contents, while increasing root and shoot Na^+ contents, and activity of non enzymatic (ascorbic acid, phenolics) and enzymatic (POD, CAT) antioxidants in both pea varieties. Application of GB as foliar spray (0, 5, 10 mM) increased dry matter of shoot, fresh weight of root and root length, stomatal conductance, NPQ, root and shoot Ca^{2+} (in Pea 09), ascorbic acid and total phenolic contents. Of various GB levels, 10 mM proved more effective in reducing Na^+ content of root and shoot and increasing key metabolites in both pea varieties under saline conditions. Possible mechanism involved in GB-mediated abiotic stress tolerance includes induction of specific genes expression that encodes enzymes that scavenge reactive oxygen species and prevent excess ROS accumulation (Chen and Murata 2011). In this study, GB might have stabilized proteins structure and maintained membrane integrity by increasing activities of non-enzymatic antioxidants that might have led to scavenging of reactive oxygen species under salt stress. Furthermore, GB might be involved in the nutrients' uptake as it is exhibited by increased root Ca^{2+} accumulation in root and shoot tissues. Overall, variety Pea 09 showed better performance in comparison to that of variety Meteor Fsd.

Author contribution Miss. Nida Nusrat performed the experiment under the supervision of Dr. Muhammad Shahbaz while the third author (Dr. Shagufta Perveen) contributed in the form of help during lab analysis and manuscript write up.

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