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Original Article

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Foliar Spray of Proline Enhanced the Photosynthetic Efficiency and Antioxidant System in *Brassica juncea*

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Abstract

The present study was carried out to screen out the best concentration of proline, applied exogenously as foliar spray, which will have beneficial effects on growth and physio-biochemical parameters in two varieties of *Brassica juncea* ('Varuna' and 'RH-30'). The proline concentrations tested in this experiment were 0, 10, 20 or 30 mM. The foliar spray was done at 29 days after sowing (DAS) and sampling at 60 DAS. Out of these concentrations, 20 mM proline was found best. The proline treatment significantly increased the growth, net photosynthetic rate and related attributes, SPAD value of chlorophyll, leaf carbonic anhydrase activity and quantum yield of photosystem II, as well as proline content and activity of antioxident enzymes also increased in both the varieties at 60 d stage of growth. The result was more pronounced in the 'Varuna' than in 'RH-30'.

Keywords: antioxidant system, chlorophyll fluorescence, leaf area, heavy metal, reactive oxygen species, quantum yield

Introduction

Various abiotic stresses (like salinity, drought, heavy metal toxicity etc.) are constantly challenging the plant species causing the oxidative stress by the generation of reactive oxygen species (ROS) (Roychoudhury, 2012). These ROS cause damage to the cells, various metabolic disorders, structural and functional loss of organelles which ultimately lead to cell death (Blokhina *et al.*, 2003). Plants have developed certain mechanisms to counteract these stresses. One such mechanism includes the accumulations of various kinds of compatible solutes like proline (Pro), sugars, polyols and betaine. Among these compatible solutes, proline is one of the most important that results in the osmotic adjustment against various stresses (Ashraf and Foolad, 2007). In addition to the role of osmo-protection, proline performs protective function by scavenging the ROS particles (Hayat *et al.*, 2012). Proline, an essential amino acid, is ubiquitously distributed in plants where it has various metabolic roles (Szabados and Savoure, 2009). Pro has been proposed to act as an osmo-protectant (Ahmed *et al.*, 2010), a protein stabilizer, a metal chelator, an inhibitor of lipid membrane peroxidation and as a ROS scavenger (Trovato *et al.*, 2008). Therefore, proline is not only an important redox signaling molecule, but also an effective quencher of ROS formed under various stress conditions in plants (Ashraf and Foolad, 2007). Proline accumulation normally occurs in cytoplasm where it functions as molecular chaperon by stabilizing the structure of proteins, buffers cytosolic pH and maintains cell redox status. It has also been proposed that its accumulation may be part of stress signal, influencing adaptive responses (Hayat *et al.*, 2012).

The exogenous application of proline has been reported to play an important role by enhancing the tolerance of plants to the stress (Wani *et al.*, 2012). Its application has improved various photosynthetic attributes (Wani *et al.*, 2012), increased the level of stress protective proteins (Khedr *et al.*, 2003). However, the effects of proline are concentration-dependent (Ashraf and Foolad, 2007). Besides these beneficial roles of proline, its higher concentrations could have negative impact on plant metabolism (Nanjo *et al.*, 2003). It becomes, therefore, important to find out the optimal Pro concentration that has beneficial effects in various plant species.

Keeping in view the above points, the present study was designed to screen out the best Pro concentration, applied as foliar spray that will have positive impact on the photosynthesis, antioxidant systems and other growth biomarkers of the plant.

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Materials and Methods

Plant material and treatments

This experiment was conducted to screen out the best dosages of proline applied as foliar spray to *Brassica juncea* plants. Two *Brassica juncea* varieties ('Varuna' and 'RH-30') were used and the experiment was conducted with 40 earthen pots (25×25 cm) in a way where each treatment had five replicates and three plants were maintained in each pot, arranged under simple randomized block design in the net-house of the Department of Botany, Aligarh Muslim University, Aligarh, India. The seeds were surface-sterilized with 0.01% mercuric chloride solution followed by the repeated washing with double distilled water (DDW) in order to remove adhered mercuric chloride particles on the seed surface. These sterilized seeds were then sown in the earthen pots filled with an equal quantity of sandy loam soil mixed with farmyard manure (9:1). Four concentrations [0 (water spray), 10, 20, or 30 mM] of proline were prepared in DDW containing 0.5% Tween-20 as surfactant and exogenously applied as foliar spray at 29 DAS. The plants were sprayed thrice and the nozzle of the sprayer was adjusted in such a way that it pumped out 1 mL of DDW or proline solution in one sprinkle. The plants were then sampled at 60 DAS to assess the following parameters.

Plant growth

At 60 DAS the plants were up-rooted and dipped in water to remove the adhered soil without injuring the roots. The root and shoot length was measured on a meter scale. The roots were then separated from the shoot, blotted and subsequently weighed to record their fresh mass and then placed in an oven at 80 °C for 72 h. The samples were weighed again to record the respective dry mass. The leaf area was ascertained by gravimetric method by tracing the outline of the leaf on graph sheet and counting the squares covered by it on graph paper.

SPAD chlorophyll and gas exchange characteristics

The SPAD chlorophyll value and gas exchange characteristics [net photosynthetic rate (P_N), stomatal conductance (g_s), internal CO_2 concentration (C_i) and transpiration rate (E)] in the intact leaves were measured by SPAD chlorophyll meter (Minolta 502) and portable photosynthetic system (LICOR-6400, Lincoln, NE, USA) between 11:00 and 13:00 h, under bright sunlight, respectively. During the photosynthesis measurement, following conditions were maintained: photosynthetically active radiation 1016±6l μmol $m^{-2}s^{-1}$, relative air humidity 60±3%, atmospheric temperature 22±1 °C and atmospheric CO_2 360 μmol mol^{-1}. The ratio of atmospheric CO_2 to intercellular CO_2 concentration was constant.

Maximum quantum yield of PSII

The maximum quantum yield of PSII (Fv/Fm) was measured on the adaxial surface of the intact leaf using portable photosynthesis system (LICOR-6400, Lincoln NE, USA). Prior to the measurement, plants were left for 30 min in dark, at room temperature. The chlorophyll molecules were excited for 10s by actinic light with a photon flux density of 40 μmol $m^{-2}s^{-1}$.

Carbonic anhydrase (CA) activity

The activity of CA was assayed by following the method of Dwivedi and Randhawa (1974). The leaf samples were cut into small pieces and suspended in cystein hydrochloride solution. The samples were incubated at 4 °C for 20 min and then filtered. The filtrate was transferred to the test tubes, containing phosphate buffer (pH 6.8) followed by the addition of alkaline bicarbonate solution and bromothymol blue indicator. The samples were incubated at 4 °C for 20 min. The reaction mixture was titrated against 0.05 N HCl after the addition of 0.2 ml of methyl red indicator.

Activity of antioxidant enzymes

The supernatant collected from centrifuging the homogenate [0.5 g fresh leaves homogenized in 5 mL of 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone] at 10080 × g for 10 min, was used as a source for enzyme assay. This whole process was carried out at 4 °C.

The method developed by Chance and Maehly (1956) was used for the assay of catalase (CAT) and peroxidase (POX). CAT activity was measured by titrating the reaction mixture [phosphate buffer (pH 6.8), 0.1 M H_2O_2, enzyme extract and 2% H_2SO_4] against 0.1 N $KMnO_4$. The activity of POX was measured by noting the change in the absorbance of reaction mixture [pyragallol phosphate buffer (pH 6.8), 1% H_2O_2 and enzyme extract], due to catalytic conversion of pyragallol to purpurogallin, at an interval of 20 s for 2 min at 420 nm on spectrophotometer (Spectronic-20D, Milton Roy, USA). A control set was prepared by using DDW, instead of the enzyme extract. The superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) by adopting the method of Beauchamp and Fridovich (1971). The reaction mixture [50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0-50 μl enzyme extract] in tubes was placed under 15 W fluorescent lamps for starting the reaction. After 10 min, the reaction was stopped by switching off the light. Non-illuminated reaction mixture was used as a blank. The absorbance was measured at 560 nm on spectrophotometer (Spectronic-20D, Milton Roy, USA) and the SOD activity was expressed as unit g^{-1} fresh mass. One unit of SOD activity was defined as the amount of enzyme that inhibited 50% of NBT photo-reduction.

Leaf proline content

The method given by Bates *et al.* (1973) was used to measure the endogenous proline content in fresh leaves. The samples were extracted in sulphosalicylic acid. To the extract, an equal volume (2 mL) of glacial acetic acid and ninhydrin solutions was added. The sample was heated at 100 °C, to which 5 mL of toluene was added, after cooling in ice bath. The absorbance by aspirated toluene layer was read at 528 nm, on a spectrophotometer (Spectronic-20D, Milton Roy, USA).

Statistical analysis

Treatment means were compared by the analysis of variance using SPSS (SPSS ver. 17, Chicago, United States). Least Significant Difference (LSD) was calculated at 5% level of probability. Standard error between the replicates was calculated.

Results

Plant growth

The foliar application of various concentrations (10, 20 or 30 mM) proline increased the length, fresh and dry mass of root and shoot and leaf area in both varieties of mustard (Figs. 1 and 2A). Out of these concentrations used, 20 mM proved best and showed maximum increase in the growth parameters which in terms of percentage was 47% and 39% (shoot length), 35% and 29% (root length), 44% and 37% (shoot fresh mass), 32% and 27% (root fresh mass), 48% and 40% (shoot dry mass), 41% and 34% (root dry mass) and 22% and 17% (leaf area) higher in both 'Varuna' and 'RH-30' plants compared to their respective control plants.

Carbonic anhydrase activity

The foliar spray of proline (10, 20 or 30 mM) increased the activity of CA by 13%, 19% and 16% in 'Varuna' and 5%, 10% and 7% in 'RH-30' respectively, compared to the control plants (Fig. 2B). The 20 mM proline proved best in increasing the activity to maximum in both the varieties ('Varuna' and 'RH'-30).

SPAD chlorophyll and photosynthetic attributes

Out of the two varieties, 'Varuna' possessed higher values for SPAD chlorophyll and photosynthetic attributes (P_N, g_s, C_i and E) than 'RH-30' at 60 DAS (Fig. 3A-E). The foliar spray of various concentrations (10, 20 or 30 mM) of proline increased the SPAD chlorophyll and photosynthetic attributes in both the varieties. 20 mM proline proved the best concentration that increased the SPAD chlorophyll by 19% and 13%, P_N 24% and 17% , g_s 41% and 37%, C_i 17% and 11%, and E 24% and 15% in 'Varuna' and 'RH-30' respectively, when compared to their respective control plants.

Maximum quantum yield of PSII (Fv/Fm)

The values for Fv/Fm were higher in 'Varuna' than 'RH-30' and were significantly improved by proline treatment (Fig. 3F). The plants that received proline (20 mM) exhibited maximum value which was higher by 11% and 8% in 'Varuna' and 'RH-30' respectively, at 60 DAS over their control plants. 'Varuna' showed better response than 'RH-30', to proline treatment.

Antioxidant system

The activity of various antioxidant enzymes (CAT, POX and SOD) and endogenous proline content increased in response to the exogenous proline (10, 20 or 30 mM) both in 'Varuna' and 'RH-30' in a concentration dependent manner (Fig. 4A-D). Out of the proline concentrations, 20 mM proved best and enhanced the CAT activity by 13% and 8%, POX 29% and 20%, SOD 15% and 10% and proline content 17% and 14% in 'Varuna' and 'RH-30' respectively at 60 DAS, over their control plants. 'Varuna' possessed higher activity of antioxidant enzymes and proline content than 'RH-30'.

Discussion

Proline accumulated by plants provides energy for their growth and stress tolerance. Out of the different concentrations (0, 10, 20 or 30 mM proline) used as foliar spray in the current study, 20 mM proved best by enhancing the growth, photosynthesis and antioxidant system in the mustard plants. The effectiveness of exogenous proline application depends on the species, tissue type and the concentration used.

In this study, foliarly applied proline enhanced the CA activity and chlorophyll (SPAD value) in the mustard plants (Fig. 2B). Proline is reported to interfere with the hydrophobic/hydrophilic interactions between side chains of the constituent amino acids of the proteins (enzymes), thereby maintaining their 3D structure of the proteins (enzymes) (Paleg *et al*., 1981) and increases the activity of enzymes. Similar type of interactions might be occurring between CA and proline to enhance its activity as observed in this study. The

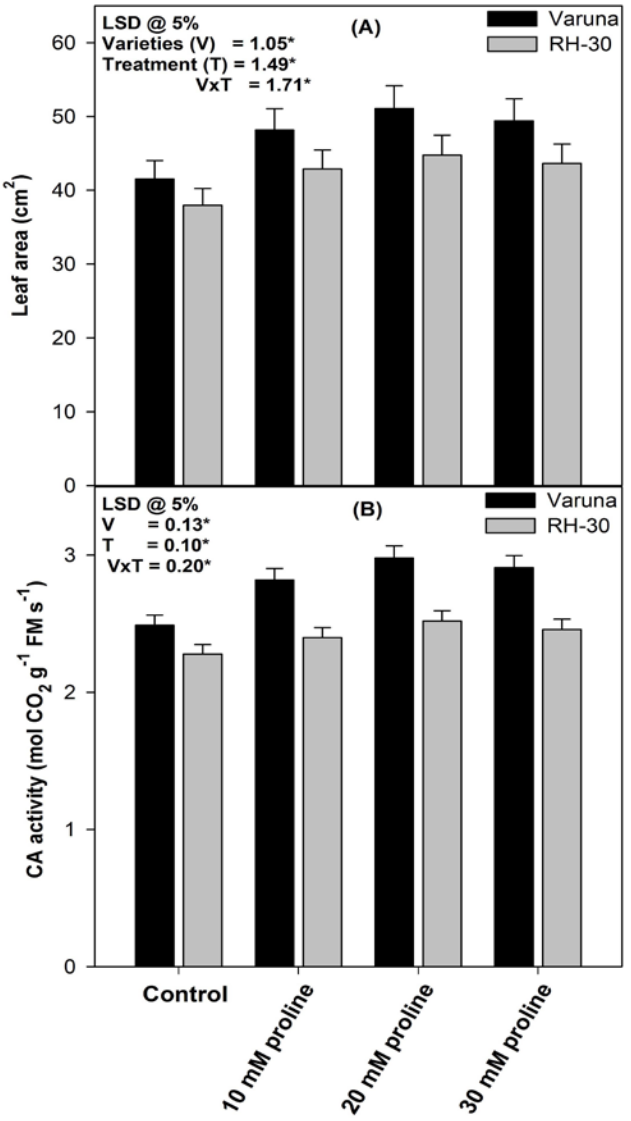

Fig. 2. Effect of proline (10, 20, or 30 mM) as foliar spray (29 d stage) on leaf area (A) and carbonic anhydrase (B) and in two varieties ('Varuna' and 'RH-30') of *Brassica juncea* (L.) Czern & Coss at 30 and 60 DAS. The symbol "*" in the figures depicts that the data is significant

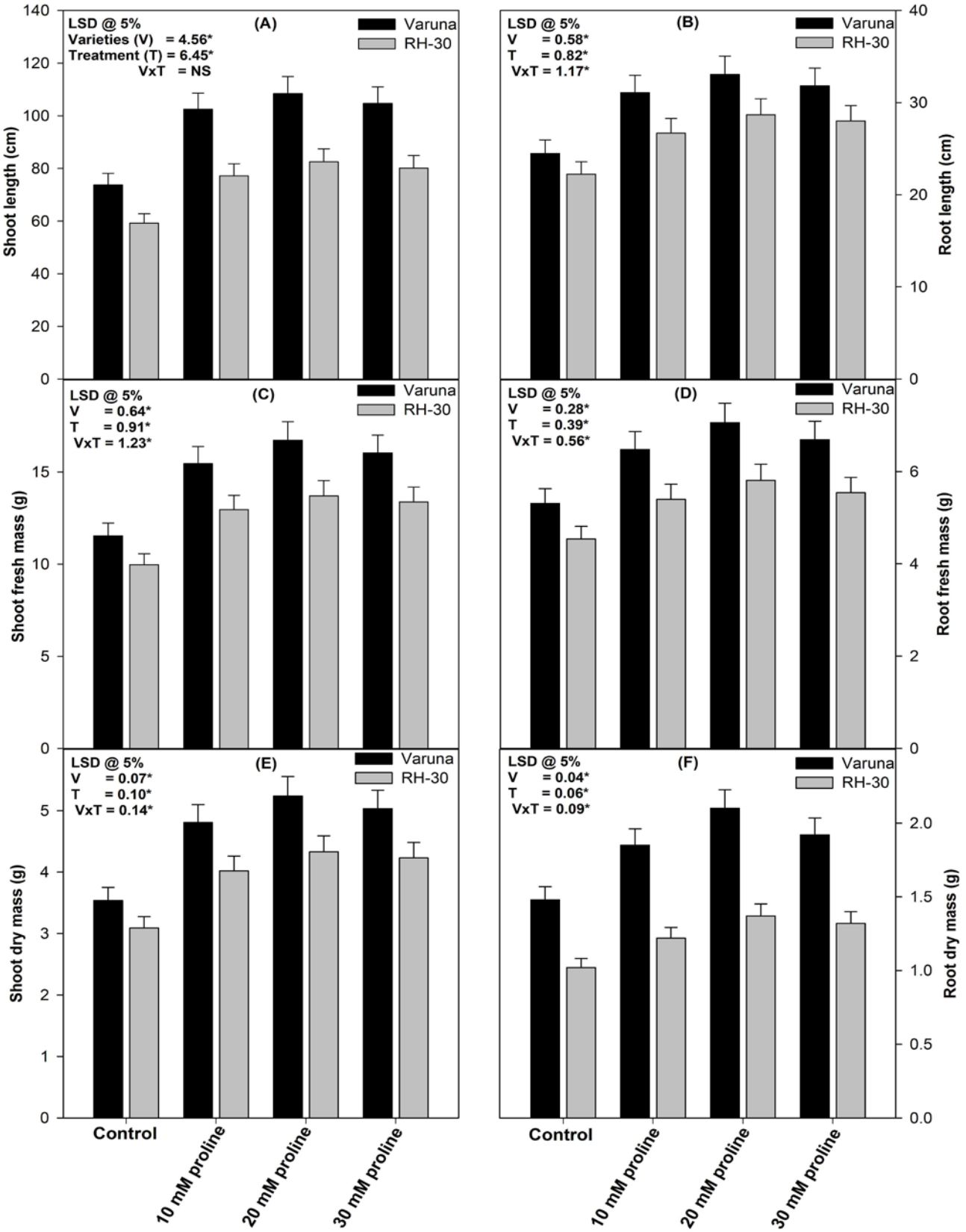

Fig. 1. Effect of proline (10, 20, or 30 mM) as foliar spray (29 d stage) on shoot length (A), root length (B), shoot fresh mass (C), root fresh mass (D), shoot dry mass (E) and root dry mass (F) in two varieties ('Varuna' and 'RH-30') of *Brassica juncea* (L.) Czern & Coss at 60 DAS. The symbol "*" in the figures depicts that the data is significant

chlorophyll molecules are the membrane bound structures whose stability depends highly on the integrity of the membrane structure which is possibly maintained in this study by proline as it acts as a membrane stabilizer (Ashraf and Foolad, 2007). These studies are in conformity with other crops (Ahmed *et al*., 2011; Aggarwal *et al*., 2011; Wani *et al*., 2012).

The photosynthesis process is mainly dependent on the stomatal movement and mesophyll cell metabolism i.e. proteins associated with PSI, PSII and chlorophyll (Lawlor and Cornic, 2002; Athar and Ashraf, 2005). Therefore, it can be inferred from present study that exogenous proline application caused increase in the stomatal conductance (Fig. 3C) by maintaining appropriate cellular turgor (Kamran *et al*., 2009) which facilitated sub-stomatal accumulation and assimilation of CO_2 at higher rate. These observations suggest that the photosynthetic enhancement primarily corresponds to the increased stomatal conductance with higher CO_2 diffusion rate within the leaves to activate P_N. Similar inferences have been proposed by Ahmed *et al.,* (2010) in young *Olea europaea* and Wani *et al.* (2012) in *Brassica juncea* plants. Moreover, higher chlorophyll contents (Fig. 3A) and CA activity (Fig. 2B) under exogenous proline application would also expectedly result in higher P_N(Fig. 3B).

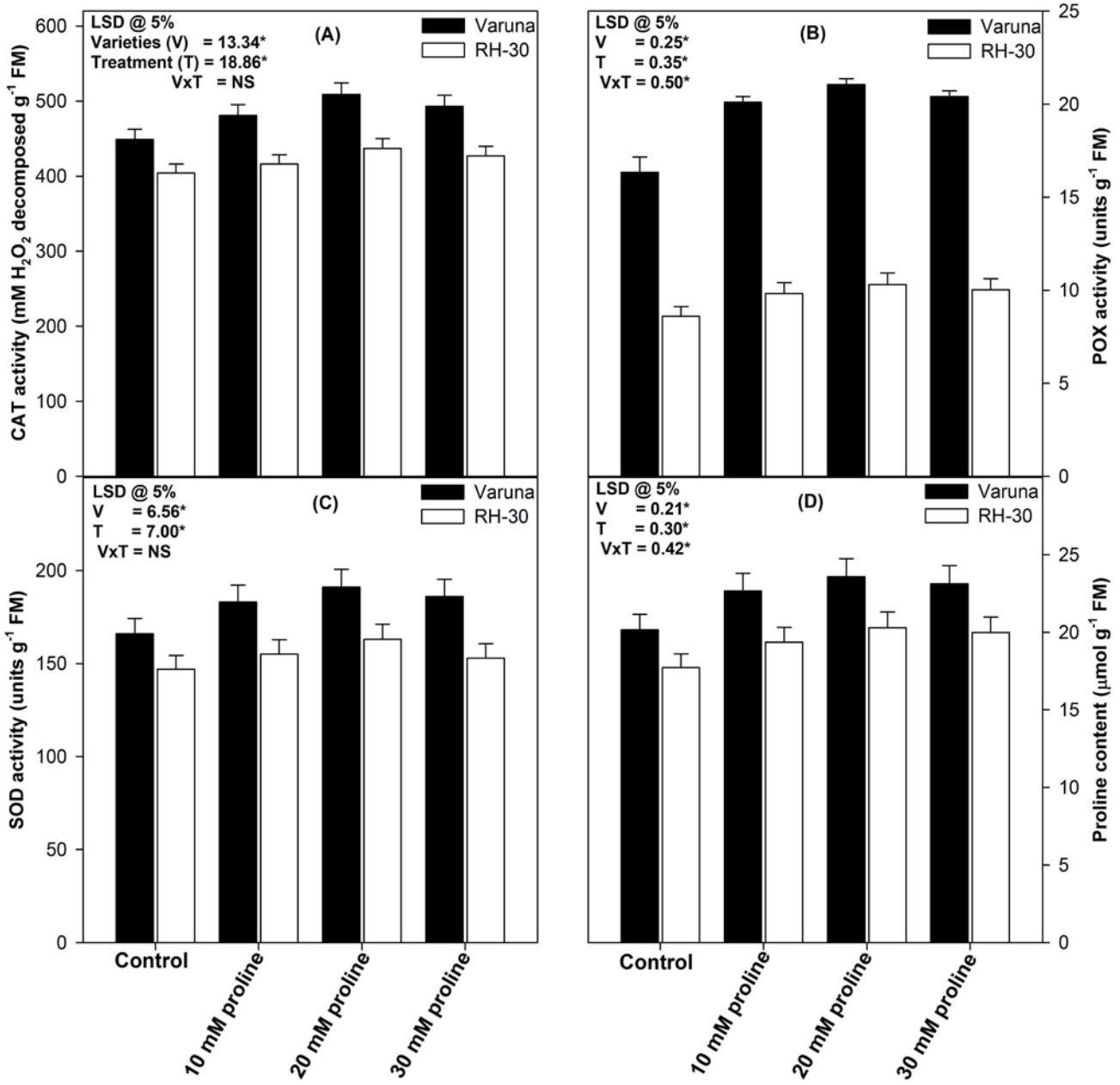

Fig. 4. Effect of proline (10, 20, or 30 mM) as foliar spray (29 d stage) on the activities of catalase; CAT (A), peroxidase; POX (B), superoxide dismutase; SOD (C), and proline content (D) in two varieties ('Varuna' and 'RH-30') of *Brassica juncea* (L.) Czern & Coss at 30 and 60 DAS. The symbol "*" in the figures depicts that the data is significant

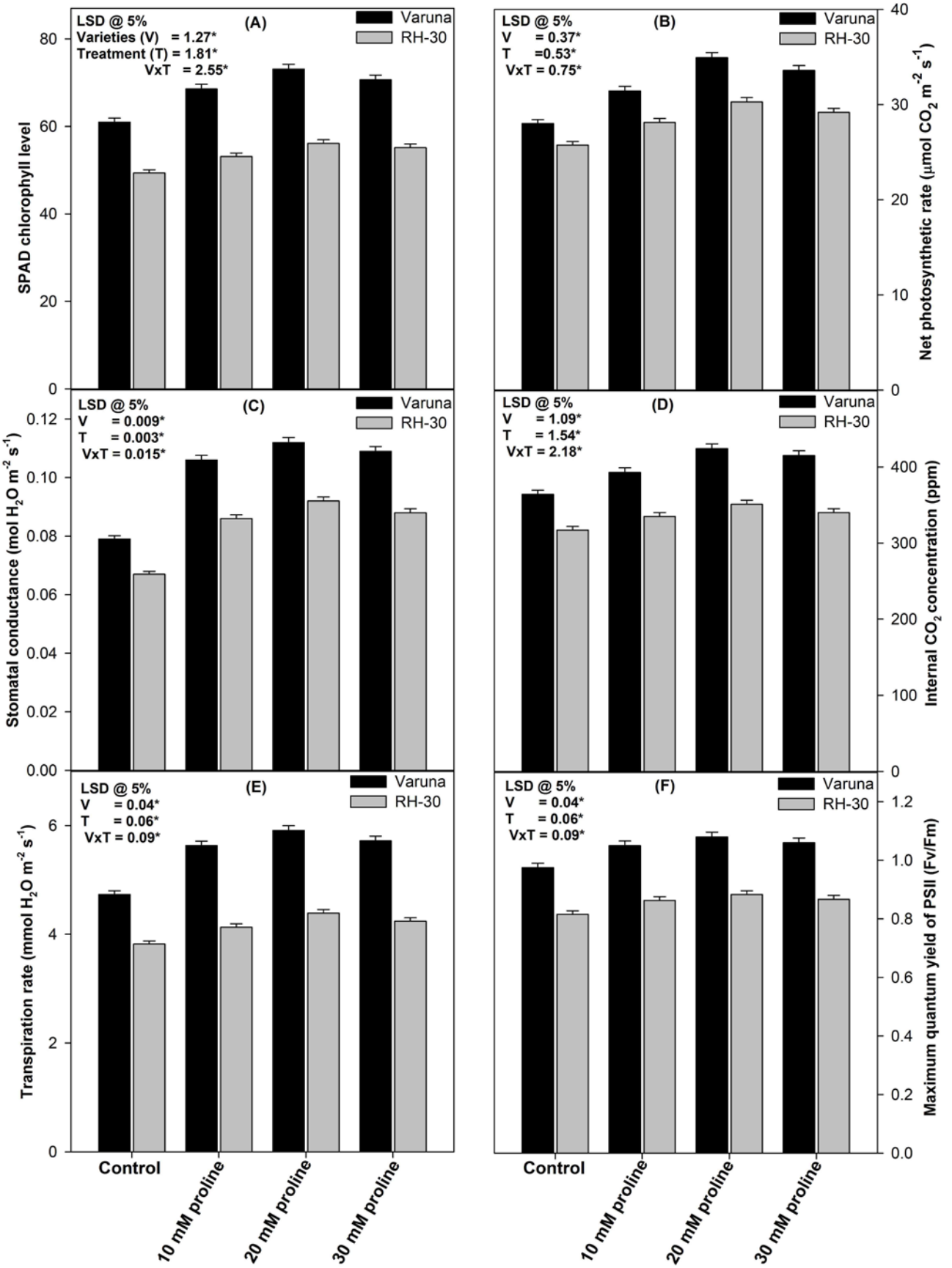

Fig. 3. Effect of proline (10, 20, or 30 mM) as foliar spray (29 d stage) on SPAD chlorophyll (A), net photosynthetic rate (B), stomatal conductance (C), internal CO_2 concentration (D), transpiration rate (E) and maximum quantum yield of PSII (F) in two varieties (Varuna and RH-30) of *Brassica juncea* (L.) Czern & Coss at 30 and 60 DAS. The symbol "*" in the figures depicts that the data is significant

Photosynthetic efficiency i.e. Fv/Fm increased with the application of proline as a foliar spray in both the varieties (Fig. 3F). The significant increase in photosynthetic pigments (chlorophyll content) by proline application is likely to directly affect the light harvesting and electron transport system in photosytem II, which is indicated by chlorophyll fluorescence parameter (Fv/Fm).

The production as well as detoxification of reactive oxygen species must be controlled in plants under non-stress conditions (Apel and Hirt, 2004), otherwise their excessive levels will cause oxidative damage to nucleic acids and chlorophyll pigment degradation, etc. (Schutzendubel and Polle, 2002). Plants have developed various antioxidant defence mechanisms which scavenge these ROS in plants (Alscher *et al*., 2002) which include antioxidant enzymes activation and production of compatible solutes such as proline, glycine betaine, etc.

Proline has been reported to be responsible for scavenging of ROS and other free radicals (Okuma *et al*., 2004). Exogenous application of proline reduces cellular ROS level indicating its free radical scavenging potential (Cuin and Shabala, 2007), which also confers enhanced activity of antioxidant enzymes, i.e., CAT, POX, and SOD (Hoque *et al*., 2007; Ahmed *et al*., 2010). Similar inferences have been observed in the present study where exogenous proline application enhanced the activity of these antioxidant enzymes (Fig. 4A-C), which was more in 'Varuna' and lesser in 'RH-30'. This is further confirmed by the findings of Cuin and Shabala (2007), Ashraf and Foolad (2007) and Wani *et al*. (2012) where proline increased activities of these enzymes by acting at the level of transcription and/or translation.

Being a membrane stabilizer, proline application results in its rapid uptake coupled with its *de novo* synthesis (Zhu *et al*., 1990; Santos *et al*., 1996), thereby increasing its endogenous level (Fig. 4D). Proline action is carried over through its involvement at transcription and/or translation level (Cuin and Shabala, 2007; Ashraf and Foolad, 2007). However, application of higher proline concentrations imposes a check on its biosynthesis through feedback inhibition thereby decreasing the endogenous proline content (Zhang *et al*., 1995; Garcia-Rios *et al*., 1997 and Fig. 4D).

The increase in the endogenous level of proline by its exogenous application in this study (Fig. 4D) protects the enzymes (Khedr *et al*., 2003) and 3-D structure of proteins (Paleg *et al*., 1981), cell organelles and membranes by checking lipid peroxidation (Okuma *et al*., 2004) and facilitates the energy supply for plant growth and survival (Hoque *et al*., 2007; Ashraf and Foolad, 2007). Therefore, higher proline content acts as an osmoregulator to provide osmo-protection to plant tissues and therefore lead to improved plant growth (Csonka and Hanson, 1991; Yancey, 1994; Figs. 1 and 2A). Growth responses were more prominent in 'Varuna' than in 'RH-30' to exogenous proline application (Figs. 1 and 2A). These findings confirm the argument of Garg (2003) that genotypes of the same species may vary in their responses to exogenous proline. Similarly, Deivani *et al*. (2011) and Wani *et al*. (2012) reported higher proline content in rice and mustard plants respectively is associated with improved growth.

Conclusions

The critical assessment of the present investigation provides some important clues about the role of proline when given as foliar spray in mustard plants. It may be summarized as: (a) 20 mM proline proved best that increased all the parameters studied (b) the photosynthetic feedback in genotypes selected in present study provided an added advantage to the growth and physiological attributes. Furthermore, the promoting effects of lower concentration dose of proline (20 mM) were more prominent in Varuna as compared to RH-30.

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