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Nitric oxide and abscisic acid protects against PEG-induced drought stress differentially in *Brassica* genotypes by combining the role of stress modulators, markers and antioxidants

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ABSTRACT

The present study was designed to see the effect of exogenous nitric oxide (NO) and abscisic acid (ABA) and their interaction on physiological and biochemical activities in leaves and roots of two Indian mustard (*Brassica juncea*) cultivars [cv. Pusa Jagannath (PJN) and Varuna (VAR)] exposed to polyethylene glycol (PEG)-induced drought stress. Seven days old hydroponically grown seedlings were treated with PEG (10%), sodium nitroprusside, a NO donor [NO (100 μM)] and abscisic acid [ABA (10 μM)], using different combinations as: Control, ABA, NO, PEG, PEG + ABA, PEG + NO, and PEG + NO + ABA. Results revealed that in response to PEG-induced drought stress leaf relative water content, chlorophyll, carotenoid and protein content decreased with increased production of $O_2^{-\bullet}$, MDA, H_2O_2, cysteine content and non-enzymatic antioxidants (including proline, flavonoid, phenolic, anthocyanin, and ascorbic acid), whereas, the enzymatic antioxidants (including SOD, CAT, APX, GR) showed the response range from no effect to increase or decrease in certain enzymes in both *Brassica* cultivars. The application of NO or/and ABA in PEG-stressed cultivars showed that both enzymatic and non-enzymatic antioxidants responded differently to attenuate oxidative stress in leaves and roots of both cultivars. Overall, PJN had the antioxidant protection mainly through the accumulation of non-enzymatic antioxidants, whereas VAR showed tolerance by the enhancement of both enzymatic and non-enzymatic antioxidant activities. Altogether, the study concluded that the independent NO and its interaction with ABA (PEG + NO and PEG + NO + ABA) were much effective than independent ABA (PEG + ABA) in lowering PEG-drought stress in *Brassica* cultivars.

1. Introduction

Drought stress is one of the most critical abiotic stresses that affect all lives of organisms in terms of health and food [1,2]. It is quite pervasive and causes economical losses problem which is exemplified with the data recorded by the United States between 1980 and 2012. The data showed that the drought alone within the agricultural production suffered an incredible loss of 50 billion dollars [3]. The losses in economic yield in various crops have been well documented, as reviewed by Farooq et al. [4]. Further, the damage from drought stress alone may be several times greater than other biotic and abiotic stress together [5]. This condition is severe all over the world, and occurs mostly in arid and semi-arid regions. Polyethylene glycol 6000 (PEG 6000) is a very commonly used reliable marker to modify the osmotic potential, and mimicking a controlled water deficit stress in the growth medium [6]. It reflected the similar type of stress in the aqueous growth medium to that imposed by drying soil in the field condition [7]. At the cellular level, exposure of plants to PEG-induced osmotic, drought or water stress triggers various interactive events such as generation of abscisic acid (ABA), reactive oxygen species (ROS), and nitric oxide (NO) in the plant cell [8–10] and, as reviewed by Santisree et al. [11]. It is a well-known fact that excess ROS could shift their elimination in the plant, which resulted in damages such as the functioning of nucleic acid (DNA and RNA) and proteins, peroxidation of membrane lipids, sugars damage, ion leakage, inhibition of enzymes and, ultimately loss of growth and biomass [12]. To protect themselves from toxic ROS, plants endowed with the physiological and biochemical antioxidant systems, which include enzymatic antioxidant components (viz., superoxide dismutase-SOD; catalase-CAT; ascorbate peroxidase-APX; glutathione reductase-GR etc.) and non-enzymatic antioxidant components (viz., proline, cysteine, ascorbic acid, anthocyanin, phenolic and flavonoid content etc.) [13]. The antioxidant defense components act in order to

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keep overproduction of ROS generation under control.

Abscisic acid (ABA), a primary stress phytohormone, and nitric oxide (NO), a freely diffusible and reactive gaseous compound have been demonstrated as extreme indispensable signalling molecules, involved in the control of various physiological aspects in plants [11,14–16]. Furthermore, both NO and ABA are considered to be complementary and call to one another to synthesize for subsequent change in redox balance and development of redox homeostasis in the plant cell against drought stress. For instance, some reports are available which explain the NO and ABA co-mediating effects on the root growth, seed dormancy, and the stomatal movement etc [17]. However, not much is known about the possible interactive effects on biochemical and physiological components under drought/water stress which still remains to be elucidated. Furthermore, it is largely unknown that how the pathways are interconnected.

Amongst the major food crops, Indian mustard (*Brassica juncea* L.) is the most affected crop by drought stress, due to the fact that it is mainly grown in arid and semi-arid areas. It is mainly cultivated for leaf and oil production, and consumed as a health-promoting food of the major importance, since it has nutritional (proteins, vitamins, and essential minerals) as well as medicinal significance (treatment for rheumatism and joint pains, indurations of liver and spleen, tooth pain and throat tumors). This amphidiploid species (AABB) is an attractive study system because of its natural genetic variation in the traits related to drought tolerance.

Scant information is available on the interactive action of NO, ABA and drought stress in *B. juncea* at physiological, biochemical and molecular level. Therefore, the present study evaluated the effects of independent and simultaneous addition of NO and ABA under PEG-induced drought stress using various stress modulators, markers and antioxidants, both of enzymatic and non-enzymatic antioxidants. In this study, the hypothesis was tested of whether enzymatic and non-enzymatic antioxidants confer a similar physiological mechanism of protection in two cultivars/genotypes of *Brassica juncea* [Pusa Jagannath (PJN) and Varuna (VAR)] with different drought resistance. This study may facilitate a better understanding of the mechanism of an operating antioxidant system along with how NO and ABA interact and enhance drought stress tolerance in different plant organs (leaves and roots) from the same and of different genotypes in *B. juncea*.

2. Material and methods

2.1. Plant material, growth conditions and treatments

Two common Indian mustard (*Brassica juncea* L.) cultivars viz., PJN and VAR with contrasting drought tolerance (as drought sensitive and tolerant, respectively) were used. PEG 6000 at 10%, a moderate concentration was added to Hoagland medium (HM) [18] to impose drought stress by the gradual decrease in its osmotic potential (OP) until −1.48 MPa, which is believed to induce low drought toxicity. The calculation of OP for 10% PEG 6000 was done according to Michel and Kaufnamm [19]. The cultivars and 10% PEG were selected based on their degree of drought susceptibility index (DSI), calculated as differences in fresh weight (FW) or dry weight (DW) under PEG-stress treatment and control condition by using formula as described by Benesova et al. [6], as follows: DSI = 1- (fresh or dry weight in stress treatment/fresh or dry weight in control treatment)/1- (mean fresh or dry weight of all cultivars in stress treatment/mean fresh or dry weight of all cultivars in control treatment), which is shown in supplementary figure (S1). As depicted in the figure that DSI increased with an increase in PEG concentrations, with more in PJN than VAR cultivar. Further, the stress effect was comparatively lower under 10% PEG, when compared with 20% and 40% PEG treatments. As such, at 10% PEG treatment, DW-DSI and FW-DSI was 56% and 52%, respectively for PJN, and 20% and 23% for VAR cultivar. Therefore, PJN was regarded as the drought sensitive and VAR was as the drought tolerant cultivar at this PEG concentration treatment.

Seeds were disinfected by surface sterilization in 30% ethanol (v/v) for 2–3 min, washed thoroughly several times with miliQ water to remove any traces of ethanol. Sterilized seeds were soaked for overnight, then equal numbers of seeds were transferred in petri-plates on the moist cotton bed, and allowed to germinate in dark at 25 °C for two days. Germinated seedlings were removed from the dark, transferred into small PVC plastic pots (4 cm height, 6 cm diameter) fixed in a tray containing HM solution. Trays were divided into seven sets i.e., set I-VII containing pots in triplicates (20 seedlings per replicate), kept into the controlled growth culture room with a day/night photoperiod of 16 h at 25 ± 2 °C, and the relative humidity of 70%. The seedlings were allowed to grow up to 7 days in HM solution. The HM solution was changed on an alternate day to prevent nutrient deficiency and pathogen growth. After 7 days, seedlings were treated with PEG 6000 (10%), sodium nitroprusside, a NO donor [NO (100 μM)], and abscisic acid [ABA (10 μM)] and grown for additional 96 h (4 days), using different combinations as: (i) Control (ii) ABA (iii), NO (iv) PEG (v) PEG + ABA (vi) PEG + NO, and (vii) PEG + ABA + NO. After 96 h, leaves and roots were separated, washed, blotted, immediately frozen and stored into −80 °C for subsequent analysis of various parameters. The experiment was set and treatments were arranged according to the complete randomized block design (CRBD). Growth parameters (root-shoot length) were measured using a metric scale (cm) and presented as Supplementary Fig. S2.

2.2. Measurements of leaf relative water content (LRWC), endogenous ABA and NO content

Leaf relative water content was measured following the method of Fukao et al. [20] as follow: LRWC (%) = [(FW - DW)/(TW - DW)] × 100, where FW indicates the fresh weight of 50 leaves taken immediately after harvest, while TW is the turgid weight of 50 leaves taken after imbibed the leaves in deionized water for 5–6 h. DW indicates the oven dried weight of 50 leaves at 70 °C to a constant weight.

The extraction and quantification of ABA in the leaves and roots were performed with HPLC system according to a previously reported method [21], with some modifications. Frozen samples (1g) were ground in fine powder with liquid nitrogen, followed by extraction in 15 ml cold 80% (v/v) methanol containing d6-ABA as an internal standard for 12 h at 4 °C. The extract was centrifuged and filtered through two layers of Whatman 1 filter paper. The filtrate was vacuum evaporated and reduced to an aqueous phase (pH 9.5 with NaOH), and partitioned two-three times with 100% (v/v) hexane. The hexane layer was discarded and an aqueous phase (pH 2.5 with 0.1 M HCl) partitioned again two to three times against ethyl acetate. The aqueous phase was discarded and the ethyl acetate phase was vacuum evaporated to an almost dry residue which was dissolved in 5–10 ml of 70% methanol (pH 8.5), and passed through a 10 ml glass syringe attached to Waters Symmetry C-18 cartridge to remove plant pigments and other non-polar compounds. Finally, C-18 elute (containing ABA) was collected and under a vacuum of 35 °C, evaporated to a dry state, then redissolved in 80% methanol and passed through the HPLC system with a C18 reverse phase column (4.6 × 250 mm, 5 μm). The absorbance was recorded at 254 nm and also the d6-ABA dissolved in acetonitrile was used as standard.

Griess's reagent method, with some modifications was used to determine the nitrite-derived NO level [22]. Plant leaves and roots (0.2g) were homogenized in 2 ml chilled 0.1 M K-phosphate buffer containing Zn-diacetate (pH 3.8) and centrifuged at 12000 × *g* for 15 min at 4 °C. A pinch of charcoal was added to the supernatant, mixed vigorously and filtered. The filtrate and Greiss reagent (a mixture of 1% sulfanilamide and 0.2% N-NED in 3 M HCl) was mixed in equal volume, incubated for 15–20min in dark at room temperature, followed by spectrophotometric absorbance monitored at 540 nm. The $NaNO_2$ was used as a standard. The content of NO was expressed μmol g^{-1} FW.

2.3. Determination of chlorophyll (Chl), carotenoid (Caro) and protein content

Total Chl and Caro content was extracted by homogenizing leaves (50 mg) in chilled 80% acetone (v/v), followed by centrifugation at 12,000 × *g* for 10 min as described by Arnon [23]. Absorbance was read at 663 nm and 645 nm for Chl, and at 480 nm and 510 nm for Caro estimation using UV-VIS spectrophotometer (OPTIZEN 3220 UVbio, Korea). Bradford's method [24] was used to estimate the protein content in fresh leaves of control and treated Indian mustard seedlings, using bovine serum albumin (BSA) as standard.

2.4. Measurements of stress markers: malondialdehyde (MDA), superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) content

A level of lipid peroxidation is determined in terms of the MDA content. The amount of MDA was calculated by subtracting the absorbance at 600 nm from the absorbance at 532 nm, using an absorption coefficient or extinction coefficient (ε) of 155 $mmol^{-1}\,cm^{-1}$, following the method of Heath and Packer [25], with some modifications. Leaves and roots (200–500 mg) were homogenized in 2 ml of 1% TCA using cold mortar and pestle placed on chilled ice. Homogenate was centrifuged at 12,000 × *g* for 5 min at room temperature. Supernatant was mixed with 4 ml of 0.5% TBA. The mixture was heated on the water bath at 95 °C for 30 min followed by cooling in an ice bath for 10 min to stop the reaction. After that, the solution was centrifuged at 10,000 × *g* for 15min, and the absorbance of the supernatant was read at 532 nm and 600 nm. The value was expressed in nmol MDA g^{-1} FW. The $O_2^{-\bullet}$ content was measured adopting the method as described by Liu et al. [26], with some modifications. Leaves and roots (200–500 mg) were homogenized in 2 ml of 65 mM phosphate buffer (pH 7.8), followed by centrifugation at 5000 × *g* for 10 min at 4 °C. Supernatant was mixed with 65 mM phosphate buffer and 10 mM hydroxylamine hydrochloride, and incubated for 20 min. Equal volume of incubated mixture, sulphanilamide (1% in 1.5 M HCl) and NED-HCl (0.02% in 0.2 M HCl) was mixed well and incubated for another 30 min. Absorbance was recorded at 540 nm using spectrophotometer and the content of $O_2^{-\bullet}$ radicals was expressed in μmol g^{-1} FW. For H_2O_2 estimation, the method of Alexieva et al. [27] was adopted. Leaves and roots (200–500 mg) were homogenized in 2 ml of 0.5% TCA, followed by centrifugation at 10,000 × *g* for 25 min at 4 °C. To an aliquot of the supernatant, 0.5 ml of 0.1 M phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide was added to initiate the reaction, and kept in dark for 1 h for reaction completion. The absorbance of the reaction solution was recorded at 390 nm and the value was expressed in μmol g^{-1} FW.

2.5. Measurements of stress modulators: cysteine (Cys) and proline (Pro) content

The leaves and roots sample (200–500 mg) of control and treated seedlings were crushed in 2 ml of chilled 5% perchloric acid and centrifuged at 10,000 × *g* for 20 min at room temperature following the procedure of Gaitonde [28]. To an equal amount of supernatant, an equal volume of ninhydrin and glacial acetic acid was added, boiled at 100 °C in a water bath for 10 min and cooled. Half of the equal volume of 95% absolute alcohol was added to the solution for colour stabilization. L-Cysteine was used as a standard and absorbance was read at 560 nm. The value of Cys was expressed in term of μmol g^{-1} FW. The Pro content was measured according to Bates et al. [29]. Plant tissues (200–500 mg) were crushed and mixed in 3% sulfosalicylic acid, centrifuged at 4000 × *g* for 10 min, and measured as described by Pandey et al. [30]. The amount of free Pro concentration was expressed as μmol g^{-1} FW.

2.6. Measurements of stress scavengers/inhibitor markers: enzymatic antioxidants

Enzyme extraction: Fresh and frozen plant tissues (200 mg) were homogenized in pre-cooled 2 ml of extraction buffer, contained 50 mM K-phosphate buffer (pH 7.0), 1 mM PMSF, 0.2 mM EDTA, 1% PVP, 0.1% triton. Besides, 1 mM ascorbate may be added for extraction of APX enzyme. Extraction mixture was centrifuged at 15,000 × *g* for 20 min at 4 °C and used for estimation of total soluble protein [24] and following enzymes.

The activity of SOD enzyme (EC 1.15.1.1) dismutase superoxide anion radical ($O_2^{-\bullet}$) into hydrogen peroxide non-radical (H_2O_2), was assayed according to nitroblue tetrazolium (NBT) photo-reduction method of Dhindsa et al. [31]. Enzyme assay was performed by adding 100 μl enzymatic extract to reaction mixture (containing 1 M sodium carbonate, 10 mM riboflavin, 2 mM L-methionine and 2.25 mM NBT). Riboflavin and NBT were added in the last to initiate the photo-reduction reaction under the light of a 30W florescent lamp and under dark for 5 min at 25 °C. An increase in the absorbance was read at 560 nm for total time 9 min at 3 min interval. The CAT activity (EC 1.11.1.6) was assayed by adding 70 μl enzymatic extract to a reaction solution (containing 2 mM H_2O_2 phosphate buffer in 50 mM K-phosphate buffer-pH 7.0), following the method as described by Aebi [32]. The reaction initiated after adding enzymatic extract, and disappearance of H_2O_2 recorded by the decrease in absorbance 1 min later for total 3 min at 240 nm. CAT activity was calculated by using extinction coefficient of 0.036 (or 36 × 10^3) $mmol^{-1}\,cm^{-1}$. The activity of APX (EC 1.11.1.11) was assessed according to the method of Nakano and Asada [33] based on the ascorbic acid-dependent reduction of H_2O_2 recorded by the decrease in absorbance of an enzyme assay. The enzyme assay (1 ml) contained 100 μl enzyme extract and reaction mixture (50 mM phosphate buffer, 3 mM EDTA, 5 mM ascorbate and 10 mM H_2O_2 phosphate buffer-pH 7.0). The reaction started after H_2O_2 phosphate buffer addition, and the decrease in absorbance was measured at 1 min interval for total 2 min at 290 nm. The molar extinction coefficient 2.8 $mmol^{-1}cm^{-1}$ was used to calculate APX activity. GR activity (EC 1.6.4.2) was evaluated by monitoring the glutathione (GSSG) dependent oxidation of NADPH method as described by Foyer and Halliwell [34]. Leaves and roots (200 mg) were extracted in 0.2 M K-phosphate buffer (pH 7.0), followed by centrifugation at 12000 × *g* for 20 min at 4 °C. Enzyme assay (1 ml) contained a reaction mixture (consisted 0.2 M Na-phosphate buffer-pH 7.0 with 2 mM EDTA, 20 mM GSSG, 2 mM NADPH) and 100 μl enzymatic extract was used to determine the oxidation of NADPH by recording the decrease in absorbance at 340 nm for total 2 min at an interval of 1 min. An extinction coefficient 6.22 × 10^6 $cm^2\,mol^{-1}$ for NADPH oxidation was used to compute GR activity. The enzyme activities were expressed in the term of EU min^{-1} mg^{-1} protein.

2.7. Measurements of stress protective metabolites: non-enzymatic antioxidants

The total flavonoid content (TFC) in different plant tissues was determined by the $AlCl_3$ method of Zhishen et al. [35], with some modifications. Briefly, leaves and roots tissues (200 mg) were extracted in hot distilled water and centrifuged at 10,000 × *g* for 15 min. To the supernatant 5% $NaNO_2$ was added, followed by the addition of 10% $AlCl_3$ and 1 M NaOH, and the final volume was maintained up to 1.25 ml using distilled water. The solution was allowed to stand for 15 min, followed by the absorbance measurement at 510 nm. Rutin was used as a standard. TFC was expressed as rutin equivalents mg g^{-1} FW. The total phenolic (TPC) content in the plant tissues was extracted by the Folin-Ciocalteu method of Ainsworth and Gillespie [36], with some modifications. Briefly, leaves and roots (100 mg) were extracted in 50% methanol, followed by centrifugation at 10,000 × *g* for 15 min. The equal proportion of the supernatant and the Folin-Ciocalteu's reagent

was mixed and kept for incubation in the boiling water-bath for 1 min, followed by cooling. The absorbance was read at 765 nm. Gallic acid was used as a standard, and TPC was expressed as gallic acid equivalents in mg g^{-1} FW. The anthocyanin content (AC) was estimated using the method of Fuleki and Francis [37], with some modifications. Plant leaves and roots (200 mg) were homogenized in 2 ml acidified ethanol, followed by centrifugation at 10,000 × *g* for 10 min. The absorbance of supernatant was measured at 535 nm to determine the anthocyanin content using following formula: AC = ($Absorbance_{at\ 535}$ × $Volume_{of\ extraction}$)/(98.2 × $FW_{plant\ tissue}$) 100. The value 98.2 was used as molar absorptivity of anthocyanin at 535 nm by Beer-Lambert plots. The computed amount of anthocyanin was expressed in mg 100 g^{-1} FW. The ascorbic acid (AsA) content was measured after following the method of Roe and Kuether [38], with some modifications. Briefly, 200 mg plant tissues (leaves and roots) were homogenized with 4% TCA, followed by centrifugation at 2000 × *g* for 10 min. To the supernatant, a pinch of activated charcoal was mixed, shaken vigorously, filtered, and again centrifuged. The supernatant was mixed well with 4% TCA, 2,4-dinitrophenyl hydrazine (DNPH) and 10% thiourea solution to a final volume of 1 ml. Mixture was incubated for 3 h at 37 °C. After 3 h, an osazone formed as a result of coupling reaction of oxidized ascorbic acid (dehydroascorbic) with DNPH, which was dissolved by adding 85% H_2SO_4. Absorbance was determined at 540 nm and the amount of total AsA content was expressed as μmol g^{-1} FW.

3. Statistical analysis

The resulted data are mean ± SE of 3 biological replicates (n = 3) taken from three independent experiments. Data were subjected to one-way analysis of variance (ANOVA) test according to the model for CRBD. Treatment means were compared for the significance difference among them (DMRT, $p \leq 0.05$). Mean values followed by the same and different letter showing the non-significance and significance difference respectively, among treatments within the same organ-type in a particular cultivar. Mean values followed by an asterisk (*) represents the significant difference among the cultivars within the same organ-type at a particular treatment.

4. Results

To explore the independent and interactive action of NO and ABA in PEG-induced drought stress and corresponding physiological and biochemical responses, two Indian mustard cultivars, PJN and VAR were compared. Results evaluated and discussed by comparison of control plants with PEG and PEG-combinations (PEG + ABA, PEG + NO and PEG + NO + ABA) rather than with ABA only or NO only. It is because of that ABA or NO treatments were statistically non-significant to control of both cultivars.

4.1. Effect of NO or/and ABA on LRWC, pigments and protein content under PEG-induced drought stress

A loss of LRWC by ~43% in PJN and ~30% in VAR was observed under PEG treatment over their controls, indicated that PJN was affected more than VAR (Table 1a). However, the application of PEG with NO or/and ABA (PEG + ABA, PEG + NO, and PEG + NO + ABA) significantly improved LRWC by ~34%, ~59% and ~48% in PJN, and ~27%, ~48%, and ~46% in VAR cultivar, respectively over their PEG alone. Significant inhibition in the total Chl content of both *B. juncea* cultivars (cv. PJN and VAR) was observed under drought-containing 10% PEG treatment by ~41% and ~21% respectively, compared to their controls (Table 1a). PEG + ABA suppressed the decline in Chl content for each *Brassica* cultivar by dropped-up the level of Chl content ~9% (PJN cultivar) and ~22% (VAR cultivar) more, compared to their controls. Furthermore, PEG + NO and PEG + NO + ABA also showed the pronounced effect on Chl content by giving ~26% and ~15% increase in PJN and ~60% and ~48% increase in VAR cultivar, respectively over their controls. Similarly, PEG treatment alone significantly reduced the Caro content by ~73% and ~22% in PJN and VAR cultivar, respectively compared to controls (Table 1a). The PEG-stressed plants when supplemented with NO or/and ABA the Caro content was increased by ~313% and ~142% at PEG + ABA, ~407% and ~252% at PEG + NO and ~386% and ~203% at PEG + NO + ABA treatment level in PJN and VAR, respectively over PEG alone. Protein content was also decreased by ~45% in PJN and ~34% in VAR cultivar under PEG treatment over untreated control plants (Table 1a). Increase in protein content by ~54% and ~120% upon PEG + ABA, ~200% and ~191% upon PEG + NO, and ~141% and ~140% upon PEG + NO + ABA treatment were observed over PEG alone in PJN and VAR cultivars, respectively. Overall, PEG-induced the loss of water content (LRWC), Chl, Caro and protein content in leaves surpassed more effectively at PEG + NO and PEG + NO + ABA rather than PEG + ABA combination treatments. Furthermore, VAR cultivar was better than PJN to adapt the drought stress condition as VAR cultivar neglected drought induces LRWC, pigments, and protein inhibition completely upon the application of NO or/and ABA.

4.2. Accumulation of ABA and NO content under PEG and PEG-combined NO and/or ABA

As shown in Table 1b, endogenous ABA and NO level was more in leaves than roots in both *Brassica* cultivars. There was no significant difference in the leaves and roots ABA levels in PJN and VAR cultivar under unstressed control conditions (control, ABA only and NO only). After PEG treatment, ABA level was significantly increased by 2.6 fold (leaves) and 2.7 fold (roots) at PEG, 3.2 fold (leaves) and 3.3 fold (roots) at PEG + ABA, 2.8 fold (leaves) and 4 fold (roots) at PEG + NO, and 3 fold (leaves) and 4.1 fold (roots) at PEG + ABA + NO treatment in PJN cultivar. In VAR cultivar, the fold increase in ABA level was 0.6 and 1 under PEG, 1 and 2.2 under PEG + ABA, 1.2 and 2.1 under PEG + NO, and 1.2 and 2.5 fold under PEG + ABA + NO in leaves and roots, respectively, but levels remained significantly lower than both PJN-leaves and roots. Similarly, the NO content was increased by 1.9, 2.3, 2.8, and 2.5 fold in PJN-leaves, and 1.4, 2.0, 2.5 and 2.3 fold in VAR-leaves, whilst 3.4, 4.2, 5.2 and 4.7 fold in PJN-roots, and 2.0, 2.4, 3.1, and 2.6 fold in VAR-roots under PEG, PEG + ABA, PEG + NO, and PEG + ABA + NO treatments (Table 1b). This result indicates that PJN cultivar accumulated more ABA and NO content following the PEG and PEG-combination treatments.

4.3. Effect of NO and/or ABA on stress markers (MDA, $O_2^{-\bullet}$ and H_2O_2) under PEG-induced drought stress

Data revealed that external PEG simulated more increase in MDA content in leaves as well as in roots, than untreated (control) and PEG-combined ABA or/and NO treated seedlings. The significant increase in the level of MDA was ~95% and ~63% in leaves, and ~109% and ~117% in roots of PJN and VAR cultivars, respectively under PEG treatment alone over their controls (Fig. 1a and b). On the other hand, PEG + ABA reduced MDA content by ~10% and ~26% in leaves, and ~12% and ~24% in roots of both PJN and VAR cultivars, respectively over independent PEG-treated seedlings. The PEG + NO treatment decreased MDA levels in leaves and roots by ~35% and ~30% in PJN, and ~42% and ~45% in VAR cultivar, respectively compared to PEG alone. Furthermore, the effect of co-applied NO and ABA with PEG (PEG + NO + ABA) was also efficacious as it showed the reduction in MDA concentration (~26% and ~37% in leaves, whereas, ~35% and ~45% in roots) in PJN and VAR cultivars respectively, when compared with PEG alone (Fig. 1a and b).

The accumulation of $O_2^{-\bullet}$ ROS was significantly high in the roots than leaves in both cultivars. PEG-stressed PJN-leaves and roots

Table 1

a) Leaf relative water content (LRWC), chlorophyll (Chl), carotenoid (Caro) and protein content measured in leaves **b)** endogenous ABA and NO content measured in leaves and roots of 7 days old seedlings of two Indian mustard cultivars, Pusa Jagannath (PJN) and Varuna (VAR) after 96 h exposure under 10% PEG-induced drought stress, with or without abscisic acid (ABA, 10 μM) and sodium nitroprusside (NO, 100 μM). Values represent mean ± SE of 3 replicate plants from three independent experiments. Values sharing different small letters and asterisk (*) indicate significant difference among treatments within a particular cultivar and among cultivars at a particular treatment, respectively at 5% significance level.

a

	LRWC(%)		Chl content (mg g^{-1})		Caro content (mg g^{-1})		Protein content (mg g^{-1})	
	PJN	VAR	PJN	VAR	PJN	VAR	PJN	VAR
Control	94.3_a*	88.2_a	$0.886 \pm 0.06_c$	$0.808 \pm 0.09_c$	$0.395 \pm 0.04_b$*	$0.256 \pm 0.02_d$	$10.69 \pm 0.59_c$*	$17.11 \pm 0.59_e$
ABA	95.6_a*	88.6_a	$0.941 \pm 0.06_{bc}$*	$0.794 \pm 0.06_d$	$0.415 \pm 0.03_b$*	$0.251 \pm 0.01_d$	$12.28 \pm 1.02_b$*	$17.64 \pm 1.02_{de}$
NO	96.1_a*	89.5_a	$0.986 \pm 0.05_b$*	$0.806 \pm 0.08_c$	$0.441 \pm 0.04_b$*	$0.251 \pm 0.01_d$	$13.25 \pm 1.48_b$*	$19.03 \pm 1.44_d$
PEG	53.4_e*	61.2_c	$0.556 \pm 0.04_d$	$0.633 \pm 0.06_e$	$0.103 \pm 0.02_c$	$0.199 \pm 0.01_e$	$5.51 \pm 0.55_d$*	$11.19 \pm 0.65_f$
PEG + ABA	71.7_d*	77.8_b	$0.993 \pm 0.02_b$*	$0.991 \pm 0.03_b$	$0.426 \pm 0.03_b$	$0.483 \pm 0.02_c$	$8.53 \pm 0.71_c$*	$24.67 \pm 0.91_c$
PEG + NO	85.4_b*	91.1_a	$1.121 \pm 0.05_a$*	$1.301 \pm 0.02_a$	$0.523 \pm 0.02_a$*	$0.702 \pm 0.03_a$	$16.58 \pm 0.54_a$*	$32.63 \pm 0.84_a$
PEG + ABA + NO	79.3_c*	89.6_a	$1.032 \pm 0.02_b$*	$1.200 \pm 0.07_a$	$0.501 \pm 0.02_{ab}$*	$0.604 \pm 0.02_b$	$13.28 \pm 0.59_b$*	$27.07 \pm 0.99_b$

b

PEG (%)	ABA (μM)	NO (μM)	ABA content (ng g^{-1} FW)				NO content (μmol g^{-1} FW)			
			PJN (Leaves)	PJN (Roots)	VAR (Leaves)	VAR (Roots)	PJN (Leaves)	PJN (Roots)	VAR (Leaves)	VAR (Roots)
0	0	0	$18 \pm 0.52_e$	$10 \pm 0.36_e$	$21 \pm 0.55_e$	$8 \pm 0.55_f$	$0.17 \pm 0.01_e$	$0.07 \pm 0.006_e$	$0.15 \pm 0.01_e$	$0.08 \pm 0.007_d$
0	10	0	$25 \pm 0.56_d$	$16 \pm 0.41_d$	$28 \pm 0.73_d$	$13 \pm 0.43_e$	$0.20 \pm 0.01_d$	$0.11 \pm 0.008_d$	$0.18 \pm 0.01_e$	$0.09 \pm 0.005_d$
0	0	100	$22 \pm 0.50_d$	$18 \pm 0.40_d$*	$25 \pm 0.77_d$	$10 \pm 0.63_d$	$0.21 \pm 0.01_d$	$0.14 \pm 0.008_d$	$0.23 \pm 0.02_d$	$0.11 \pm 0.008_d$
10	0	0	$65 \pm 0.74_c$*	$37 \pm 0.55_c$*	$34 \pm 0.82_c$	$16 \pm 0.59_c$	$0.50 \pm 0.02_c$*	$0.31 \pm 0.02_c$*	$0.36 \pm 0.04_c$	$0.24 \pm 0.01_c$
10	10	0	$77 \pm 0.79_a$*	$43 \pm 0.50_b$*	$41 \pm 0.78_b$	$26 \pm 0.90_b$	$0.57 \pm 0.02_b$*	$0.37 \pm 0.02_b$*	$0.46 \pm 0.04_b$	$0.27 \pm 0.01_b$
10	0	100	$70 \pm 0.71_b$*	$50 \pm 0.50_a$*	$50 \pm 0.91_a$	$25 \pm 0.83_b$	$0.65 \pm 0.03_a$*	$0.44 \pm 0.03_a$*	$0.53 \pm 0.03_a$	$0.33 \pm 0.02_a$
10	10	100	$77 \pm 0.80_a$*	$51 \pm 0.50_a$*	$52 \pm 0.88_a$	$28 \pm 0.99_a$	$0.60 \pm 0.02_b$*	$0.40 \pm 0.03_b$*	$0.50 \pm 0.04_a$	$0.29 \pm 0.01_b$

accumulated $O_2^{-\bullet}$ 49% and 98%, whereas $O_2^{-\bullet}$ content was 29% in leaves and 72% in roots of VAR cultivar over their unstressed control plants, indicated that PJN was affected much more than VAR (Fig. 1c, d). PEG-accelerated $O_2^{-\bullet}$ accumulation was prevented upon the supplementation PEG + ABA, PEG + NO, and PEG + NO + ABA by ~8%, ~23% and ~19% in PJN-leaves and ~10%, ~28% and ~20% in PJN-roots, whereas, 10%, 28% and 20% in VAR-leaves and ~18%, ~39% and ~36% in VAR-roots, compared to PEG alone. It indicated that $O_2^{-\bullet}$ was recovered at a partial level in PJN, whilst completely in VAR cultivars which suggest VAR cultivar is better than PJN to withstand $O_2^{-\bullet}$ induced oxidative stress.

A significantly higher concentration of another ROS-H_2O_2 was observed in seedlings exposed to PEG-induced drought stress (Fig. 1e and f). Upon independent PEG treatment, PJN cultivar was influenced more than VAR cultivar, as such H_2O_2 concentration was increased ~120% in PEG-stressed PJN-leaves and ~132% in PEG-stressed PJN-roots over their respective control. Whilst the accumulation of H_2O_2 was ~46% more in VAR-leaves and ~74% more in VAR-roots over their controls. On the other hand, PEG-stressed seedlings when supplemented with exogenous ABA or/and NO substantially decreased PEG-enhanced H_2O_2 content in leaves of PJN and VAR by ~18% and ~16% at PEG + ABA, ~38% and ~32% at PEG + NO, and ~27% and ~26% at PEG + NO + ABA, respectively. In case of roots, the effect of PEG combination treatments were statistically equal to one another, drop-down or reduced PEG-elevated H_2O_2 content in PJN-roots by ~41%, ~32% and ~22%, and in VAR-roots by ~54%, ~43% and ~31% respectively.

4.4. NO and/or ABA responsive differential change in Cys and Pro under PEG-induced drought stress

Both PJN and VAR seedlings subjected to independent PEG treatment possessed higher amount of Cys, more in roots than leaves (Fig. 2a and b). In leaves, PEG-stressed cultivar PJN showed more than ~85%, whilst the cultivar VAR gave only ~35% higher Cys content over their controls. Similarly, PEG-simulated PJN-roots were observed with more than ~191%, and VAR-roots with ~89% increases in Cys accumulation over controls. PEG solution enriched with ABA (PEG + ABA), NO (PEG + NO) and combined (PEG + NO + ABA) reduced Cys content ~12%, ~24% and ~20% in PJN-leaves; ~16%, ~41% and ~34% PJN-roots, whilst being ~17%, ~35% and ~29% in VAR-leaves; and ~15%, ~37% and ~27% in VAR-roots, respectively over their respective PEG treatment alone.

The PEG-simulated ~17% and ~41% increase of Pro in leaves, and ~118% and ~132% in roots of PJN and VAR seedlings over untreated (control) seedlings, indicating that roots were more expressed and affected by PEG-induced drought treatment. Furthermore, the leaves of both cultivars behaved differently upon the supplementation of combination treatments. In PJN-leaves, at PEG + NO and PEG + NO + ABA treatment level, a further increase in Pro content was found, compared to control as well as PEG alone. However, the effect of both combination treatments was statistically non-significant ($p = 5\%$). Although PEG + ABA treatment did not significantly enhance the Pro level but maintained the level almost equal to PEG and control (Fig. 2c). On the other hand, in the case of VAR-leaves, PEG + NO, PEG + ABA and PEG + NO + ABA decreased PEG-induced higher Pro accumulation by ~25%, ~12% and ~21%, respectively, as depicted in Fig. 2d. Roots of each cultivar behaved in a similar fashion for all combination treatments, reduced PEG-accelerated high level of proline (Fig. 2c and d) by ~20% and ~19% in PEG + ABA, by ~41% and ~36% in PEG + NO, and by ~31% and ~30% in PEG + NO + ABA for PJN and VAR cultivars respectively.

4.5. Differential response in enzymatic antioxidants to NO and/or ABA against PEG-induced drought stress

The SOD activity was different in two Indian mustard cultivars. In cultivar PJN, leaf-SOD activity did not change under PEG-treatment, and it was decreased even under PEG + ABA, PEG + NO, and PEG + NO + ABA treatments by ~70%, ~74%, and ~51% respectively, compared to control as well as PEG alone treatment (Fig. 3a). This pattern of change in SOD activity was not evident in the case of PJN-roots. In contrast, in roots, there were significant increases in SOD activity by ~57%, ~113% and ~77% at PEG + ABA, PEG + NO, and

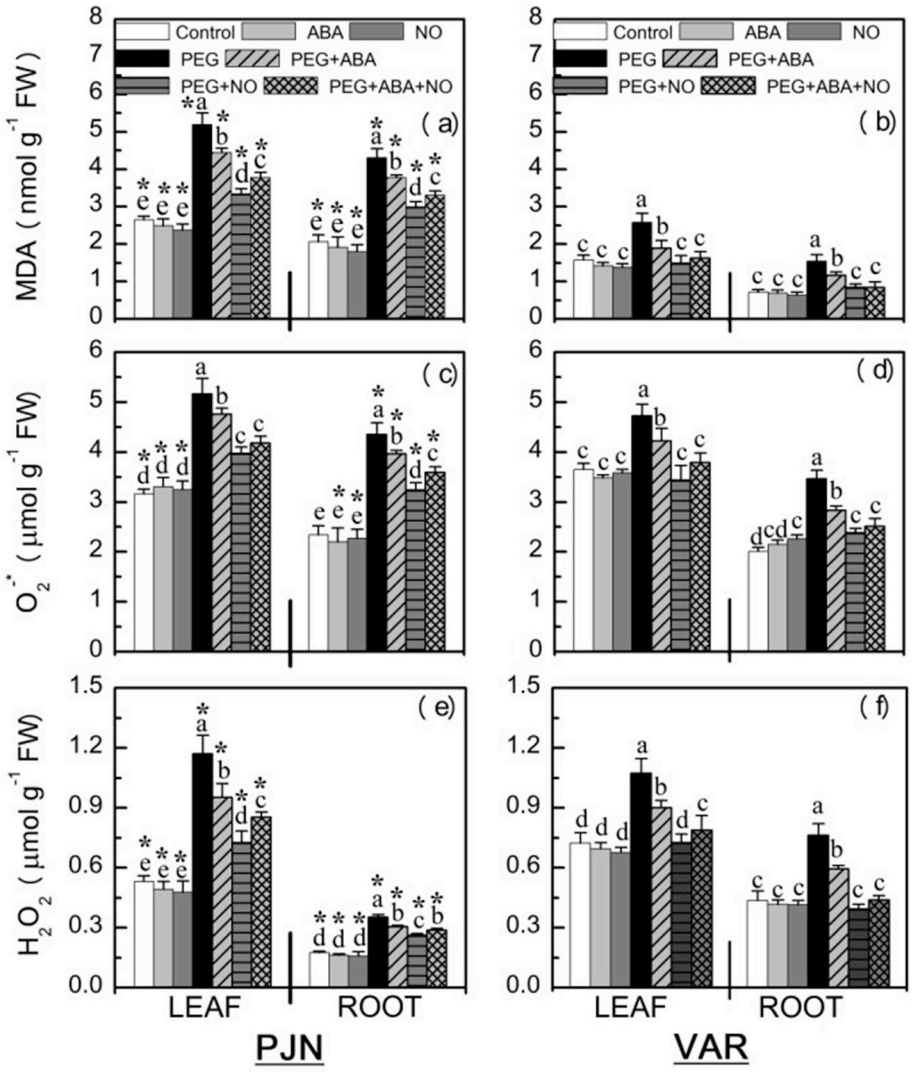

Fig. 1. Malondialdehyde (MDA) (a, b), superoxide ($O_2^{-\bullet}$) (c, d) and hydrogen peroxide (H_2O_2) (e, f) content measured in leaves and roots of 7 days old seedlings of two Indian mustard cultivars, Pusa Jagannath (PJN) and Varuna (VAR) after 96 h exposure under 10% PEG-induced drought stress, with or without abscisic acid (ABA, 10 µM) and sodium nitroprusside (NO, 100 µM). The vertical data bars represent mean values ± SE of 3 replicate plants from three independent experiments. Values sharing different small letters and asterisk (*) above the bars indicate significant difference among treatments within a particular cultivar and among cultivars at a particular treatment, respectively at 5% significance level.

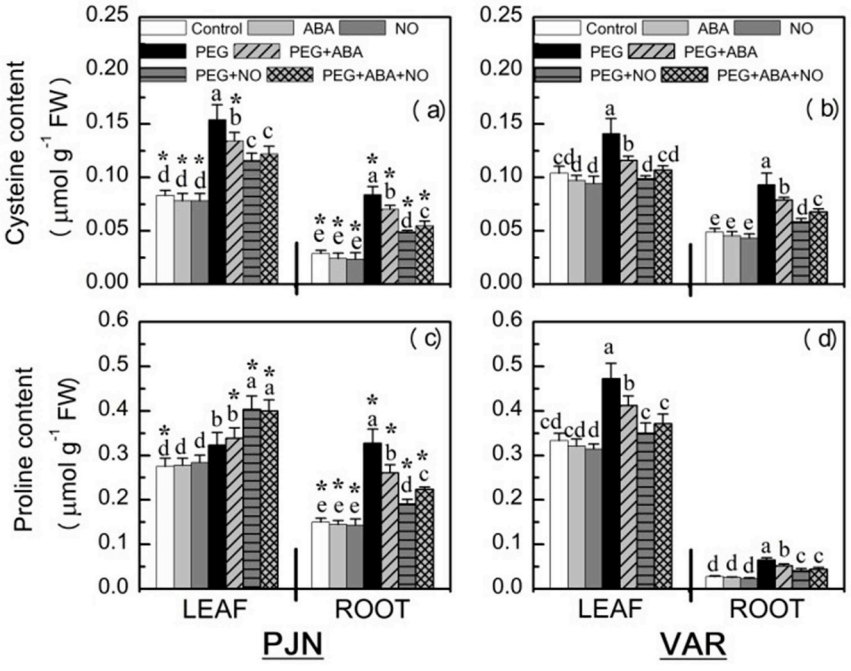

Fig. 2. Cysteine (Cys) (a, b) and proline (Pro) (c, d) content measured in leaves and roots of 7 days old seedlings of two Indian mustard cultivars, Pusa Jagannath (PJN) and Varuna (VAR) after 96 h exposure under 10% PEG-induced drought stress, with or without abscisic acid (ABA, 10 µM) and sodium nitroprusside (NO, 100 µM). The vertical data bars represent mean values ± SE of 3 replicate plants from three independent experiments. Values sharing different small letters and asterisk (*) above the bars indicate significant difference among treatments within a particular cultivar and among cultivars at a particular treatment, respectively at 5% significance level.

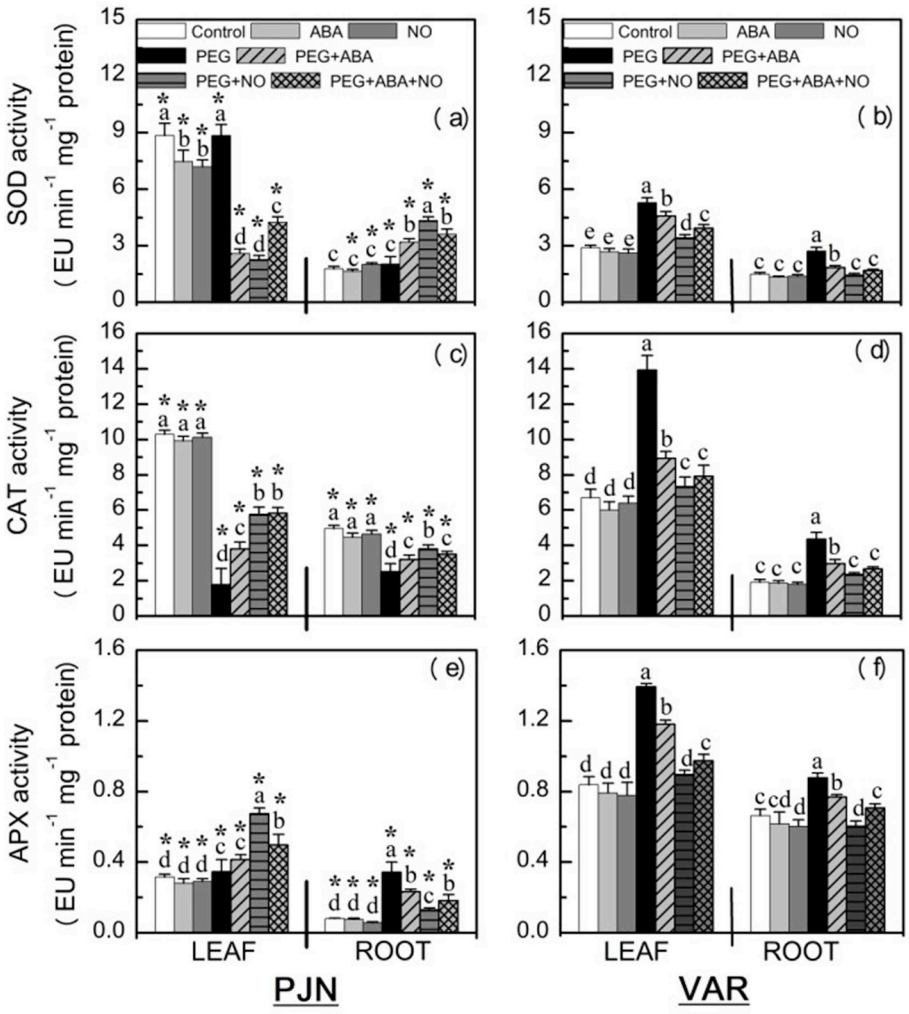

Fig. 3. Superoxide dismutase (SOD) (a, b), catalase (CAT) (c, d) and ascorbate peroxidase (APX) (e, f) activities measured in leaves and roots of 7 days old seedlings of two Indian mustard cultivars, Pusa Jagannath (PJN) and Varuna (VAR) after 96 h exposure under 10% PEG-induced drought stress, with or without abscisic acid (ABA, 10 μM) and sodium nitroprusside (NO, 100 μM). The vertical data bars represent mean values ± SE of 3 replicate plants from three independent experiments. Values sharing different small letters and asterisk (*) above the bars indicate significant difference among treatments within a particular cultivar and among cultivars at a particular treatment, respectively at 5% significance level.

PEG + NO + ABA treatment, respectively compared to control as well as PEG. Cultivar VAR was entirely different from cultivar PJN to SOD activity (Fig. 3b). In both VAR-leaves and roots, SOD activity was elevated by ~82% in leaves and ~84% in roots, after treatment with 10% PEG. PEG-induced enhancement in SOD was managed upon PEG-combination treatments, which dropped the SOD level slightly higher to the control (in leaves) or almost up to control (in roots).

Similar to SOD activity, CAT activity was different in both PJN and VAR cultivars. As depicted in Fig. 3c and d, CAT activity decreased in PJN cultivar by ~82% in leaves and ~49% in roots under PEG alone, with respect to their controls. However, the combination treatments i.e., PEG + ABA, PEG + NO and PEG + NO + ABA improved CAT activity by ~114%, ~225%, and ~229% respectively, in leaves and ~27%, ~50%, and ~38% respectively, in roots compared to PEG alone. The effect of combination treatments was statistically equal to one another (Fig. 3c). Contrastingly, in the case of VAR cultivar, PEG treatment increased the activity of CAT by ~107% and ~126% of control plants in leaves and roots, respectively, indicated a tolerance mechanism with this cultivar (Fig. 3d). Furthermore, the application of PEG + ABA, PEG + NO, and PEG + NO + ABA maintained the CAT activity by reducing the CAT level near to that of respective controls by decreasing ~35%, ~47%, and ~43%, respectively in leaves, and ~31%, ~45%, and ~38%, respectively in roots compared to PEG alone.

The APX activity also differed in both cultivars imposed to PEG-stress (Fig. 3e and f). In case of PJN cultivar, leaf-APX activity was unchanged, where on the other hand the root-APX activity increased approximately ~380% in PEG alone in comparison to their controls. Contrastingly, in the case of VAR cultivar, APX activity in both leaves and roots increased by ~66% and ~32% upon PEG treatment, compared to respective controls. The PEG-induced unchanged APX activity improved by ~11%, ~95%, and ~23% in PJN-leaves, whereas, PEG-induced higher APX activity and was maintained by reducing ~52%, ~66%, and ~39% in PJN-roots, ~15%, ~35%, and ~30% in VAR-leaves, and ~12%, ~31%, and ~19% in VAR-roots, when PEG-stressed seedling was supplemented with ABA and NO, either separately or in combination (PEG + ABA, PEG + NO and PEG + ABA + NO, respectively) (Fig. 3e and f).

The treatment of PEG alone enhanced the activity of GR in both *Brassica* cultivars, with more in leaves than roots (Fig. 4a and b). Seedlings treated with PEG solution were recorded with the significant increase of ~72% and ~111% in leaves, and ~101% and ~71% in roots of PJN and VAR cultivars, respectively over their controls. PEG solution co-applied with ABA (PEG + ABA) or NO (PEG + NO) or combination (PEG + ABA + NO) of both to the seedlings showed the pronounced effect but statistically equal to one another, and therefore reverted the PEG-induced GR activity level almost equal to control (in PJN leaves and VAR-roots) or slightly higher to control (in VAR-leaves), or slightly lower to control (in PJN-roots).

Overall, PEG + NO proved good, then followed by PEG + ABA + NO and PEG + ABA for their ability to both either improve or maintain the unchanged and unbalanced level of SOD, CAT, APX, and GR activities accordingly to mitigate $O_2^{-\bullet}$ and H_2O_2 induced stress.

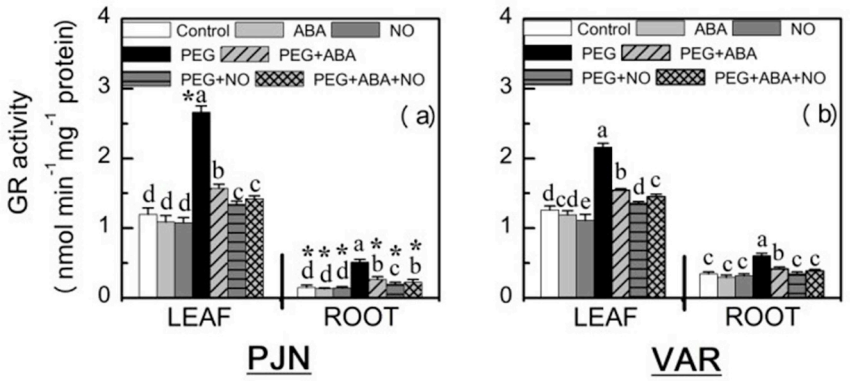

Fig. 4. Glutathione reductase (GR) (a, b) activity measured in leaves and roots of 7 days old seedlings of two Indian mustard cultivars, Pusa Jagannath (PJN) and Varuna (VAR) after 96 h exposure under 10% PEG-induced drought stress, with or without abscisic acid (ABA, 10 μM) and sodium nitroprusside (NO, 100 μM). The vertical data bars represent mean values ± SE of 3 replicate plants from three independent experiments. Values sharing different small letters and asterisk (*) above the bars indicate significant difference among treatments within a particular cultivar and among cultivars at a particular treatment, respectively at 5% significance level.

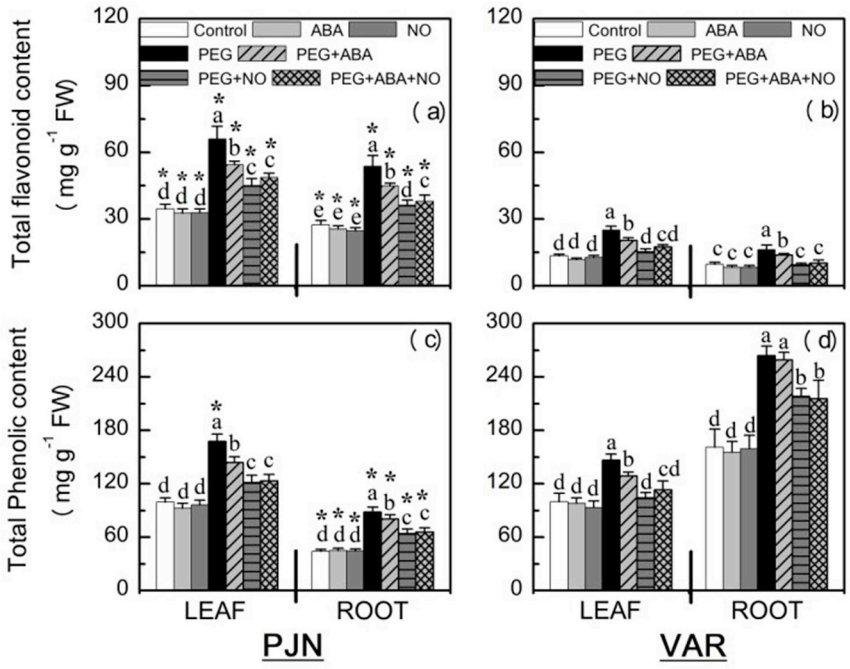

Fig. 5. Total flavonoid (a, b) and phenolic (c, d) content measured in leaves and roots of 7 days old seedlings of two Indian mustard cultivars, Pusa Jagannath (PJN) and Varuna (VAR) after 96 h exposure under 10% PEG-induced drought stress, with or without abscisic acid (ABA, 10 μM) and sodium nitroprusside (NO, 100 μM). The vertical data bars represent mean values ± SE of 3 replicate plants from three independent experiments. Values sharing different small letters and asterisk (*) above the bars indicate significant difference among treatments within a particular cultivar and among cultivars at a particular treatment, respectively at 5% significance level.

4.6. Effective response of non-enzymatic antioxidants to NO or/and ABA against PEG-induced drought stress

The TFC was more in the cultivar PJN than the VAR in both leaves and roots (Fig. 5a and b). Independent PEG treatment significantly increased TFC in leaves and roots, where the increase was ~90% and ~95% in PJN, and ~84% and ~67% in the VAR cultivar, respectively over their controls. Combination treatments reduced/dropped-down the PEG-TFC level, with a remarkable reduction under PEG + NO (~31% and ~36% in PJN-leaves and roots, and ~39% and ~43% in VAR-leaves and roots, respectively) and PEG + ABA + NO (~26% and ~32% in PJN-leaves and roots, and ~30% and ~36% in VAR-leaves and roots, respectively) rather than with PEG + ABA (~17% and ~16% in PJN-leaves and roots, and ~18% and ~15% in VAR-leaves and roots, respectively). Furthermore, the reduction in PEG-induced TFC content reached almost the same level of control in the VAR cultivar, whereas, it was higher to control level in PJN cultivar at combination treatments, indicating that VAR cultivar was more efficient than PJN to maintain or recover from stress condition.

The AsA was observed to accumulate more in leaves than roots of both *Brassica* cultivars. As compared to control, non-significant increase (~3%) in AsA was recorded at 10% PEG alone treatment in cultivar PJN-leaves. Whereas, a significant increase with ~77% in roots AsA at 10% PEG alone over their control treatment (Table 2). In the case of VAR, an increase of ~95% in roots AsA and ~51% in leaves AsA was observed at the same PEG concentration over control (Table 2). Regarding PEG-combination treatments, the PEG + ABA, PEG + NO and PEG + ABA + NO treatments reduced AsA by ~16%, ~43%, and ~37% in PJN-leaves, and ~20%, ~28%, and ~25% in PJN-roots, respectively over independent PEG treatment. In case of VAR, PEG + ABA, PEG + NO and PEG + ABA + NO treatments reduced AsA content by ~17%, ~31%, and ~26% in leaves, and ~25%, ~48%, and ~42% in roots, respectively over PEG alone.

The AC was greater in leaves than in roots of both cultivars PJN and VAR (Table 2). An increase in anthocyanin accumulation of ~64% in leaves, and ~71% in the roots of PJN cultivar, while ~47% in leaves and ~31% in the root of VAR cultivar was observed in response to PEG alone, with their controls. Regarding combination treatments, PEG + ABA, PEG + NO, and PEG + ABA + NO decreased the PEG-accelerated high AC to ~13% (leaves) and ~15% (roots), ~27% (leaves) and ~31% (roots), and ~22% (leaves) and ~23% (roots), respectively compared to PEG-stressed plants in PJN cultivar. In the VAR cultivar, PEG + ABA, PEG + NO and PEG + ABA + NO reduced AC by ~17%, ~29% and ~21% in leaves, and ~6%, ~22% and ~14% in roots, respectively over independent PEG treatment. It indicated that the VAR-leaves and roots were almost recovered from PEG-affected anthocyanin content (Table 2).

Table 2
Ascorbic (AsA) and anthocyanin (AC) content measured in leaves and roots of 7 days old seedlings of two Indian mustard cultivars, Pusa Jagannath (PJN) and Varuna (VAR) after 96 h exposure under 10% PEG-induced drought stress, with or without abscisic acid (ABA, 10 μM) and sodium nitroprusside (NO, 100 μM). Values represent mean ± SE of 3 replicate plants from three independent experiments. Values sharing different small letters and asterisk (*) indicate significant difference among treatments within a particular cultivar and among cultivars at a particular treatment, respectively at 5% significance level.

	AsA (μmol g^{-1} fw)				AC (mg 100 g^{-1} fw)			
	PJN (Leaves)	PJN (Roots)	VAR (Leaves)	VAR (Roots)	PJN (Leaves)	PJN (Roots)	VAR (Leaves)	VAR (Roots)
Control	435.8 ± 11.1$_c$*	74.5 ± 3.7$_d$*	583.5 ± 15.8$_d$	164.6 ± 8.9$_d$	2.67 ± 0.12$_e$	1.60 ± 0.08$_c$	2.31 ± 0.08$_d$	1.38 ± 0.04$_c$
ABA	426.7 ± 19.9$_c$*	71.1 ± 1.6$_d$*	575.7 ± 25.6$_d$	152.5 ± 9.2$_d$	2.54 ± 0.14$_e$	1.48 ± 0.07$_c$	2.19 ± 0.07$_d$	1.29 ± 0.04$_c$
NO	407.3 ± 22.8$_c$*	72.9 ± 1.4$_d$*	572.5 ± 28.6$_d$	148.7 ± 8.8$_d$	2.41 ± 0.15$_e$	1.40 ± 0.10$_c$	2.25 ± 0.08$_d$	1.25 ± 0.08$_c$
PEG	449.2 ± 30.4$_c$*	132.3 ± 11.2$_a$*	884.2 ± 20.6$_a$	321.2 ± 20.5$_a$	4.39 ± 0.20$_a$*	2.74 ± 0.21$_a$*	3.41 ± 0.08$_a$	1.81 ± 0.12$_a$
PEG + ABA	525.1 ± 42.2$_b$*	105.5 ± 6.0$_b$*	728.1 ± 26.1$_b$	276.3 ± 11.4$_b$	3.78 ± 0.17$_b$*	2.31 ± 0.14$_a$*	2.83 ± 0.20$_b$	1.70 ± 0.10$_a$
PEG + NO	645.2 ± 35.7$_a$	94.9 ± 2.4$_c$*	609.5 ± 29.5$_c$	190.3 ± 14.4$_c$	3.20 ± 0.10$_d$*	1.87 ± 0.16$_b$*	2.41 ± 0.07$_c$	1.40 ± 0.08$_{bc}$
PEG + ABA + NO	616.6 ± 25.7$_a$	98.4 ± 4.1$_c$*	654.2 ± 33.1$_{cd}$	212.9 ± 20.1$_c$	3.41 ± 0.13$_c$*	2.09 ± 0.12$_b$*	2.68 ± 0.07$_c$	1.54 ± 0.07$_b$

5. Discussion

Initially, amongst various physiological and biochemical components, the change in LRWC was observed as an important parameter that evaluates the plant tolerance. Declined LRWC in both *Brassica* cultivars (cv. PJN and VAR) during the PEG treatment indicated the loss of water content from the plant cells and faced osmotic disturbance, as reported by other studies [39,40]. However, LRWC was restored by the exogenous application of independent and combined NO and ABA under PEG which could be due to increased endogenous ABA or/and NO content, as evidenced by partially or fully improved LRWC in PJN and VAR, respectively. Differential improvement in LRWC may be due to a significant variation in accumulation of endogenous ABA and NO content. During PEG and PEG-combination treatments the VAR cultivar accumulated less ABA and NO in their leaves and roots which is associated to drought resistance/tolerance, whereas cultivar PJN accumulated more ABA or/and NO which is considered sensitive to drought stress. The result supported by earlier studies reported genotypic variation for capacity to ABA and NO accumulation and variable recovery of relative water content [41–45]. The difference in ABA and NO accumulation (less in VAR and more in PJN) as a result of PEG and PEG-combination treatments may point to the reason that tolerant genotypes react quicker to changes/maintain the endogenous ABA or/and NO levels, whilst it continued to increase in the sensitive genotypes, as suggested in time-course experiments in wheat and rice [46,47]. A predominant inhibition in total Chl content of both *B. juncea* cultivars indicates oxidative stress damage, similar to earlier reports in other plants such as cotton, maize etc [40,48]. The Chl loss was supposed to be due to the drought-induced changes in plant-water relations (i.e., LRWC), leading to a disruption in the chloroplast's biochemistry (such as photo-oxidation and chlorophyll degradation) [49]. A recovery in dramatic loss of Chl content after application of NO or/and ABA in plants subjected from low to severe drought stress have been reported in many studies [39,50,51]. The Caro pigment is a C40 intrinsic lipophilic component of the chloroplast that acts as a first-line non-enzymatic antioxidant that quenches singlet active oxygen species ($^1O_2$) as well as its source i.e., triplet excited chlorophyll ($^3Chl^*$) thereby it protects from the formation of $O_2^{-\bullet}$ radical and related lipid peroxidation [52]. Present data showed a decreased Caro and indicated overproduction of ROS is evident from increased $O_2^{-\bullet}$ content in plants exposed to PEG treatment. However, the level of Caro was enhanced when PEG-treated seedlings were supplied with NO or/and ABA in both cultivars, and high Caro levels have been considered to be a measure of drought tolerance as suggested by Zhou et al. [51] and Cechin et al. [53]. In PEG-induced drought response, leaf total protein content decreased in both *Brassica* cultivars, which can be similarly observed in drought-stressed *Phoebe bournei and Vitisvinifera* [54,55]. However, the exogenously applied NO and/or ABA ameliorated the PEG-drought stress by an increase in the leaf total protein content. Similar observation reported by Zheng et al. [56], that NO donor increased the total protein content in wheat seedlings subjected to salt stress. MDA as a poisonous end product of free radicals, attacking lipids is always considered as a suitable biomarker/bio-indicator/bio-index of lipid peroxidation during various abiotic stresses including drought. Increased MDA content in both organs (leaves and roots) in both cultivars under PEG stress indicated the extent of ROS damage to membrane lipids which is similar to other reports [44,51,53,54]. Increase in MDA level was correlated with lack of induction in optimum CAT and APX $OH^{\bullet}$ scavenging activity, and resulted in excess generation of $O_2^{-\bullet}$ and H_2O_2. Suppression in MDA level by NO or ABA administration in PEG stress has been supported by other studies [15,51,53]. However, in the present study, NO showed the best protective role, whereas, ABA had the same function when it was additionally supplied with NO under PEG. It is also evident from the increased NO content as a result of PEG + ABA + NO greater than PEG + ABA, and suggested that the increased NO level probably required through reacting with scavenging lipid peroxyl radicals (indicated antioxidant property itself) or inhibiting the peroxidation enzymes by stimulating antioxidant enzyme (CAT and APX) activity (indicated signalling molecule itself), as documented by Shi et al. [57] and Yildiztugay et al. [58]. Furthermore, the level of MDA remained higher from control (cv. PJN) or almost equal to control (cv. VAR) as a result of combination treatments suggested their varietal differences, but a feature of drought tolerance [42,59]. High $O_2^{-\bullet}$ and H_2O_2 level is also the characteristic feature of oxidative stress, and categorized as stress indicator/marker. Excess accumulation of $O_2^{-\bullet}$ and H_2O_2 was prevented by the application of ABA or/and NO with PEG. Our findings are supported by the authors working on drought stress related to ABA [15,60] and NO [58]. Decreased $O_2^{-\bullet}$ and H_2O_2 content were correlated with efficient and coordinated role of SOD, CAT and APX together. The Cys, an end-product of sulpfhdryl moiety, is mainly needed for synthesis of sulphur-enriched compounds like glutathione, iron-sulphur clusters etc, for their anti-stress activity. In the present study, PEG treatment increased the Cys accumulation in leaves and roots of both cultivars, which is similar to other abiotic stresses such as salinity [61]. In addition, independent NO or combined with ABA under PEG stress displayed their best role to maintain the PEG-induced higher Cys level by reaching towards their control level. It was associated with endogenous NO content as a result of PEG-combination treatments, ultimately increased NO efficacy to covalently attach with thiol (-SH) group of Cys residue to form S-nitrothiol (SNO) [62]. Thus, NO may have effectively blocked the lipid and protein peroxidation, as evidenced from the lower level of MDA and increased protein content upon PEG + NO and PEG + ABA + NO in the present study. The Pro is protective compatible solute and acts as an osmoprotectant. It also serves as a potent and non-enzymatic antioxidant by scavenging the free radicals [59]. A significant concentration of Pro has been considered to facilitate water absorption to maintain the osmotic water potential and turgidity close to the cell optimum level. Hence, it protects proteins and other macro-molecule structure to damage during hyper-osmotic stress induced by drought condition [63]. Under stress

condition, increased Pro accumulation rather than total Pro content has been suggested as a sign of drought tolerance [64], as shown in many plants, that tolerant plant accumulated more Pro than sensitive ones upon stress [42,44]. This trend is also consistent with our present results, which showed that PEG-stimulated increase in Pro content was higher in VAR cultivar than PJN cultivar, for both leaves and roots. Furthermore, roots as a primary organ that sense water stress at an early stage, showed increased Pro level, compared to leaves in both cultivars, also observed for another plant like chickpea [65]. Surprisingly, the role of NO and ABA application under PEG-stress was found to be regulated differentially according to organ/tissue, which could be related to different genetically determined ability to drought tolerance [66]. As such, drought-stressed PJN leaves induced Pro accumulation, which increased further on the treatment of NO or ABA with PEG, as also reported earlier [51,53]. However, this trend was not supported to PJN-root and VAR-leaves and roots, where treatments of NO or ABA either alone or in combination stimulated PEG-produced high Pro level to decrease down near to optimum level. A reason may be corroborated with a fact, that PJN-leaves were required free proline to further accumulate in stress adaptive manner, whilst the PJN-roots and VAR-leaves and roots maintained Pro accumulation in the stress tolerance manner, as evidenced by partial recovery and complete recovery in LRWC in PJN and VAR cultivars, respectively. It was in agreements with the studies, indicated that NO can induce a significant increase or decrease in Pro accumulation in different plant organ in response to various abiotic stresses including dehydration/drought stress [67,68], depending on the concentration of exogenous applied/endogenously produced NO or/and ABA under drought stress [53,69]. Furthermore, all these discrepancies of results might be related to many factors such as species and their genotypes, severity and duration of stress involved. SOD is the first line enzymatic antioxidant that plays a crucial role by scavenging first ROS radical i.e., $O_2^{-\bullet}$(superoxide anion), and convert it into comparatively less toxic ROS (H_2O_2). CAT may act in line after SOD dismutation of superoxide anion radical, thus eliminates the H_2O_2 by converting it into H_2O and O_2. APX performs the same function as CAT does. As a central component of Ascorbate-Glutathione (Asc-Glu) cycle, APX enzyme uses two molecules of ascorbic acid to scavenge H_2O_2, and convert into H_2O along with DHA. In the present study, activities of SOD, CAT and APX differed in response to drought stress ranging from no effect, an increase or decrease in their activity, which was also evidenced by Cechin et al. [53], Ozfidan et al. [60], Tanou et al. [70], and Uzilday et al. [71]. The reason may be due to contrast behaviour of different plant cultivars with different plant organs (leaves and roots) to stress condition, for allowing towards tolerance response. Many studies showed an increase or decrease in antioxidant activities, depending on the duration of stress imposed, plant species, state of the plant, plant age etc. [44,72,73]. It has been documented that NO or ABA increases antioxidant activities, and mediated drought tolerance [8,44,53,60]. In the present study, the activities were differential upon NO and/or ABA treatments with PEG and mediated protection either by enhanced or maintained SOD, APX and CAT activity, except in PJN-leaves (decreased SOD). This decreased SOD activity was, however negatively correlated with LRWC, Chl, MDA and $O_2^{-\bullet}$ level, and indicated that the lack of scavenging capacity of SOD even upon the application of NO or/and ABA to PEG treatment could not affect the plant physiology. The reason was associated with the availability of enhanced activities of non-enzymatic antioxidants (such as TFC, AC and AsA), that might have replaced the function of SOD, and scavenged $O_2^{-\bullet}$ directly at the same PEG-combination treatments, as evidenced by lowered MDA and $O_2^{-\bullet}$ level, as compared to PEG alone. GR is the flavo-protein based enzymatic antioxidant involved in H_2O_2 removal through the glutathione-ascorbate (GSH-ASC) cycle. The main function of GR is to catalyze a reaction using NADPH and converts oxidized glutathione (GSSG) to reduced glutathione (GSH), and maintain GSH level in plant cells. GR activity was found to be increased under PEG-stimulated drought stress condition, and our result is similar to Pyngrope et al. [73], reported an increase in GR activity in both sensitive and tolerant rice seedlings subjected to PEG-simulated drought stress. The involvement of the secondary metabolites (non-enzymatic antioxidants) such as phenolic compounds (TPC and TFC), AC and AsA have also been considered to play the important role in diminishing the H_2O_2 and the singlet oxygen generated in response to oxidative stress condition. The present study showed an enhanced concentration of all these non-enzymatic antioxidants accelerated after PEG-induced drought stress, which were similar to the results documented [74–77]. Such stress-related enhancement may occur to cope with drought toxicity, particularly in that condition where enzymatic antioxidants could not activate or decrease under stress condition. In the present study, the leaves and roots exposed to PEG-stress led to an increase in TPC, TFC, AsA, and AC, the reverse being observed with decreased or inactivated SOD, CAT and APX activities in PJN cultivar. Our outcome is in accordance with some previous studies, which showed the accumulation of antioxidant flavonoid derivatives and anthocyanin is inversely correlated to SOD and CAT activities under abiotic stress [78,79]. ROS homeostasis might be, therefore, mediated by the accumulation of secondary metabolites when the pool of enzymatic antioxidants is declined as a consequence of stress condition. It suggested that, rather, enzymatic antioxidants represent the first-line of ROS defense system, but their action needs to be complemented by another non-enzymatic antioxidant ROS scavenging system (secondary metabolites). The present study showed a notable maintenance in TPC, TFC, AsA, or/and AC levels near optimum (PJN) or control (VAR) upon the application of NO or/and ABA with PEG. This is presumably due to the key functional role of NO independent or together with ABA rather than ABA alone to PEG-stress to keep the concentration of ROS (such as $O_2^{-\bullet}$ and H_2O_2) at the non-lethal level, and sending a signal to these metabolites so that they don't accumulate/synthesize anymore. However, the mechanism(s) by which NO independent, in coordination with other hormones such as ABA, mediates functions of secondary metabolic compounds often remains unclear, particularly under drought stress. In addition, accumulation of these metabolites at differential concentration appears to be associated with genetic basis of natural variation in both the cultivars of *Brassica*.

6. Conclusions

The gathered data suggests that NO and ABA independent as well as concomitant action during PEG-induced drought stress could attenuate oxidative stress in both cultivars, either by mediating/modulating or maintaining antioxidant enzymes and secondary metabolic compounds. Numerous reports are available on antioxidant protections during oxidative stress initiated by drought. Contrary, in case of PJN cultivar in the present study, the induction of enzymatic antioxidants activity did not appear to be the major player as depicted by lowered or not pronounced SOD, CAT and APX activities (mainly in leaves), under PEG-imposed drought stress. However, this cultivar was able to improve its tolerance mechanism as indicated by pronounced activities when NO and/or ABA were supplied with PEG. Regardless, the compatible solutes and non-enzymatic antioxidants (including proline, phenolics, flavonoid, ascorbic acid etc) played well and confirm protection from ROS-induced oxidative stress initiated by PEG treatment in both cultivars. The present study supports the established belief, that during lack of pronounced activity of enzymatic antioxidants including SOD, CAT and APX, the non-enzymatic antioxidants may have an equivalent role towards stress alleviation. In fact, non-enzymatic antioxidants were evolved as back-up antioxidants system towards tolerance to drought stress in PJN cultivar. Although, it is important for different plant organs to work with a well-coordinated manner, and regulate combined function/action of a whole operating antioxidant system to have best tolerance status against stress condition, as found in the VAR cultivar. Of course, functions of all these antioxidants related to the defense role in PEG-induced drought stress require further validation through their

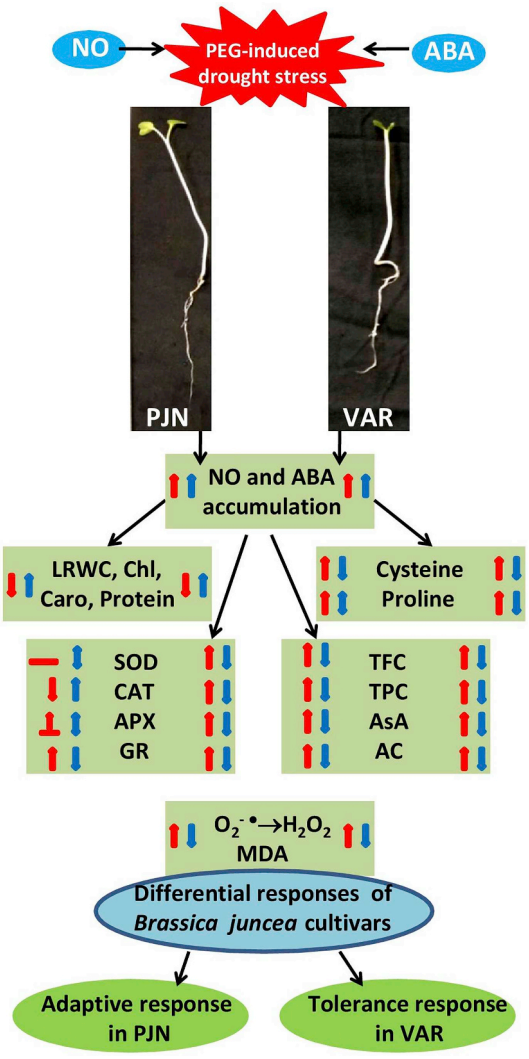

Fig. 6. Diagrammatically representation of a model showing the role of independent and combined NO and ABA action in mediating the drought-induced oxidative stress responses (adaptation and tolerance), is associated with differentially regulated physiological and biochemical activities in two cultivars of *Brassica juncea* (cv. PJN and VAR). The upwards (⇑), downwards (⇓), non-directional —, up-down (⇕) and upwards-non-directional ((⊥)) arrows indicate an increase, decrease, no change, increase-decrease (leaves-roots), and increase-no change (leaves-roots) responses, respectively mediated by PEG-induced drought stress as compared control (red arrow) and PEG combined with NO or/and ABA, compared to PEG alone (blue arrow).

gene expression analysis in both cultivars. PEG supplied with NO alone or NO with ABA confronted PEG-induced drought stress much more efficiently than by PEG with ABA alone suggested the balanced endogenous change in NO levels and ABA level as a result of exogenous PEG + ABA, PEG + NO, and PEG + NO + ABA are interacted to mitigate the oxidative stress in different *Brassica* cultivars. Based on the present results, we outlined a model for the physiological and biochemical mechanism of NO-ABA mediated PEG-induced oxidative responses (i.e., adaptive mechanism in PJN and tolerance mechanism in VAR) which are associated with differential changes in activities of stress markers, stress modulators and stress protectors (i.e., enzymatic and non enzymatic antioxidants) (Fig. 6).

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Author contributions

Sahay S. and Gupta M. conceived the concept and design of the study. Sahay S. and Khan E. contributed to perform the experiment, analysis and interpretation of data. All authors have discussed the results and contributed equally to the final version of the submitted manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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