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Research Article

PUTRESCINE AND ASCORBIC ACID MEDIATED ENHANCEMENT IN GROWTH AND ANTIOXIDANT STATUS OF *ERUCA SATIVA* VARIETIES

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ABSTRACT
The objective of this study was to investigate the effect of plant growth regulators (putrescine and ascorbic acid) on seed germination and seedling growth and various biochemical parameters concerned with antioxidative defense system in *Eruca sativa* varieties with variable seed germination (RTM 1301, RTM 314, RTM 1359 and RTM 1356). Germination percentage, speed of germination, coefficient of germination, mean germination time, shoot and root length, fresh and dry matter and vigor index were significantly affected by the two treatments. Major decline in the electrolyte leakage, malondialdehyde and reactive oxygen species level was recorded when putrescine and ascorbic acid were applied exogenously. Proline content, total phenols, activities of ascorbate peroxidase, catalase, superoxide dismutase and pyrogallol peroxidase increased with treatments. A variety dependent effect was observed. The results of the present study indicate that putrescine and ascorbic acid alleviate the detrimental effects of reactive oxygen species in *E. sativa* by improving the antioxidative defense system.

Key Words: *Antioxidants, Ascorbic Acid, Eruca sativa, Putrescine, Vigor Index*

INTRODUCTION
Eruca sativa Mill. (fam. Brassicaceae), is an important oilseed crop, commonly known as "Taramira" or "Arugula". It is an annual herb, widely distributed across Southern Europe, North Africa, Western Asia and India. Taramira remains greatly valued in many countries of the Mediterranean region such as Italy, Greece and Turkey where it is consumed mainly in salads for their hot pungent taste. Phytochemical analysis of this plant is reported to contain alkaloids, cardiac glycosides, flavonoids, phenolics, glucosinolates, ascorbic acid, saponins and tannins (Barillari *et al.*, 2005; Gulfraz *et al.*, 2011). Traditionally, its use as astringent, diuretic, digestive, emollient, tonic, laxative, depurative, rubefacient and stimulant is well documented (Al-Qurainy, 2010). It has varied medicinal and therapeutic properties including inhibition of tumorigenesis (Lynn *et al.*, 2006), hepatoprotective (Rafatullah *et al.*, 2008) activities and anti-ulcer (Alqasoumi *et al.*, 2008, 2009). The essential oil extracted from seeds of *E. sativa* contains 67 volatile compounds which constitutes 96.52% of the oil (Mitsuo *et al.*, 2002). The seed oil of *E. sativa* has various pharmacological efficacy and potential bio-active compounds as compared to different aerial and root plant extracts (Khoobchandani *et al.*, 2010).

Plant growth regulators (PGRs) are organic compounds that play an important role in various physiological and molecular processes of plants (Al-Whaibi *et al.*, 2012). Polyamines (PAs) are small aliphatic nitrogenous compounds, including putrescine (Put), spermidine (Spd) and spermine (Spm) present in all living organisms (Slathia *et al.*, 2012). PAs are known to play significant role in the regulation of cell division, cell differentiation, embryogenesis, cellular ionic environment, reproductive organ development and root growth (Hussain *et al.*, 2011). They are also considered as a secondary messenger in signaling pathways (Kusano *et al.*, 2008). PAs protect the membranes from oxidative damage as they act as free radical scavengers (Verma and Mishra, 2005). Ascorbic acid is a crucial substance in the network of plant antioxidants, plays multiple roles in plant growth, functioning in cell division, cell wall expansion, and other developmental processes (Behairy *et al.*, 2012). It acts as a primary substrate in the cyclical pathway for detoxification and neutralization of superoxide radicals

($O_2^{\bullet}$), singlet oxygen ($^1O_2$) and hydroxyl radical ($^{\bullet}OH$) (Ekmekci and Karaman, 2012). The antioxidant activity of AA is associated with resistance to oxidative stress and longevity in plants (Khan *et al.*, 2011). The objective of the present investigation was to determine the effect of putrescine and ascorbic acid on seed germination, seedling growth and biochemical parameters associated with antioxidative defense system.

MATERIALS AND METHODS

Seeds of *Eruca sativa* varieties (RTM 1301, RTM 314, RTM 1359, RTM 1356) were secured from the Department of Plant Breeding & Genetics. S.K.N. College of Agriculture, Jobner. Uniformly selected seeds were sterilized with 5 % sodium hypochlorite (NaClO) for 2 min and then repetitively washed under running tap water followed by distilled water. Ten seeds were sown in each Petri dish and incubated in BOD incubator set at 25°C.

For each treatment sterilized seeds were soaked daily with putrescine (2.5 mM) and ascorbic acid (2 mM). Seed germination parameters were computed regularly up to 5 days and seedling growth parameters were made after 5 days of sowing.

Different biochemical traits were analyzed after 6 days of sowing. Each treatment was replicated three times and the data represented as average values.

Seed Germination Parameters

Percentage Germination (%)

The per cent germination was recorded daily up to five days. Seeds were taken as germinated when radicle had emerged from seed coat.

$$\text{Percentage germination (\%)} = \frac{\text{Number of germinated seeds} \times 100}{\text{Total number of seeds}}$$

Speed of Germination (SOG)

It is defined as the maximum daily seed germination reached at any time. Number of germinated seeds in each variety was counted daily and the speed of germination was calculated as per the formula suggested by Maguire (1962):

$$\textbf{SOG} = \frac{X_1}{Y_1} + \frac{X_2 - X_1}{Y_2} + \ldots\ldots\ldots + \frac{X_n - (X_n - 1)}{Y_n}$$

Where, X1, X2 and X_n = number of germinated seeds on 1st 2nd and nth day, respectively

Y1, Y2 and Y_n = number of days from sowing to 1st, 2nd and nth count, respectively.

Coefficient of Germination (COG) (%)

The coefficient of germination is an index of rapidity or the rate of germination of seeds. It was calculated using the formula given by Copeland (1976):

$$\textbf{COG}\ (\%) = \frac{A_1 + A_2 + \ldots\ldots\ldots A_n}{A_1T_1 + A_2T_2 + \ldots\ldots\ldots A_nT_n} \times 100$$

A = number of seeds germinated; T = time (days) corresponding to A; n = number of days to final count

Mean Germination Time (MGT) (Ellis and Roberts, 1981):

$$\textbf{MGT} = \frac{\Sigma\, d_i}{\Sigma\, n_i}$$

n_i = number of germinated seeds in every count; d_i = day of counting

Seedling Growth Parameters

Root and Shoot Length

Five normal seedlings were selected at random and used for measuring root and shoot length.

Mean root and shoot length was expressed in centimeters.

Fresh and Dry Matter

Five seedlings from each Petri dish were taken and their fresh matter was recorded after 5th day. For dry matter seedlings were oven dried overnight at 80°C. The dried seedlings were weighed to estimate the dry matter and the mean values were expressed in g.

Seedling Vigor index (VI)

The seedling vigor index was computed by adopting the method suggested by Abdul-Baki and Anderson (1973) and expressed as an index numbers.

Seedling Vigor Index = [Root length (cm) + Shoot length (cm)] × Germination (%)

Biochemical Parameters

Electrolyte Leakage (EL)

Electrolyte leakage analysis was determined according to the method of Zhang *et al.*, (2006). Percentage of electrolyte leakage was estimated from the equation:

EL (%) = (Xi / Xi + Xt) × 100

Malonaldehyde (MDA)

The level of lipid peroxidation was determined as the content of malondialdehyde (MDA), by the method of Heath and Packer (1968). The absorbance of supernatant was read at 532 nm. MDA content was calculated by the extinction coefficient of 155 mM^{-1} cm^{-1} expressed as per gram of fresh weight.

Total Phenols

The spectrophotometeric method of Swain and Hills (1959) was used for the determination of total phenols. In the extract Folin-Ciocalteu reagent was added and content shaken vigorously and added sodium carbonate solution. The test tubes were kept in dark for one hour after which absorbance was measured at 725 nm against 80 % ethanol as a blank.

Proline Content

The proline content was estimated by the method of Bates *et al.*, (1973). The reaction mixture consisting of 2 ml supernatant, 2 ml acid ninhydrin and 2 ml of glacial acetic acid was boiled at 100°C for 1 hr to develop colour. After termination of the reaction in ice bath, the reaction mixture was extracted with 4 ml toluene and the absorbance was read at 520 nm against toluene as a blank.

Ascorbate Peroxidase (APX)

APX activity was determined based on the oxidation of ascorbate as a decrease in absorbance at 290 nm (Nakano and Asada, 1981). The reaction mixture containing 50 mM phosphate buffer, 0.1 mM EDTA, 0.5 mM ascorbic acid, and enzyme extract.

The change in A290 was recorded at 30 seconds intervals after addition of H_2O_2. The rate constant was calculated using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

Catalase (CAT)

Catalase activity was determined by the method of Aebi (1984). The reaction was initiated by adding H_2O_2 and enzyme activity was determined following degradation of at H_2O_2 at 240 nm for 2 min. The catalase activity is expressed as μM $ml^{-1}min^{-1}mg^{-1}$ protein.

Superoxide Dismutase (SOD)

The activity of SOD was estimated by the method of Dhindsa *et al.*, (1981). The photo-reduction of NBT resulted in the formation of purple formazon.

The absorbance was read at 560 nm and the total SOD activity of the sample was estimated by measuring its ability to inhibit the photochemical reduction of nitro-blue-tetrazolium (NBT). The activity of SOD is expressed as change in OD $min^{-1}ml^{-1}mg^{-1}$ protein.

Pyrogallol Peroxidase (PPX)

PPX activity was assayed by the method of Kar and Mishra (1976). Reaction mixture contained 200 mM sodium phosphate buffer (pH 7.5), 0.1 M pyrogallol, distilled water and crude enzyme extract at 4°C. This was incubated for 5 min at 25°C after which the reaction was stopped by adding H_2SO_4. The amount of purpurogallin formed was determined by taking the absorbance at 420 nm against buffer as blank. The enzyme activity is expressed in μg g^{-1} FW.

Reactive Oxygen Species (ROS)

ROS production was measured as described by Able *et al.,* (1998). The reaction mixture contained 50 mM K-phosphate buffer (pH 7.8), 0.5 mm XTT and supernatant. The reaction of XTT was determined at 470 nm for 3 min. ROS production is calculated by using extinction coefficient of 2.163104 $M^{-1}cm^{-1}$.

RESULTS AND DISCUSSION

The four varieties specified above were used to compare different seed germination, seedling growth parameters and biochemical parameters associated with antioxidative defense system and effect of two PGRs (Put and AA).

Seed Germination and Seedling Growth Parameters

The effect of Put and AA on seed germination and seedling growth parameters is shown in tables 1 and 2. The obtained results showed that Put enhanced seed germination parameters (GP, SOG, COG) and seedling growth parameters (SL, RL, FM, DM, VI) except MGT. It has been reported that Put stimulates the germination of seeds in several species, for instance *Oryza sativa* (Prakash and Pratapasenan, 1988), *Hordeum vulgare* (Locke *et al.*, 2000), *Citrullus lanatus* (Korkmaz *et al.*, 2004), *Cucumis melo* (Korkmaz *et al.*, 2005) and *Medicago sativa* (Zeid and Shedeed, 2006). Put was highly effective in RTM 1359 with regards to SL, RL, DM, and VI. Sedaghat and Rahemi (2011) reported that PAs significantly increased hypocotyls- radicle length of germinated seeds in *Pistacia vera*. Effect of exogenously applied polyamines on physiological processes of plants and growth is well documented (Gupta *et al.,* 2003; Farooq *et al.,* 2009).

The present results demonstrate that ascorbic acid increased germination percentage and other parameters except MGT in all the varieties. The maximal germination percentage was 70 % as compared with control in RMT 1359 (table 1). The improved effect of AA on germination percentage was also observed in sunflower and rape seeds (Dolatabadian and Modarressanavy, 2008). Moreover, the positive impact of AA was also reported by Shaddad *et al.,* (1990) and Arab and Ehsanpour (2006). Interestingly, increase was most pronounced pertinent to SOG, SL, RL and VI. AA treatment may influence range of diverse processes in plants, comprising seed germination, membrane permeability, ion uptake and transport (Dolatabadian and Modarressanavy, 2008).

From the data set in table 2 it can be inferred that AA stimulated root and shoot growth and increased fresh and dry matter in all the varieties. Razaji *et al.,* (2012) found that AA increased root and shoot length in treated seeds of *Carthamus tinctorius*. Enhancement of root and shoot length by AA might be due to cell division and differentiation of meristem cells (Liso *et al.,* 1988). The effect of exogenous AA on plant survival is associated with the partial inhibition of a few interactions in production of reactive oxygen species (Shalata and Neumann, 2001).

Biochemical Parameters

The electrolytic leakage (EL) indicated the membrane damage. Exogenously applied Put showed significant decrease in EL in all the varieties of *E. sativa*. The influence of Put in EL indicated their involvement in the stabilization of cellular membranes. Our data for EL agree with that of Amooaghaie (2011) in soybean seeds and their results showed that in comparisons with the water deficit control, putrescine, spermidine and spermine all decreased electrolyte leakage from embryonic axes and suggested protection of membrane integrity.

Lipid peroxidation is used as indicator of oxidative damages caused by various environmental stresses in plants. Reactive oxygen species react with lipid and leads to formation of highly reactive peroxyl radical, which in turn starts chain propagation of lipid peroxidation. The results showed decline in MDA content with Put when compared with control (table 3). Slathia *et al.,* (2012) observed that put reduced MDA content in *Lycopersicon esculentum.* Several investigators have concluded that, the exogenously applied of PAs reduced the auto-oxidation of membrane lipids and stabilize the membrane in wheat (HuiGuo *et al.,* 2006); and maize (Todorov *et al.,* 1998).

Put treatment increased total phenols and proline content in all the varieties. Proline has been suggested to act as a compatible solute that adjusts osmotic potential of the cytoplasm, and also participates in radicle detoxification and enzyme protection (Ashraf and Foolad, 2007). Plants are able to protect their tissue from harmful effects of reactive oxygen species using various antioxidant enzymes (SOD, CAT, APX and PPX) (Shallan *et al.,* 2012).

In the present investigation we encountered that, exogenously applied Put enhanced the activities of APX, CAT, SOD and PPX as compared with control (table 4). These findings could be supported by the observations that PAs could increase the activities of APX, CAT and SOD in cucumber under salt stressed and controlled conditions (Duan *et al.,* 2007). In cotton plants under drought stress, Put induced all antioxidant enzyme activities and increased total antioxidant capacity (Shallan *et al.,* 2012). Decline in MDA content by Put application could be linked with enhanced activities of CAT, APX SOD and PPX enzymes which successively scavenge ROS species and reduce the negative impact of ROS on membrane damage.

Table 1: Effect of putrescine and ascorbic acid on seed germination parameters

Varieties	Treatment (mM)	Germination Percentage (%)	Speed of Germination	Coefficient of Germination (%)	Mean Germination Time
RTM 1301	**Control**	30	0.58± 0.08	20.69± 2.6	2.16± 0.41
	Put (2.5)	50	1.23± 0.13	22.50± 3.1	1.33± 0.63
	AA (2)	50	1.32± 0.17	23.26± 2.7	1.2±0.32
RTM 314	**Control**	50	1.18± 0.11	22.22± 1.9	1.5± 0.37
	Put (2.5)	60	1.57± 0.09	23.08± 2.8	1± 0.28
	AA (2)	70	1.68± 0.05	22.22± 4.2	1± 0.25
RTM 1359	**Control**	40	0.95± 0.12	20± 3.3	1.8± 0.53
	Put (2.5)	40	1.03± 0.19	22.86± 2.5	1.5± 0.46
	AA (2)	70	1.68± 0.08	22.22± 3.4	1± 0.38
RTM 1356	**Control**	30	0.75± 0.14	20.69± 3.1	2.17± 0.35
	Put (2.5)	50	1.37± 0.2	23.40± 2.3	1.09± 0.42
	AA (2)	60	1.52± 0.22	22.92± 3.9	1.09± 0.47

Values are in terms of mean ± SE after triplicate analysis

Table 2: Effect of putrescine and ascorbic acid on seedling growth parameters after 5 days of germination

Varieties	Treatment (nm)	Shoot length (cm)	Root length (cm)	Fresh matter (g)	Dry matter (g)	Vigor index
RTM 1301	**Control**	1.89± 0.27	1.9± 0.25	0.19± 0.031	0.012± 0.0035	113.7± 1.24
	Put (2.5)	2.43± 0.35	2.15± 0.37	0.208± 0.037	0.022± 0.0027	229± 2.19
	AA (2)	2.5± 0.37	2± 0.24	0.212± 0.027	0.018± 0.0024	225± 2.05
RTM 314	**Control**	1.9± 0.4	1.92± 0.32	0.184± 0.014	0.012± 0.003	191± 2.57
	Put (2.5)	2.4± 0.31	2.1± 0.35	0.213± 0.017	0.017± 0.0037	270± 3.16
	AA (2)	2.53± 0.45	3.87± 0.27	0.223± 0.032	0.02± 0.0041	448± 2.97
RTM 1359	**Control**	2.45± 0.28	2.6± 0.4	0.199± 0.035	0.013± 0.0039	202± 3.25
	Put (2.5)	2.8± 0.37	2.69± 0.21	0.216± 0.024	0.017± 0.0054	219.6± 3.74
	AA (2)	3.8± 0.42	5.4± 0.37	0.231± 0.023	0.019± 0.0042	644± 2.63
RTM 1356	**Control**	2± 0.24	1.63± 0.34	0.184± 0.019	0.013± 0.0034	108.9± 2.23
	Put (2.5)	2.3± 0.32	1.78± 0.24	0.207± 0.03	0.016± 0.0031	204± 1.84
	AA (2)	2.46± 0.47	2.1± 0.29	0.208± 0.027	0.018± 0.0049	273.6± 2.85

Values are in terms of mean ± SE after triplicate analysis

Table 3: Effect of putrescine and ascorbic acid on electrolyte leakage, malondialdehyde, total phenols and proline content after 6 days of sowing

Varieties	Treatment (mM)	Electrolyte Leakage (%)	Malondialdehyde (n mol g^{-1} FW)	Total phenols (µg g^{-1} FW)	Proline (µ mol g^{-1} FW)
RTM 1301	**Control**	48.71± 1.18	31.2± 1.18	3.54± 0.1	2.41± 0.052
	Put (2.5)	37.5± 2.13	30.5± 0.92	3.69± 0.055	2.56± 0.04
	AA (2)	35.49± 3.33	29.7± 1.09	3.75± 0.062	2.69± 0.036
RTM 314	**Control**	43.21± 1.83	33.5± 1.02	3.96± 0.055	2.59± 0.07
	Put (2.5)	32.04± 2.44	32.1± 0.83	4.05± 0.075	2.66± 0.036
	AA (2)	29.61± 2.22	29.3± 0.75	4.12± 0.056	2.8± 0.041
RTM 1359	**Control**	44.16± 1.99	32.9± 1.04	3.9± 0.04	2.77± 0.041
	Put (2.5)	35.08± 2.29	30.7± 1.17	3.98± 0.055	2.89± 0.037
	AA (2)	23.52± 1.93	28.5± 1.15	4.05± 0.051	2.95± 0.058
RTM 1356	**Control**	45.67± 1.84	30.5± 0.98	3.42± 0.075	2.74± 0.036
	Put (2.5)	36.78± 2.24	27.2± 0.32	3.51± 0.03	2.86± 0.035
	AA (2)	28.57± 1.89	25.8± 1.26	3.59± 0.043	2.92± 0.05

Values are in terms of mean ± SE after triplicate analysis

Table 4: shows activities of various enzymatic antioxidants (ascorbate peroxidase, catalase, superoxide dismutase, pyrogallol peroxidase) and reactive oxygen species with Putrescine and ascorbic acid treatment

Varieties	Treatment (mM)	APX (µ mol ml^{-1} min^{-1} mg protein)	CAT (µ mol $ml^{-1}min^{-1}mg^{-1}$ protein)	SOD (Change in OD $min^{-1}ml^{-1}mg^{-1}$ protein)	PPX (µg g^{-1} FW)	Reactive Oxygen Species (µM g^{-1}FW)
RTM 1301	**Control**	0.19± 0.015	17.52± 1.13	0.39± 0.02	0.52± 0.021	77.45± 3.12
	Put (2.5)	0.23± 0.04	17.87± 0.6	0.42± 0.026	0.56±0.026	63.29 ±3.55
	AA (2)	0.25± 0.041	18.05±0.56	0.43± 0.021	0.55±0.023	61.02± 3.82
RTM 314	**Control**	0.21±0.026	19.23± 1.3	0.34± 0.02	0.58±0.032	70.65± 3.71
	Put (2.5)	0.26± 0.04	19.56± 0.63	0.37±0.025	0.62±0.02	57.32 ± 4.19
	AA (2)	0.27± 0.045	19.82± 1.34	0.39± 0.015	0.62±0.024	54.97± 3.15
RTM 1359	**Control**	0.24±0.017	19.68± 1.22	0.36± 0.021	0.49±0.018	79.54± 2.8
	Put (2.5)	0.27±0.03	20.07± 0.98	0.39± 0.026	0.53±0.025	72.36 ± 3.56
	AA (2)	0.3± 0.037	20.3± 1.02	0.41± 0.026	0.52±0.021	54.87± 4.18
RTM 1356	**Control**	0.25±0.017	16.29± 1.53	0.31± 0.027	0.52±0.017	74.69± 2.57
	Put (2.5)	0.29±0.036	16.67±0.69	0.35± 0.023	0.56±0.024	62.35 ± 4.91
	AA (2)	0.31± 0.037	16.72± 0.99	0.35± 0.25	0.58±0.023	56.24± 4.09

Values are in terms of mean ± SE after triplicate analysis

EL in leaves of *E. sativa* decreased markedly in all the varieties when treated with AA (table 3). These results indicated that AA could recover the membrane function. Similar results were observed and previously reported by Tuna *et al.,* (2013) who reported that AA significantly reduced the EL in salt stressed maize plants. MDA is the major end product of lipid peroxidation. The present results revealed the reduction in MDA content in all the varieties with ascorbic acid treatment. Maximum response was observed in RTM 1359 (table 3). Our data for MDA agree with those of Dolatabadian and Modarressanavy (2008) in sunflower and rape seed. Dolatabadian and Jouneghani (2009) showed that ascorbic acid treatment prevented lipid peroxidation and decreased production of malondialdehyde, a final product of peroxidation of membrane lipids, in bean plants. AA also increased total phenolic contents in all the varieties. Leaf proline content in the AA treated seedlings was higher than that of control plants (table 3). Ejaz *et al.*, (2012) observed increase in proline contents of sugarcane plants after AA application under salt stress. Same results have been reported in sorghum (Azooz *et al.*, 2004), maize (Hussein *et al.*, 2007), rice (Gurmani *et al.*, 2006), and tobacco (Celik and Atak, 2012) where exogenous application of several growth regulators increased the proline contents of plants. It has been proposed that plants accumulate higher amounts of proline as a protective strategy against stressful conditions (Turan *et*

al., 2009) and also acts as a component of the antioxidative defense system rather than purely as an osmotic adjustment mediator (Molinari *et al.,* 2007). The activity of APX increased with AA treatment. Similar trend was observed with CAT, SOD and PPX activities (table 4). In sunflower seedlings ascorbic acid treatment increased catalase activity (Dolatabadian and Modarressanavy, 2008). CAT is tetrameric heme containing enzyme that is rich in the glyoxysomes of lipid storing tissues. It converts the toxic O_2, H_2O_2 to water and molecular oxygen, preventing the cellular damage under unfavourable condition (Razaji *et al.,* 2012). Tuna *et al.,* (2013) and Ejaz *et al.,* (2012) observed that exogenously applied ascorbic acid significantly improved the activity of SOD. The formation of reactive oxygen species (ROS) occurs naturally as a by-product of metabolism but environmental stresses increase ROS to toxic levels (Mittler *et al.,* 2004), overcoming protective ROS scavenging mechanisms and resulting in severe damage to cellular structures and cell death (Sharma *et al.,* 2012). Plant can detoxify ROS by up-regulation antioxidant enzymes, such as SOD, CAT, APX and PPX as well as some non- enzymatic antioxidant compounds. The ascorbic acid neutralizes the ROS, defending the plant tissue from harmful effects of ROS and thus improving plant resistance (Behairy *et al.,* 2012).

The present investigation demonstrates the effects of putrescine and ascorbic acid on *Eruca sativa* varieties showing poor germination. Accordingly Put and AA improved GP, SOG, COG, SL, RL, FM, DM and VI. The exogenously applied PGRs enhanced proline content and antioxidant enzymes alleviated reactive oxygen species possibly due to protection of membranes and minimization of oxidative damage. A variety dependent effect was observed with both the PGRs.

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