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RESEARCH ARTICLE

Sodium nitroprusside mediates attenuation of paraquat-mediated oxidative stress in *Eruca sativa* in vitro

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Abstract Paraquat (PQ) causes oxidative stress, the main source of damage in plants subjected to adverse environmental factors. Sodium nitroprusside (SNP), a signaling molecule, alleviates oxidative damage. The present study was carried out to investigate the role of exogenous SNP in the amelioration of PQ-mediated oxidative stress effects on *Eruca sativa* plantlets cultured in MS basal media. Firstly, MS medium supplemented with 6-BA was found to be the best basal medium for seed germination. Then, a rapid micropropagation protocol was designed to produce *E. sativa* plantlets by using nodal segments as explants, and 0.25 mg/L 6-BA in combination with 0.1 mg/L IBA was found to be the most favorable for shoot proliferation of *E. sativa*. Four weeks old plants were applied with or without SNP (100 µM) and exposed to oxidative stress induced by 2.5 µM PQ. The SNP application decreased membrane damage, hydrogen peroxide, and proline contents, and increased relative water, pigments, ascorbate and total phenolic contents, and some antioxidant enzyme activities in seedlings under PQ stress compared to PQ stress alone. These results suggested that exogenous SNP could protect *E. sativa* plantlets propagated in vitro with PQ stress through modulation of proline and phenolics biosynthesis and antioxidant defense system.

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Keywords *Eruca sativa* · Micropropagation · Sodium nitroprusside · Paraquat stress · Antioxidant defense system

Introduction

The paraquat, one of the most widely used herbicides, which catalyzes the production of superoxide radicals ($O_2^{\bullet -}$) is a strong electron acceptor in photosystem I, are converted to hydrogen peroxide (H_2O_2), leading to oxidative stress (Varadi et al. 2000; Moustakas et al. 2016). Paraquat can also cause rapid chlorophyll loss, inhibition of photosynthesis, necrosis, lipid peroxidation, protein denaturation, and electrolyte leakage (Beligni and Lamattina 1999; Verma et al. 2013). Plants have tolerance mechanisms comprising of cellular mechanisms including osmoprotectants, enzymatic and non-enzymatic antioxidant defense systems to protect from the harmful effects of oxidative stress. The enzymatic antioxidant defense system consists of superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPOD), while non-enzymatic defense system consists of compounds such as ascorbate (AsA), carotenoid, and total phenolic (Sharma et al. 2012). Osmotic regulation relates to the synthesis of osmoprotectants such as proline, soluble sugars, sugar alcohols, organic acid, and some protective compounds, thus ensuring the maintenance of water potential in plant cells. Paraquat stress extremely increases the proline level, and this increase in proline is one of the widespread stress responses in plants (Hasanuzzaman et al. 2018). Moreover, secondary metabolites such as phenolics, which are regarded as potent nonenzymatic antioxidants are accumulated in response to paraquat-mediated oxidative stress

and contribute significantly to oxidative stress tolerance (Cvetković et al. 2015).

Sodium nitroprusside (SNP) is used to produce nitric oxide (NO), which is a signaling molecule that plays a role in a variety of physiological responses to biotic and abiotic stresses, can function as a growth (Kováčik et al. 2019). In previous studies, it has been well reported that NO acts a role in improving plant tolerance against salt and metal-induced oxidative stresses (Khator and Shekhawat 2020a, b).

E. sativa is an important industrial oil-yielding crop that can grow in the arid and semiarid regions. The oil content of *E. sativa* ranges from 32 to 37% percent, and it is used as a protein meal supplement (Fagbenro 2004). The leaves of this plant have been found to contain substantial amounts of phytochemicals with strong antioxidant activities (Grami et al. 2018). Also, *E. sativa* production has continuously increased, because of the high demand for volatile oils in the pharmaceutical industry (El-Fadaly et al. 2017). Seed oil is used as hair oil, massage oil, lubricant, vesicant, illuminant, hair oil, and massage oil. It is also used as cow feed, and powdered seeds have antimicrobial properties (Jakhar et al. 2002).

Production of plants with economic value by the micropropagation method has gained a great acceleration in recent years. In addition to the used basal media, the plant growth regulators and concentrations are also important in the micropropagation of these plants. Micropropagation of *E. sativa* plantlets by using cotyledonary segments as explant growing in the India flora was studied by Sharma et al. (2012). Although, some morphological, physiological, and biochemical responses of *E. sativa* exposed to various stresses have been well-documented (Ozdener et al. 2010; Mahawar et al. 2018; Santiago et al. 2020), no attempts have not yet been made on its stress tolerance mechanisms from *E. sativa* plantlets propagated in vitro. It has been well reported that NO has a role in improving plant tolerance against salt and metal-induced oxidative stresses (Khator and Shekhawat 2020a, b). There were few studies on the function of NO in alleviating PQ toxicity in various plants (Morán et al. 2010; Hasanuzzaman et al. 2018), while the role of SNP in tolerance to paraquat stress of micropropagated *E. sativa* plantlets has not been clarified yet. The aim of the present study is to determine an influential procedure for the micropropagation of the seedlings of *E. sativa* by using nodal segments as explants, and to clarify the role of SNP in tolerance mechanisms of *E. sativa* seedlings under PQ-mediated oxidative stress.

Materials and methods

Plant material, growth conditions, and treatments

E. sativa seeds were subjected to a pre-washing process with fresh tap water for nearly 30 min, followed by the 70% ethanol treatment, the most common pre-disinfection process, were applied to these seeds for 30 s. Following removal of ethanol, seeds were disinfected with 20% commercial bleach (Domestos) for 10 min to prevent fungal and bacterial contamination. Finally, bleach residues left on the surface of the seeds were removed with autoclaved sterile distilled water. This process was repeated 3 times and each repetition took 15 min. The sterilized seeds were transferred to pre-prepared glass magenta culture vessels with approximately 30 mL of fresh nutrient medium. Cultured seeds were placed in an optimum plant growth chamber until the beginning of germination.

For seed germination, the most preferred two different basal media MS (Murashige and Skoog 1962) and B5 (Gamborg et al. 1968) each supported with 1.0 mg/L 6-BA and without PGR (control) were tested to determine the best effective basal medium. After four weeks of incubation, two important parameters such as the percentage of seed germination and shoot length of germinating seeds were evaluated for each treatment.

For shoot proliferation, nodal segments were excised from the shoots of seedlings at the end of the third subculture and cultured on MS basal medium. Sucrose (Duchefa Biochemie, Haarlem, The Netherlands), and agar (Duchefa Biochemie, Haarlem, The Netherlands), ratios of this components were 2% (w/v) sucrose 0.8% (w/v), respectively. Different types of plant growth regulators (PGRs) such as N6-[2-isopentenil] adenine (2iP), 6- Benzyladenine (6-BA), kinetin (KIN), and thidiazuron (TDZ) (1.0 mg/L, each) individually in combination with 0.1 mg/L indole-3- butyric acid (IBA) were added in this medium. Filter sterilization was applied to all tested PGRs with 0.22 µm filters except for 6-BA, and they were added to the cooled basal media after autoclaving. 1 N NaOH and HCl were preferred to adjust the pH of the media to 5.8. All cultures were maintained at 24 ± 2 °C under a 16 h light and 8 h photoperiod. To provide these conditions, daylight fluorescent lamps with 50 µmol m^{-2} s^{-1} photosynthetic flux were quite ideal. The ideal subculturing procedure was done according to the reaction of the explants under the culture conditions, and this period was usually four weeks. The shoot proliferation performance of the different media was assessed by calculating the number of shoots per explant, length of shoots, number of leaves, leaf width and biomass yield based on the fresh and dry weight on each shoot. Different 6-BA concentrations (0.25, 0.5 and

2.0 mg/L) in combination with 0.1 mg/L IBA or without PGR were tested in MS basal media for determining the best PGR concentration.

After the most suitable basal medium was determined, at the age of four weeks, a set of plants were pretreated with 100 µM SNP (Sigma Aldrich, USA) for 12 h and then exposed to oxidative stress induced by 2.5 µM PQ (Sigma Aldrich, USA) for 5 days. Another set of plants was applied with 2.5 µM PQ only for 5 days. Plants cultured only in MS media containing 6-BA/IBA (0.25/0.1 mg/L) were planned as control. In our study, the lowest membrane damage and the best water status were determined at an SNP concentration of 100 µM. Plant tissues were harvested to determine various stress parameters, changes in protective compound contents and antioxidant enzymes activities. The experiments were carried out in three replicates.

Determination of thiobarbituric acid reactive substances (TBARS) content

TBARS contents were measured according to method of Heath and Packer (1968). 0.1 g of fresh leaf tissues were homogenized in trichloroacetic acid (0.1%) by a homogenizer. The homogenate was centrifuged at 15,000 × g for 5 min. The mixture of thiobarbituric acid and 20% TCA was added to the supernatant and was heated at 95 °C for 30 min and then quickly cooled. The absorbance of the supernatant was measured at 532 and 600 nm after 10 min of centrifugation at 10,000 × g.

Determination of hydrogen peroxide (H_2O_2) content

The extract obtained from fresh leaf samples crushed with activated charcoal in TCA was centrifuged, then 1 mL of supernatant was taken and potassium phosphate (10 mM) buffer and potassium iodide (1 M) were added. The absorbance was then read at 390 nm (Velikova et al. 2000).

Determination of total chlorophyll and carotenoid contents

Fresh leaf tissues weighing 0.1 g were ground with liquid nitrogen and homogenized in a cold acetone solution (80%). At room temperature, the homogenate was centrifuged at 5000 rpm for 10 min and read at 450, 645, and 663 nm. The total chlorophyll and carotenoid contents were measured According to Arnon (1949) and Witham et al. (1971), respectively.

Determination of proline and total phenolic contents

Dried leaves were homogenized with sulfosalicylic acid (3%) for proline content then filtered. The filtrate was centrifuged at 4000 × g for 5 min. Acetic acid and ninhydrin were added to the supernatant. The mixture was then put in tubes and heated at 100 °C for 1 h. Toluene (3 mL) was added to the cooled samples. Then absorbance was read at 520 nm (Bates et al. 1973).

According to Singleton et al. (1999), total phenolic content was determined. Methanol (95%) was used to homogenize fresh leaf samples (0.1 g). After homogenate was centrifuged, Na_2CO_3 (7.5%) and 2 N Folin–Phenol Ciocalteu's Reagent were added to the diluted supernatant. The samples were held in the dark for 30 min before being measured at 765 nm using a gallic acid standard curve.

Determination of relative water content (RWC)

The plant leaves (0.1 g) were weighed to determine the fresh weight (FW), and then the leaves were placed in water and rehydrated for 12 h to estimate water saturated weight (SW). The leaves were dried at 75 °C for 48 h to determine their dry weight (DW).

Determination of ascorbate (AsA) content

Ascorbate concentration was determined by Liso et al. (1984). Fresh leaves (0.1 g) were homogenized with 5% (w/v) m-phosphoric acid. The extract was centrifuged at 10,000 × g for 4 min. The sample 70 (µL) was added to 3 mL of reaction medium containing 0.1 M citrate-0.2 M phosphate buffer (pH 6.2). Initial absorbance to be read at 265 nm, ascorbate concentration was determined by reading the reduction occurring 5 min after 2 units the ascorbate oxidase were added to the reaction medium. Then, 2.5 mM DTT was added to the mix. Following reduction (3 min) with DTT, the absorbance was read again at 265 nm.

Determination of changes in antioxidant enzymes

Fresh plant tissues (0.1 g) were ground to a fine powder in liquid nitrogen and were extracted in 5 mL of extraction buffer (50 mM K_2HPO_4, 1 mM EDTA, 1% PVPP, pH 7.0). The extracts were centrifuged at 20,000 × g for 20 min at 4 °C. Ascorbic acid (5 mM) was added to the buffer for APX. The activity SOD was measured using the Beauchamp and Fridovich (1971) method. Riboflavin (2 mM) was added to the reaction mix involving potassium phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), L-methionine (13 mM), nitro blue tetrazolium, and 50 mL extract to start the reaction. After 10 min of exposure to white light at 375 µmol m^{-2} s^{-1}, the absorbance values at 560 nm were determined. The activity CAT was determined according to Aebi (1983) method. The enzyme activity was measured at 240 nm in a reaction mixture

(1 mM) containing 50 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2, and 20 µL enzyme extract. The activity of APX was assayed by the decrease in absorbance at 290 nm (Nakano and Asada 1981).

Protein determination

Protein content was measured by method of Bradford (1976). Bovine serum albumin standards were prepared and protein complex with Coomassie Brillant Blue G250 dye was measured at 595 nm. Protein concentration was calculated in mg and used to express enzyme activities.

Statistical analysis

Each treatment was performed in triplicates and included four magenta culture vessels (each containing four explants) for shoot multiplication. All data were analyzed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). For shoot multiplication, mean shoot length, mean number of shoots, mean number of leaves, mean number of leaf width, mean number of fresh and dry weight, were analyzed using analysis of variance (ANOVA) with Duncan's multiple range test (DMRT, 95% confidence level). Values are means ± standard deviation (SD). Shoot-forming capacity (SFC) (Lambardi et al. 1993) was also calculated to determine the percentage of shoots regeneration as follows:

$$\text{SFC index} = (\text{average number of shoots per regenerating explant}) \times (\% \text{ of regenerating explant})/100$$

The obtained data were evaluated with one-way ANOVA variance analysis tests (Duncan Multiple Comparison Test, $P < 0.05$).

Results

Seed germination

E. sativa seeds were separately germinated in MS and B5 media supplemented with 1.0 mg/L 6-BA or cytokinin-free medium for 30 days. The most effective germination percentage was obtained in the MS containing 1.0 mg/L 6-BA with 90.00 ± 3.33%. These percentages were determined as 88.89 ± 1.92% in B5 medium supplemented with 1.0 mg/L 6-BA and also 76.67 ± 3.33% in cytokinin-free MS medium. The lowest germination percentage was also in cytokinin-free B5 medium with 73.33 ± 3.34% (Table. 1). Among all tested basal germination media, significant statistical differences were performed in terms of germination performance ($P < 0.05$).

The average maximum shoot length obtained from germinated seeds was 29.80 ± 2.22 mm in MS medium supplemented with 1.0 mg/L 6-BA followed by 24.36 ± 1.32 mm in B5 medium supplemented with 1.0 mg/L 6-BA. These values were calculated as 15.72 ± 1.33 and 14.87 ± 1.07 mm in cytokinin-free MS and B5 media, respectively. These values also showed that there were significant statistical differences between the other tested media and MS containing 1.0 mg/L 6-BA in terms of shoot length (Table 1). Due to lower germination percentage and weak shoot efficiency B5 medium was not preferred for subsequent shoot proliferation studies.

Shoot proliferation

When the four different cytokinins mentioned above were combined individually with IBA (0.1 mg/L) and without PGR (control) in MS basal medium, 6-BA was found to be most suitable than the cytokinin/auxin combinations (Fig. 1). Therefore, several concentrations of 6-BA (0.25, 0.5 and 2.0 mg/L) were tested to determine the optimal cytokinin concentration together with IBA (0.1 mg/L) again. Among all these tested combinations and concentrations, the highest shoot number and leaf number was calculated in MS basal media fortified 1.0/0.1 mg/L 6-BA/IBA with 3.50 ± 0.38 and 24.83 ± 2.06 per explant, respectively. Although MS medium supplemented with 1.0/0.1 mg/L 2iP/IBA gave the highest shoot length and leaf width compared to the other tested media with 39.29 ± 2.84 mm and 7.08 ± 0.55 mm per explant, respectively, it was not considered to be the most suitable cytokinin due to its low values for other tested parameters (Table 2). Because the number of leaves of the shoots obtained from MS media supplemented with this growth regulator was low and the shoots were weak. This situation was an undesirable result for the *E. sativa* whose leaf was edible. These data were a strong indicator of significant statistical differences between 2iP and the other tested cytokinins ($P < 0.05$). In micropropagation studies, fresh and dry weight obtained per explant are the two important parameters in the production of plants whose leaves are consumed or evaluated. In this context, the highest fresh and dry weights were calculated from MS basal medium containing 0.25 mg/L 6-BA with 1.152 ± 0.180 g and 0.061 ± 0.009 g per explant, respectively. As a result, MS medium supplemented with 0.25 mg/L 6-BA together with 0.1 mg/L IBA was found to be the most suitable for shoot proliferation of *E. sativa*.

TBARS content

In the case of PQ stress, TBARS content increased in *E. sativa*, but SNP application prevented this increase. Namely,

Table 1 The effects of different basal media supplemented with the same cytokinin concentration and without cytokinin on seed germination of *E. sativa*

	MS		GB-5	
	Control	1.0 mg/L 6-BA	Control	1.0 mg/L 6-BA
Shoot length (mm)	$15.72^{c} \pm 1.33$	$29.80^{a} \pm 2.22$	$14.87^{d} \pm 1.07$	$24.36^{b} \pm 1.32$
Germination percentage (%)	$76.67^{b} \pm 3.33$	$90.00^{a} \pm 3.33$	$73.33^{b} \pm 3.34$	$88.89^{a} \pm 1.92$

Data were recorded 4 weeks after the culture with a total of 3 replicates of 30 seeds per treatment

^{a,b,c,d}Values having the same letter(s) in the same line are not significantly different according to Duncan's multiple range test at $P < 0.05$

Fig. 1 **A** In vitro micropropagation of *E. sativa*. **B** Shoot proliferation after 4 weeks; **B**1 on culture medium from nodal explant on MS free-medium. **B**2 supplemented with 1.0/0.1 mg/L TDZ/IBA. **B**3 with 1.0/0.1 mg/L 6-BA/IBA. **B**4 with 1.0/0.1 mg/L. 2iP/IBA (**B**5) with 1.0/0.1 mg/L KIN/IBA Bar = 1.57 cm

E. sativa seedlings treated with PQ stress alone (3.83 ± 0.04 nmol g^{-1} FW) resulted in rising TBARS content as compared to the control group (2.11 ± 0.09 nmol g^{-1} FW). However, the treatment of SNP decreased TBARS content in seedlings exposed to PQ stress (2.50 ± 0.03 nmol g^{-1} FW). Moreover, TBARS content (1.42 ± 0.05 nmol g^{-1} FW) was low in the SNP alone treatment (Fig. 2A).

H_2O_2 content

It was found that H_2O_2 content (0.38 ± 0.009 mM g^{-1} FW) in *E. sativa* seedlings was induced by PQ stress compared to control (0.29 ± 0.01 mM g^{-1} FW). SNP application decreased H_2O_2 content (0.35 ± 0.009 mM g^{-1} FW) in seedlings exposed to PQ stress. Moreover, H_2O_2 content did not differ between control plants (0.29 ± 0.01 mM g^{-1} FW) and those treated with SNP (0.28 ± 0.009 mM g^{-1} FW) ($P < 0.05$) (Fig. 2B).

Total chlorophyll and carotenoid contents

As present in Fig. 2C, D, total chlorophyll and carotenoid contents declined in PQ-treated *E. sativa* seedlings than that of control seedlings. The inhibitory effects of PQ stress on the contents of total chlorophyll and carotenoid were reduced by the treatment of SNP. The total chlorophyll and carotenoid contents were 1.12 and 1.06-fold higher than that of the PQ alone treatments.

Proline and total phenolic contents

The changes in proline and total phenolic contents in *E. sativa* seedlings were illustrated in Fig. 3. PQ stress triggered the accumulation of proline content (207.85 ± 1.43 mg g^{-1} FW) as compared to control (143.54 ± 1.21 mg g^{-1} FW). Proline content did not have a statistically significant difference between control (143.54 ± 1.21 mg g^{-1} FW) and SNP treatment (145.45 ± 1.30 mg g^{-1} FW) ($P < 0.05$). Also, under PQ stress, SNP application decreased proline content (159.11 ± 1.53 mg g^{-1} FW) as compared to PQ alone treatment (207.85 ± 1.43 mg g^{-1} FW) (Fig. 3A).

Total phenolic content increased in *E. sativa* seedlings under PQ stress (8.90 ± 0.30 mg 100 g^{-1} FW) t in comparison to the control (6.30 ± 0.10 mg 100 g^{-1} FW). Also, it was found that total phenolic content increased in *E. sativa* seedlings treated with the combination of SNP and PQ

Table 2 The effects of different cytokinin in the presence of IBA (0.1 mg/L) combination and control group on shoot proliferation of *E. sativa*

Control	TDZ	2iP	KIN	6-BA	IBA	Shoot number/ explant	Shoot length/ explant (mm)	Leaf number/ explant	Leaf width/ explant (mm)	Fresh weight/ explant (g)	Dry weight/ explant (g)	Shoot forming capacity (SFC)
Cytokinin (mg/L)												
0.0	0.0	0.0	0.0	0.0	0.0	1.37 ± 0.29e	36.05 ± 4.02bc	5.31 ± 0.8f	4.63 ± 0.72e	0.395 ± 0.051e	0.032 ± 0.005d	1.28
	1.0				0.1	3.02 ± 0.43bc	32.27 ± 2.15d	22.88 ± 1.44b	5.19 ± 0.58d	0.844 ± 0.066c	0.058 ± 0.007ab	2.64
		1.0			0.1	1.83 ± 0.42d	39.29 ± 2.84a	10.92 ± 1.83e	7.08 ± 0.55a	0.458 ± 0.075e	0.034 ± 0.005d	1.83
			1.0		0.1	2.96 ± 0.51bc	36.52 ± 2.17bc	21.25 ± 1.78d	6.44 ± 0.53b	0.730 ± 0.099d	0.053 ± 0.005c	2.96
				0.25	0.1	3.38 ± 0.89ab	37.89 ± 2.84ab	19.56 ± 1.21d	6.50 ± 0.82b	1.152 ± 0.180a	0.061 ± 0.009a	3.38
				0.5	0.1	3.06 ± 0.68bc	35.90 ± 1.59c	21.38 ± 1.54c	5.06 ± 0.77de	0.996 ± 0.099b	0.057 ± 0.008bc	3.06
				1.0	0.1	3.50 ± 0.38a	35.86 ± 2.63c	24.83 ± 2.06a	5.94 ± 0.52c	1.144 ± 0.121a	0.060 ± 0.008ab	3.50
				2.0	0.1	2.88 ± 0.62c	27.25 ± 1.29e	21.50 ± 1.51c	5.00 ± 0.82de	0.927 ± 0.099b	0.053 ± 0.006c	2.88

Data were recorded 4 weeks after the culture and represents a total of 3 replicates of 16 plants per treatment on MS. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at $P < 0.05$

(9.50 ± 0.20 mg 100 g^{-1} FW) in comparison to PQ stress (8.90 ± 0.30 mg 100 g^{-1} FW). Moreover, total phenolic content in the SNP-treated seedlings (7.60 ± 0.30 mg 100 g^{-1} FW) was higher than the control (6.30 ± 0.10 mg 100 g^{-1} FW) (Fig. 3B).

RWC

Paraquat stress decreased the leaf RWC (65.13%) in comparison to control (74.20%). However, SNP treatment increased the leaf RWC (71.36%) in seedlings under PQ stress. There was no statistically significant difference between control and SNP alone treatment (77.36%) ($P < 0.05$) (Fig. 3C).

AsA content

It was found that the AsA content (1.09 ± 0.014 µg mL^{-1} FW) increased under PQ stress in comparison to the control group 0.80 ± 0.015 µg mL^{-1} FW). Moreover, AsA content increased in SNP-applied seedlings under unstressed (0.89 ± 0.012 µg mL^{-1} FW) and stressed (1.23 ± 0.014 µg mL^{-1} FW) conditions (Fig. 4A).

Changes in antioxidant enzymes

The activity of SOD was induced by PQ stress (16.29 ± 0.40 unit mg^{-1} protein) compared to control (11.81 ± 0.50 unit mg^{-1} protein) (Fig. 4B). The SNP application increased the activity (17.65 ± 0.30 unit mg^{-1} protein) in seedlings under PQ stress as compared to PQ stress (16.29 ± 0.40 unit mg^{-1} protein). On the other hand, SOD activity was not a statistically significant difference between control (11.81 ± 0.50 unit mg^{-1} protein) and SNP alone treatment (12.21 ± 0.40 unit mg^{-1} protein) ($P < 0.05$) (Fig. 4B).

The activity of CAT increased under PQ stress (0.98 ± 0.03 unit mg^{-1} protein) as compared to control (0.81 ± 0.02 unit mg^{-1} protein). CAT activity increased in SNP-treated seedlings under PQ stress as compared to PQ stress. The activity increased from 0.98 ± 0.03 unit mg^{-1} protein (PQ) to 2.04 ± 0.04 unit mg^{-1} protein (SNP + PQ). SNP treatment also increased the enzyme activity (1.66 ± 0.02 unit mg^{-1} protein) in comparison to control (Fig. 4C).

The activity of APX (52.23 ± 1.7 unit mg^{-1} protein) was enhanced by PQ stress compared to control (45.99 ± 1.5 unit mg^{-1}). The highest APX activity was measured in SNP- treated seedlings under PQ stress (78.86 ± 1.5 unit mg^{-1} protein) (Fig. 4D).

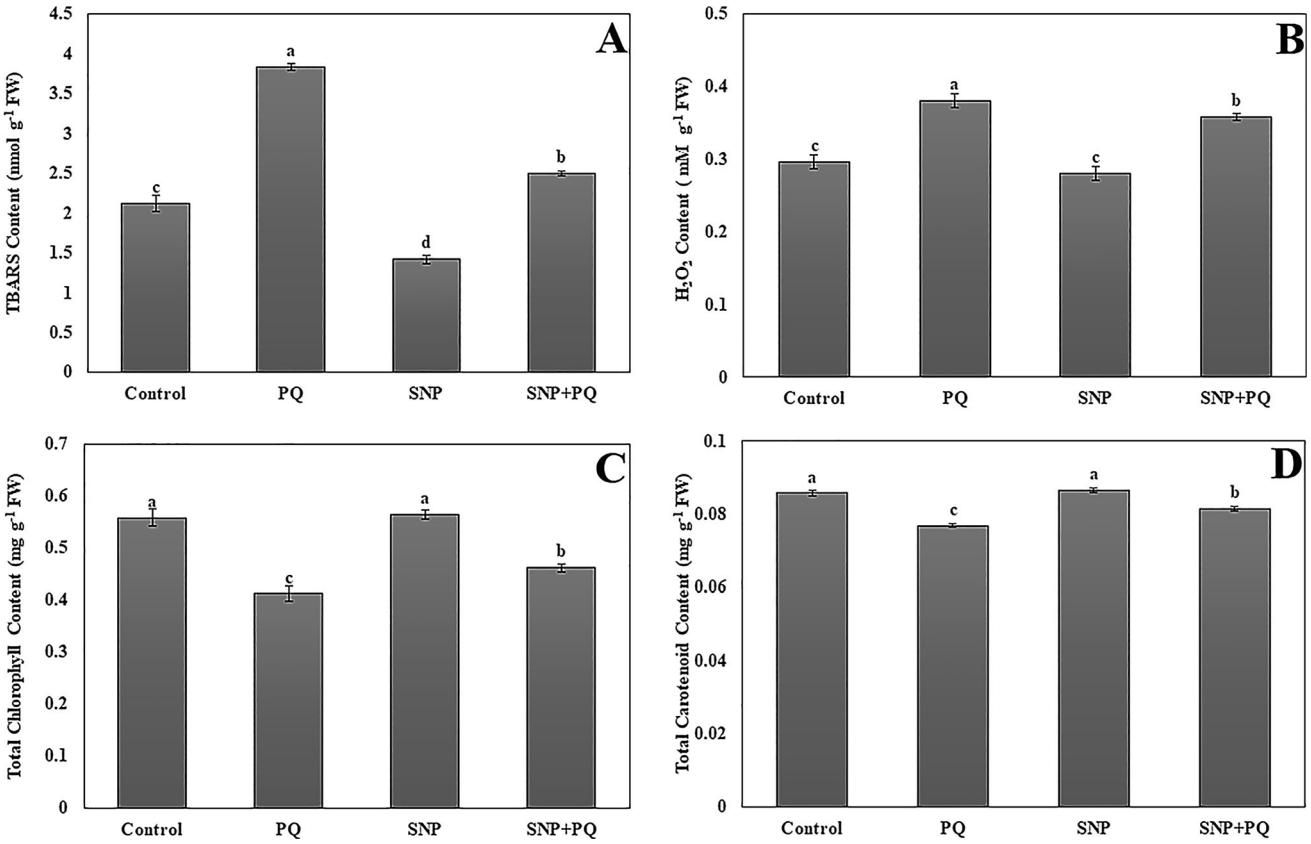

Fig. 2 Thiobarbituric acid reactive substances (TBARS) content (**A**), Hydrogen peroxide (H_2O_2) content (**B**), Total chlorophyll content (**C**), Total carotenoid content (**D**) in SNP-treated *E. sativa* seedlings under PQ stress. Vertical bars represent standard deviations of the means of three replicates. Different letters denote significant differences among all treatments at $P < 0.05$

Discussion

Depending on the rapid population growth, the need for food is also increasing rapidly nowadays. To meet this demand, researchers generally focus on higher quality, faster and higher production per unit area. The determination of the most suitable and fast production method is very crucial in the production of plants. Biotechnological production methods offer many advantages such as rapid and mass commercial production regardless of time and place on a year round basis, shortening the growth cycle, well-adapted clonal multiplication, controlled environment, or production of healthy and stress-resistant plant materials (Suman et al. 2012; Suman 2017). All these positive factors have led researchers to create effective micropropagation methods of different plant species that have medicinal, aromatic, and also economic value nowadays (Zhang et al. 2005; Cüce et al. 2017; Bekircan et al. 2018). The researchers were preferred seeds or cotyledonary nodes as explant in their studies on *E. sativa*. They tested the different concentrations of BAP and kinetin alone or in combination with different concentrations of IAA, IBA and NAA and were found BAP combination with IAA superior to other applications. These researchers have reported that kinetin applications alone were not suitable for micropropagation of *E. sativa* (Sharma et al. 2012). Although there were little differences between this report and our data, these results support our findings in general terms. The occurrence of these differences is based on many factors such as explant source, explant type, explant collection time, environmental conditions of the explant, media types, and culture conditions in micropropagation studies (Ružić et al. 2012; Cüce and Sökmen 2017; Cüce et al. 2019).

At the present time, many plant species are cultivated in vitro and regenerated into full plants. Plant tissue culture including various techniques like micropropagation offers grand opportunities for plant propagation and the growth of plants with desirable agronomic traits such as increased yield, environmental stress (salt, frost, drought, herbicide) tolerance, and plant engineering (Jan et al. 2018). Recently, changes in some physiological, biochemical and molecular parameters of plantlets grown in vitro in tolerance to various stresses have been reported (Arshad et al. 2014; Jan et al. 2018; Simsek et al. 2018; Muchate et al. 2019). However, to our knowledge there is no study about stress

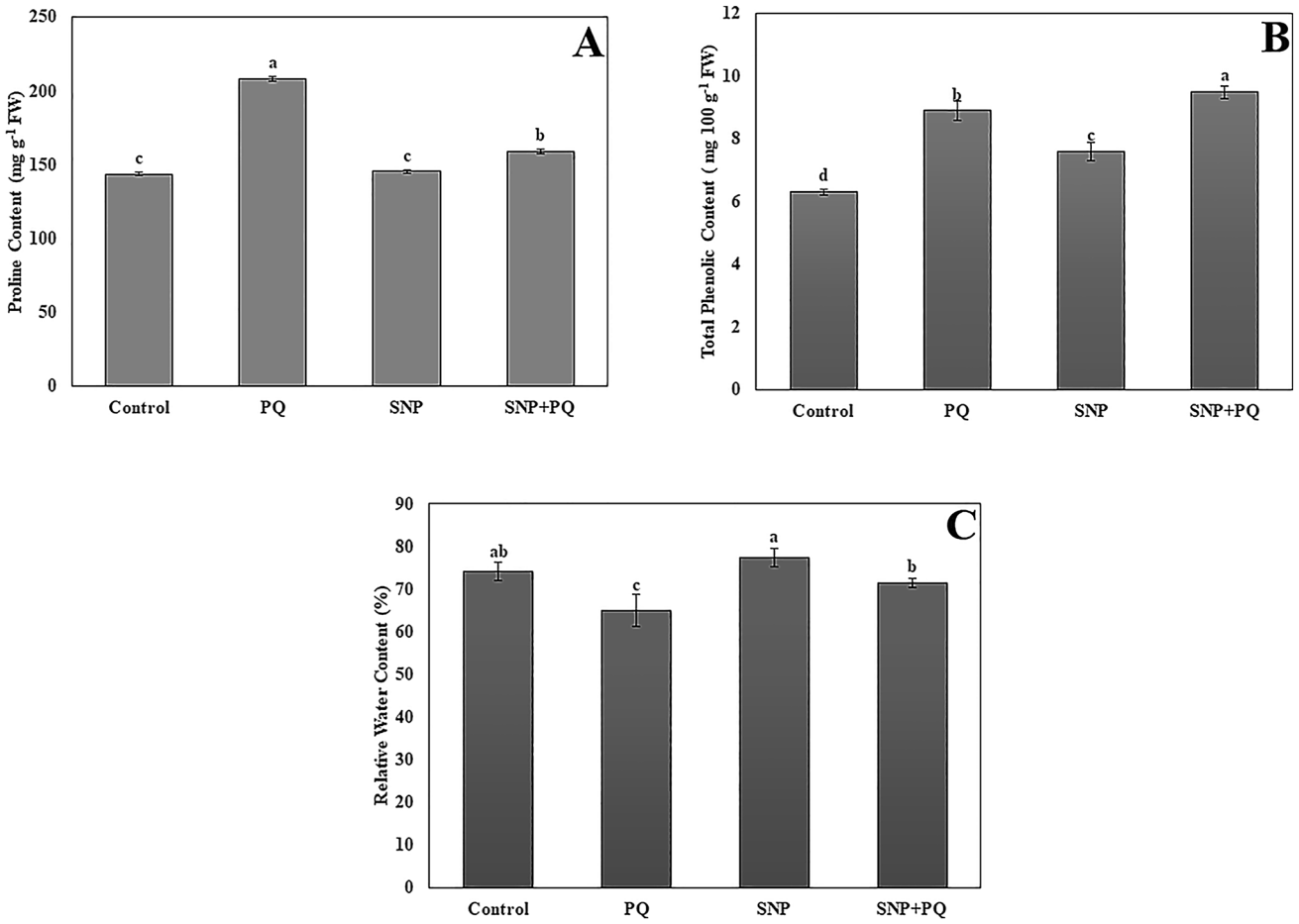

Fig. 3 Proline content (**A**), Total phenolic content (**B**), Relative water content (RWC) (C) in SNP-treated *E. sativa* seedlings under PQ stress. Vertical bars represent standard deviations of the means of three replicates. Different letters denote significant differences among all treatments at $P < 0.05$

tolerance of *E. sativa* plantlets grown in vitro in the literature. Therefore, the role of SNP in PQ-mediated oxidative stress tolerance in *E. sativa* plantlets grown in vitro was investigated in the current study.

After the most suitable basal medium was determined, to understand how PQ-mediated oxidative stress was damaging *E. sativa* plantlets propagated in vitro, we analyzed the TBARS, H_2O_2, RWC, and total chlorophyll contents. The contents of TBARS and H_2O_2 increased in *E. sativa* under PQ stress, possibly due to cell membrane damage caused by ROS-mediated oxidative damage. The previous study conducted by Khator and Shekhawat et al. (2020b) in *B. juncea* seedling revealed that the cytoprotective roles of SNP against cadmium stress to lipid membrane by reducing the production of free radicals. In the present study, the decrease in TBARS and H_2O_2 levels in SNP-treated *E. sativa* may have a role in the induction of antioxidant defense system that scavenges H_2O_2. Similar results from other PQ studies corroborate the current study's oxidative harm trend in seedlings (Moustaka et al. 2015; Takeda et al. 2016). Paraquat stress decreased total chlorophyll and carotenoids contents in *E. sativa,* which may be due to oxidative damage to photosynthetic pigments. Moreover, it was found that exogenous SNP inhibited the degradation of photosynthetic pigments in *E. sativa* seedlings under PQ stress. The application of SNP was documented to be enhanced the level of photosynthetic pigments in plants under salt induced oxidative stress (Khator and Shekhawat 2020a).

In order to reduce the negative effect of PQ stress, plants trigger to increase production of proline level. In the present study, similar trend of proline accumulation was determined in *E. sativa* treated with PQ stress. The proline level decreased when SNP was supplemented with PQ application, indicating that stress was alleviated. The findings of this study also support the findings of Hasanuzzaman et al. (2018). The application of SNP (NO donor) increased the accumulation of proline in salt stressed plants (Ahmad et al. 2016). In our study on *E. sativa* plants showed SNP application had a useful effect under

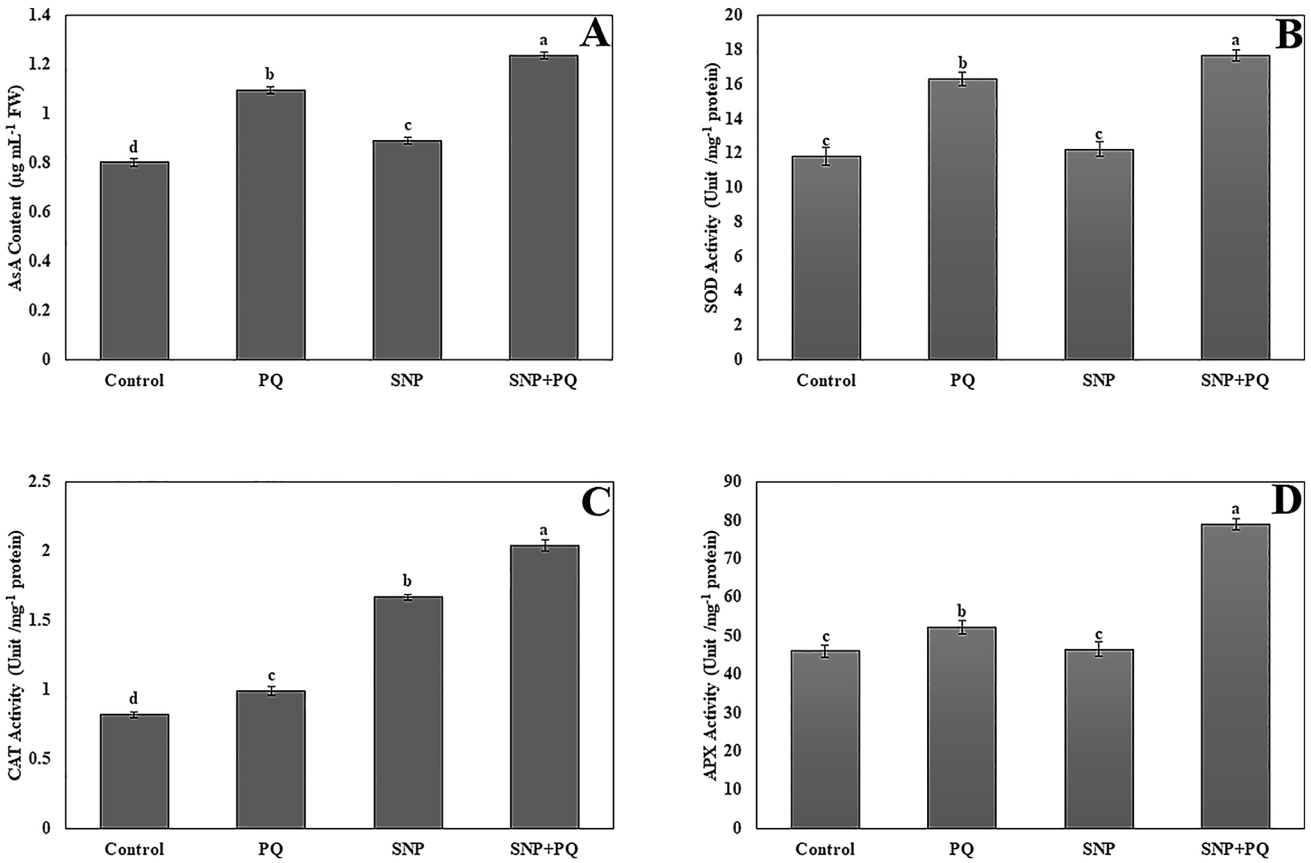

Fig. 4 Ascorbate (AsA) content (*A*), Superoxide dismutase (SOD) activity (*B*), Catalase (CAT) (*C*), Ascorbate peroxidase (APX) activity (*D*) in SNP-treated *E. sativa* seedlings under PQ stress. Vertical bars represent standard deviations of the means of three replicates. Different letters denote significant differences among all treatments $P < 0.05$

PQ stress: SNP application arranged the proline level. The total phenolic contents of *E. sativa* seedlings subjected to PQ was higher than that of control. In comparison to the regulation, exogenous SNP application increased the phenolic contents. In comparison to PQ treatment alone, combining SNP with PQ improved phenolics contents even more. Previous research has shown that salt stress increased the total phenolic compounds and increase even further when SNP is applied to the salt stress treatment (Mohsenzadeh and Zohrabi 2018).

Paraquat-affected *E. sativa* seedlings showed a decrease in RWC in comparison to control, however, RWC increased after SNP application. The improved RWC may be due to a variety of factors. In plants, SNP reduces solute potential while rising water potential, assisting in the increase of RWC under osmotic stress (Ke et al. 2013).

AsA, the most plentiful antioxidant in plants, protects plants against oxidative stress by scavenging a wide variety of ROS (Liu et al. 2009). In the present study, similar to the findings of Hasanuzzaman et al. (2018), increased AsA content under PQ stress was increased by SNP application. The upregulation of the antioxidant defense system is an important tolerance mechanism to minimize the ROS-induced toxicity in stress-affected plants. In the current study, the activities of antioxidant enzymes were determined to investigate the role of SNP in the tolerance of PQ-mediated oxidative stress in *E. sativa* seedlings. The activities of SOD, CAT and APX were induced by PQ stress compared to control. Moreover, exogenous SNP increased the activities of these enzymes in seedlings under PQ stress as compared to PQ alone treatment, showing an increase in the H_2O_2 scavenging process. The activities of antioxidant enzymes varied under PQ stress have been reported by Hasanuzzaman et al. (2018) in *B. napus* seedlings. Similarly, SNP application improved the activity of CAT in salt treated *B. Juncea* (Khator and Shekhawat 2020b). Given these results, the study demonstrated that SNP regulated antioxidant defense system in *E. sativa* plants during PQ stress and provided cellular protection against PQ induced oxidative stress by detoxification of ROS, like H_2O_2.

Conclusion

It was determined that *E. sativa* plantlets by using nodal segments as explants showed high micropropagation performance in MS media containing 6-BA/IBA (0.25/0.1 mg/L) in the present study. Moreover, the role of SNP in micropropagated plantlets of *E. sativa* under PQ stress was investigated for the first time in optimum in vitro culture conditions. As a result, it was revealed that the role of SNP in alleviating PQ-induced oxidative stress in *E. sativa*. The application of SNP (100 μM) decreased TBARS level and it had a beneficial effect on *E. sativa* under PQ stress: SNP was able to adjust the proline level and rise the RWC in plants. The study also demonstrated that SNP could act as a ROS scavenger, like H_2O_2, provide cellular protection against PQ-induced stress either directly or through enhancing antioxidant protection system components like AsA; raised the activities of SOD, CAT, and APX in *E. sativa*. Here, we suggested for the first time that exogenous SNP could protect *E. sativa* plantlets propagated in vitro from PQ stress through modulation of proline and phenolics biosynthesis and antioxidant defense system.

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Author contributions MC designed the research. MC and ASM conducted the experiments, analyzed all data and wrote manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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