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Role of Nitric Oxide in Stress mitigation in Fenugreek seedlings during the Post-germination Phase under Salinity stress

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Abstract

In the present study the exogenous sources of nitric oxide (SNP) and its scavenger (c-PTIO) were applied for studying their effect on the alteration of various biochemical parameters along with their antioxidant defence system during germination stages. The pre-treated seeds were exposed to saline condition and their various morphological, physiological and biochemical attributes were determined. Our results suggest that priming of fenugreek seeds with exogenous source of nitric oxide enhanced the morphological and biochemical attributes along with the antioxidant defence system under saline condition, which was further substantiated by the occurrence of adverse effects of salinity on the seeds which were unprimed and also those primed with c-PTIO, a nitric oxide scavenger. Therefore, the enhancement in the enzymatic as well as non-enzymatic components might be due to the involvement of nitric oxide leading to tolerance towards salinity accompanied with better growth and development. Additionally, an uninterrupted influx of these signal molecules is very much essential for better growth and salinity stress management in fenugreek seedlings, which can be hypothesized on the basis of the tremendous deterioration of the growth parameters as well as oxidative stress management status of the fenugreek seedlings revealed in the present study.

Key words : Antioxidant enzymes, salinity stress, nitric oxide, stress tolerance.

The environmental factors are known to have significant impact on the morphological, biochemical attributes along with the growth and development of plants. When any of these factors exceed the tolerance level, a stress is imposed on the plant which influences its development and structural, physiological and biochemical processes[10]. The increase in the salt content above optimum level, which creates salinity stress, is considered one among these environmental factors which are responsible for threatening the crop productivity worldwide[17].

The deleterious effect of salinity which affects the normal growth and development of the plant is attributed to a reduced osmotic potential, specific ion toxicity and nutrient deficiency of the substratum[16]. To protect themselves from the oxidative stress mediated damages, plants are found to develop scavenging mechanisms of these destructive free radicals. This involves detoxification processes regulated by an integrated system of non-enzymatic antioxidants such as ascorbic acid and glutathione[27], and the enzymatic system which comprises of efficient antioxidants such as catalase (CAT) ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR)[22]. Nitric oxide (NO) a bioactive gaseous signalling molecule, exhibits central role in mediating several physiological processes and responses towards biotic and abiotic stresses including salinity[33].

Trigonella foenum-graecum also known as fenugreek, the plant parts has been reported to possess wide pharmacological and folkloric significance. In addition, its seeds are found to possess potential hypocholesterolemic effect, antioxidant property and also have been effective in the treatment and/or prevention of diabetic disorders[18].

However, literature suggests that reports on the role of nitric oxide and its effect on growth and metabolism of fenugreek during the germination phases under salinity stress are not studied till date. Considering this fact, the present study was undertaken to investigate the impact of exogenous nitric oxide on saline mediated alterations in the morphological, biochemical and antioxidant defense system of fenugreek during post-germination phase.

Elicitation process and germination :

The fenugreek seeds were subjected to surface sterilization with 0.1% sodium hypochlorite solution. The sterilized seeds were washed thrice with distilled water and pre-treated with the solutions of 5mM sodium nitroprusside (SNP) as an exogenous source of nitric oxide; 100 µM of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CP), a nitric oxide scavenger. For control set, seeds were primed with normal water and placed in a rotary shaker along with the treated seeds. After priming for 24h, the seeds were washed thrice with sterile water and kept in the seed germinator for germination. To provide saline conditions the NaCl at the level of (0dS m^{-1}, 4dS m^{-1} and 8dS m^{-1}) was applied to the seeds for 5 days.

Measurement of growth parameters :

The growth performance of the seedlings was assessed by calculating various morphological parameters. The average length of the roots and shoots of the seedlings of each

experimental set up was recorded along with their fresh weight and dry weight.

Relative water content :

The relative water content (RWC) of the seedlings was calculated using the following equation[7]:

RWC (%) = (Fresh weight - Dry weight) / (Turgid weight - Dry weight) x 100.

Stress tolerance index :

The stress tolerance index (STI) of the seedlings was calculated using the following equation[26]:

STI = [(DWc - DWs) /DWc] × 100

where DWc: Dry weight of control seedlings and DWs: Dry weight of seedlings under stress

Determination of glutathione content :

Total glutathione content in fenugreek seedlings was determined according to Griffith[8] with some modifications. The resulted glutathione content was expressed as μg g^{-1} fw using glutathione standard curve.

Determination of ascorbic acid content :

The content of ascorbic acid was analyzed by the spectrophotometric method described by Mukherjee and Choudhary[20]. Ascorbic acid content was expressed as μg g^{-1} fresh weight tissue (fw).

Estimation of carbohydrates :

Total soluble sugar content was estimated following the anthrone method[31]. Reducing sugars were measured by following the standard DNS method[24].

Estimation of proline content :

The proline content was analyzed by the spectrophotometric method described by Bates *et al*.[3]. The obtained proline content was expressed as mg g^{-1} fw.

Preparation of enzyme extracts :

To determine enzyme activities, approximately 1 g of seedlings from both treated and control set were finely ground with a mortar and pestle under liquid nitrogen in 10 ml of cold 50 mM sodium phosphate buffer (pH 7.5) containing 1% (w/v) polyvinylpolypyrrolidone (PVP). The resulting homogenate was then centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant obtained was directly utilized as a crude extract for enzyme assays.

Assay of enzyme activities :

Catalase (EC. 1.11.1.6) :

Catalase activity was determined following the method outlined by Upadhyaya and Panda[32], quantifying the decomposition of H_2O_2 through measurement at 240 nm. The enzyme activity was measured using the extinction coefficient ε = 2.8 mM^{-1} cm^{-1}. The enzyme activity was expressed as μM/min/gFW.

Superoxide dismutase (EC. 1.15.1.1) :

The SOD enzyme activity was assessed by observing the inhibition of the photochemical reduction of NBT, following the protocol outlined by Dhindsa *et al*.[4] with minor adjustments. Absorbance readings were taken at 560 nm, and one unit of activity was defined as the enzyme amount needed to inhibit 50% of the NBT reduction rate in controls.

Ascorbate peroxidase (EC.1.11.1.11) :

The ascorbate peroxidase activity was

evaluated using the method of Nakano and Asada[21], measuring the reduction in absorbance at 290 nm. The enzyme activity was calculated using extinction coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$ and was expressed as μM/min/gFW.

Glutathione reductase (EC 1.6.4.2) :

Glutathione reductase activity was determined by monitoring the change in absorbance at 340 nm due to GSSG-dependent oxidation of NADPH[25]. The enzyme activity was calculated using extinction coefficient $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$ and was expressed as μmole of NADPH oxidized μM/min/gFW.

Statistical analysis :

The statistical tools such as MS Excel 2007 (Microsoft, Redmond, WA, USA), DSAASTAT software (version 1.002; DSAASTAT, Peruglia, Italy), Smith's Statistical Package version 2.5 (prepared by Gary Smith, CA, USA) and Multivariate Statistical Package (MVSP 3.1) were used for various statistical analysis of data.

Since germination being the initial stage of plant development defines the quality of yield and development of plant; therefore, the plants must be provided with best condition during initial stages for better germination. It has been well known that saline environment constrains the growth and development of plant by virtue of their adverse effect on the various physiological and biochemical processes which includes osmolytes accumulation and metabolism along with the antioxidant enzyme system[14]. The basic criteria for a stress tolerant plant is said to be survived under stress; maintenance of biomass production; growth performance, especially elongation of root and accumulation of biochemical markers such as proline, soluble sugars, polyamines, amino acids and also reduced level of lipid peroxidation[12]. As a result, it was observed that the growth performance of fenugreek seedlings was extensively affected by the saline condition as it was evident by the reduction in the fresh weight and dry weight of the seedlings (Figure 1).

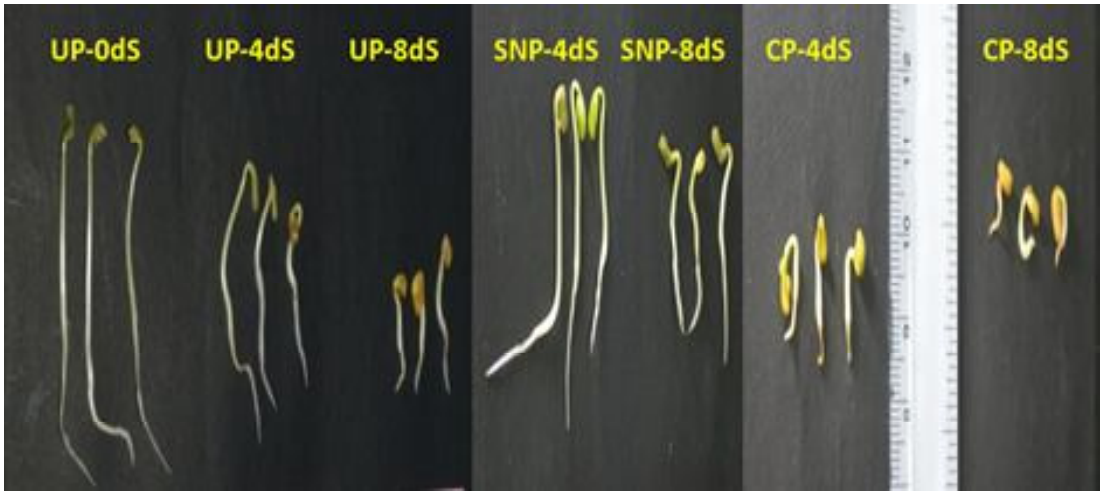

Figure 1. Fenugreek seedlings under the influence of priming agents during salinity.

In comparison to control seedlings, the fresh weight of the unprimed seedlings was found to be reduced by 35% and 49% and dry weight by 12% and 23% (Table-1) at 4dS m^{-1} and 8ds m^{-1} salinity respectively with respect to control (0dS m^{-1}) seedlings. A decrease in the reduction of the growth performance was observed in the seedlings subjected to SNP priming. Another important parameter, relative water content was calculated and it was observed to be 90.06% for control, 80.56% and 70.32% for unprimed seedlings, 87.26% and 75.13% for SNP primed and 65.89% and 51.78% for those primed with CP at 4dS m^{-1} and 8dS m^{-1} salinity respectively (Table-1). The parameter such as relative water content has been considered as one of the vital factors for the assessment of the extent of salinity induced effects and the degree of tolerance in plants towards stress environment. Our findings suggest the positive effect of exogenous nitric oxide priming on the RWC of fenugreek seedlings under salinity stress was in agreement to previous studies on several plants[2]. The seedlings subjected to nitric oxide priming exhibited minimal reduction in root elongation during saline stress, which is considered to be one the major physiological parameter for salinity tolerance[29].

For studying the effect of exogenous nitric oxide under the salinity regime on the biochemical status of fenugreek seedlings, various biochemical attributes were taken under consideration. The biochemical attributes estimated in the present study were proline, glutathione, ascorbate and sugars. It was observed that salt stress has affected the biosynthesis of almost all of these important biochemical molecules stated earlier.

The role of proline in providing the osmotolerance to the plant system during the stress environmental conditions has been widely suggested by various authors[30]. The result exhibited that the accumulation of proline was induced by salinity which was found to

Table-1. Effects of different treatments on the growth parameters of fenugreek seedlings under saline conditions

Treatment	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Fresh weight (mg)	Dry weight (mg)	RWC (%)	STI (%)
UP (0ds m^{-1})	2.8±0.16a	1.83±0.04a	4.63±0.23a	114.8±6.7a	11.4±0.15a	95.06±2.41a	98.96±1.06a
UP (4ds m^{-1})	2.02±0.11c	1.12±0.05cd	3.14±0.29cd	74.7±4.4c	10.1±0.12bc	80.56±3.36c	88.01±1.24c
UP (8ds m^{-1})	1.27±0.19e	0.97±0.08d	2.34±0.38e	58.6±2.3d	8.8±0.24cd	73.32±2.76c	75.67±0.96e
SNP (4ds m^{-1})	2.17±0.16b	1.67±0.06b	3.84±0.25b	83.2±3.2b	10.9±0.13b	87.26±3.38b	92.98±1.18b
SNP (8ds m^{-1})	1.73±0.12d	1.2±0.05cd	2.93±0.16d	66.1±2.9c	9.5±0.11c	75.13±2.96c	80.25±1.56d
CP (4ds m^{-1})	1.13±0.06f	0.33±0.05e	1.46±0.12f	37.9±1.5e	6.7±0.16e	65.89±3.12d	67.54±0.66f
CP (8ds m^{-1})	0.72±0.07g	0.30±0.04e	1.02±0.15f	31.1±2.2f	5.1±0.12f	51.78±2.18e	55.69±0.92g

Results are expressed as a mean of 10 seedlings each (Abbr. used: UP: unprimed; SNP: sodium nitroprusside; CP: c-PTIO). Different small letters within the same column indicate significant difference at $p<0.05$.

be further increased under the influence of the signalling molecule. The enhancement in the proline content of unprimed seedlings was about 35% and 47% under 4dS m^{-1} and 8dS m^{-1} condition, respectively in comparison to control seedlings. On the other hand, SNP primed seeds exhibited significant increase in the accumulation of proline at both 4dS m^{-1} and 8dS m^{-1} condition (Table-2). Likewise, various authors have also reported an enhancement in proline content upon priming with nitric oxide of other plants under various stress condition including salinity[2]. Accumulation of proline is considered to be one of the most frequently reported defense mechanism that is adapted by plants during salinity stress. In agreement to the statement which claims proline is actively involved in maintenance of water content[14], it was observed that the relative water content of the seedlings was highly correlated to their proline content. This indicates that the signalling molecules (nitric oxide) further aids in acquiring this adaptive feature in maintaining the water balance under saline condition. However, there was a huge increase in the accumulation of proline in the seedlings subjected to priming with antagonist (CP) and the probable reason behind such excessive accumulation of proline might be the increase in the degradation of protein molecules[11] in those seedlings due to enormous stress experienced by the seedlings under the salinity stress as well as inhibition in the function of the signalling molecule in presence of their antagonists.

Our results showed that the total soluble sugar content was significantly affected by salinity stress which is in agreement with the author who reported that under saline conditions the activity of enzymes involved in the sucrose-starch partitioning are either inhibited or delayed[23] which might lead to retarded growth. Accordingly in our studies the sugar content was reduced in unprimed as well as seedlings subjected to antagonist

Table-2. Biochemical attributes of fenugreek sprouts pre-treated with different priming agents under salinity stress

Treatment	Glutathione (µg/g FW)	Ascorbic acid (µg/g FW)	Total sugar (µg/g FW)	Reducing sugar (µg/g FW)	Proline (mg/g FW)
UP (0ds m^{-1})	24.04±0.05b	162.07±0.93b	192.05±1.62b	72.52±1.56ab	0.223±0.08e
UP (4ds m^{-1})	21.61±0.82c	160.45±2.42b	197.98±0.54b	63.51±1.28c	0.302±0.11c
UP (8ds m^{-1})	15.93±1.12e	155.71±2.12c	158.02±1.16d	60.98±0.48c	0.328±0.16c
SNP (4ds m^{-1})	29.01±1.12a	191.6±0.96a	206.6±2.04a	73.23±0.66a	0.391±0.13d
SNP (8ds m^{-1})	24.54±0.78b	165.56±1.28b	179.48±1.34c	74.22±0.58a	0.636±0.27b
CP (4ds m^{-1})	17.47±0.86de	78.43±1.13d	121.04±2.02e	44.42±1.15d	0.548±0.19b
CP (8ds m^{-1})	9.83±0.76f	61.34±2.91e	105.21±0.98f	35.12±0.96d	1.36±0.32a

Values represent mean ± SD (n = 3). Different small letters within the same column indicate significant difference at $p<0.05$

priming, a maximum loss of about 37% and 45% approximately for CP primed seedlings at 4ds m^{-1} and 8ds m^{-1} salinity level respectively; however an increase in the sugar accumulation was observed in unprimed seedlings at lower salinity (4dS m^{-1}) which was found to exhibit significant decline at higher salinity level (8dS m^{-1}). The fluctuation in reducing sugar content closely paralleled the overall trend observed in total sugar levels. (Table-2).

Plants have evolved various defense mechanisms and strategies for minimizing the adverse effects of these free radicals and stress condition. These defense strategies are known to comprise of both enzymatic and non-enzymatic mechanisms as mentioned earlier. The major enzymes responsible for removal of excess ROS in plant system are CAT, SOD, APX and GR[5], which are reported to exist in different cellular compartments as isozymes in chloroplast and mitochondria[1]. In the present study the non enzymatic antioxidants namely reduced glutathione and ascorbate along with enzymatic antioxidants were determined.

The glutathione content was found to be adversely affected by salinity and the antagonist, but the recovery in the glutathione pool during salinity was observed under the influence of exogenous nitric oxide. However under the influence of nitric oxide scavenger the loss of glutathione was found to be further induced. Similar trend was observed in variation of ascorbic acid content in fenugreek seedlings subjected to various treatments (Table-2).

Glutathione reductase helps in maintaining the total thiol pool in the cell system which is very much essential for plants. The maintenance of optimum ratio of GSH: GSSG (oxidized glutathione) in the thiol pool is very much important for the plant system for their defense against biotic and abiotic stress conditions[6, 28]. In our study it was observed that the GR activity was significantly reduced under salinity in fenugreek seedlings, the activity of GR enzyme in unprimed seedlings was found to be decreased by 10% and 31% with respect to control seedlings at 4dS m^{-1}

Table-3. Effects of different treatments on the activity of antioxidant enzymes in fenugreek seedlings under saline conditions

Treatment	APX activity (μM/min/gFW)	CAT activity (μM/min/gFW)	GR activity (μM/min/gFW)	SOD activity (U/min/gFW)
UP (0ds m^{-1})	11.24±0.19b	3.03±0.06d	5.38±0.12c	4.47±0.10d
UP (4ds m^{-1})	8.68±0.55d	3.45±0.13c	4.59±0.15d	4.45±0.16d
UP (8ds m^{-1})	11.92±0.70b	3.06±0.11d	4.41±0.11d	5.15±0.30c
SNP (4ds m^{-1})	13.66±0.66a	4.46±0.18a	6.95±0.20b	6.95±0.16a
SNP (8ds m^{-1})	9.32±0.58cd	3.61±0.12bc	6.02±0.30a	5.90±0.22b
CP (4ds m^{-1})	5.65±0.26e	2.32±0.10e	3.92±0.27e	3.05±0.18ef
CP (8ds m^{-1})	4.12±0.59e	2.08±0.07e	3.14±0.22f	2.74±0.32f

Values represent mean ± SD (n = 3). Different small letters within the same column indicate significant difference at $p<0.05$

and 8dS m^{-1} respectively. This indicates the activity of GR decreased with increase in the concentration of salt also it was observed that the activity decline during further germination stages. Such decrease in GR activity was also reported by Kang *et al*.[13] in cucumber seedlings subjected to hypoxia stress; further Swamy *et al*.[28] observed such reduction in GR activity in fenugreek under cadmium stress along with GSH content. The application of exogenous nitric oxide as priming agents resulted in elevation in the GR activity by 29% at 4ds m^{-1} and 12% at 8dS m^{-1} approximately (Table-3).

For the assessment of the effect of salinity stress on antioxidant system of fenugreek seedlings, the activities of other important antioxidant enzymes were determined. The other antioxidant enzymes considered for our study were CAT, SOD, and APX. The reactive oxygen species and other free radicals produced in plant system are scavenged by either the antioxidant enzymes and/or water and lipid soluble compounds. Further among these, the antioxidative enzymes are considered to be the most effective against oxidative damage caused by these free radicals[9]. From the result, it was observed that the activities of these enzymes in fenugreek seedlings were significantly altered by the salinity stress as well as the elicitors of nitric oxide signalling. Interestingly, it was found that the activities of almost all assessed enzymes were significantly increased with the increase in the extent of salinity (4dS m^{-1}) and later decreased with further rise in the salt stress (8dS m^{-1}) (Table 3). The plant species and cultivars with tolerant response towards stress conditions have been associated with maintenance of enhanced expression of antioxidant enzymes[15,19]. However significant decline in the antioxidant enzyme activities was observed under the influence of nitric oxide scavenger, APX being most affected by 63% decline. Thus, indicating actively involvement of nitric oxide in regulation of antioxidant enzyme system during salinity stress management.

In conclusion, our results suggest that priming of fenugreek seeds with exogenous source of nitric oxide enhanced the morphological and biochemical attributes along with the antioxidant defense system under saline condition, which was further substantiated by the occurrence of adverse effects of salinity on the seedlings which were unprimed and also those primed with the antagonist of this signalling molecule. Therefore, the enhancement in the enzymatic as well as non-enzymatic components might be due to the involvement of nitric oxide leading to tolerance towards salinity accompanied with better growth and development.

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