Original Article

Free Radical Scavenging Activity and Histochemical Localisation of Reactive Oxygen Species in Fenugreek Sprouts Primed with Nitric Oxide Donors

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ABSTRACT

Objective: Nitric oxide (NO) is considered as an important signal molecule in plant system. In the present study, the effect of some elicitors namely, sodium nitroprusside (SNP), sodium nitrite (NN) and potassium ferricyanide (FCN) on reactive oxygen species localisation and the antioxidant activity of *Trigonella foenum-graecum* L. was studied during germination.
Method: The antioxidant activity and reactive oxygen species localisation were evaluated up to 3 days at an interval of 24h. The *in vitro* antioxidant property was determined by the following spectrophotometric methods: scavenging of DPPH and $ABTS^{+}$ radicals, ferric reducing power and metal chelating property; also phytochemicals like phenol and flavonol contents were determined.
Results: The results demonstrated that the donors of nitric oxide treated seeds showed increase in their antioxidant activity from 24h to 48h and then after declined at stage of 3 days. Overall, the seeds treated with potassium ferricyanide and sodium nitroprusside exhibited higher antioxidant property when compared with that of control. It was evident from the study of histochemical localization of H_2O_2, lipid peroxidation and plasma membrane integrity that sodium nitroprusside and potassium ferricyanide exhibited protective role in the germinating seedlings of fenugreek.
Conclusion: Our result supported that nitric oxide plays an important role to elicit antioxidant components in plants.

Keywords: Antioxidant, germination, histochemical localisation, nitric oxide, *Trigonella foenum-graecum*.

INTRODUCTION

Free radicals, such as reactive nitrogen and oxygen species, are fundamental part of normal physiology. Oxidative stress occurs due to the imbalance of the antioxidant defence system and free radical formation[1,2]. These highly unstable free radicals on reaction with various biomolecules can cause cellular damages. This may lead to the development of several disorders and chronic diseases such as cancers, Alzheimer's and Parkinson's diseases and those related to cardiac and cerebrovascular systems[3]. Antioxidant compounds play vital role in preventing the cellular damages, against the highly unstable chemical components such as free radicals and reactive oxygen species (ROS) which are constantly produced by the cell metabolism and their concentration increases under stress conditions[4]. Phenolics and flavonoids are considered to possess significant antioxidant potential[5], which are found to be very efficient in prevention of human oxidative damages [6].

Nitric oxide (NO) is a short life, highly diffusible gas and a ubiquitous bioactive molecule. Its chemical properties make nitric oxide a versatile signalling molecule that functions through interactions with various cellular components[7]. It is also considered as a reactive nitrogen species and its concentration-dependent impacts on different cell types were reported to be either protective or toxic[8]. In recent years there has been increasing evidences of the involvement of NO in many key physiological processes of plants, such as abiotic stress tolerance[9], plant defense system[10], germination[11], growth and development of plants[12] etc. In earlier studies, the regulatory roles of NO have been reported at different developmental stages of various crops and also have been found to exhibit beneficial role during seed germination in several plants[13,14]. In addition to its regulatory role in plants, there have been reports for NO providing stress tolerance against biotic and abiotic stresses, such as drought[15] and salt stress[16].

The main objective of the present work was to study the alterations in the ROS generation as well as their quenching during post-germination phases under the influence of NO donors. Investigations were performed with fenugreek sprouts at different post-germination stages after priming with standard NO donors namely sodium nitroprusside, potassium ferricyanide and sodium nitrite and the tissue specific localization of ROS along with plasma membrane integrity was determined. Free-radical scavenging activity of those sprouts were also evaluated simultaneously for measuring the elicitation potential of NO donors.

MATERIALS AND METHODS

Materials and treatment

The sterilisation of fenugreek seeds was done with 0.1% sodium hypochlorite solution. The sterilized seeds were primed with different nitric oxide donors: sodium nitroprusside (SNP), potassium ferricyanide (FCN), and sodium nitrite (NN) for 24h. For control set, seeds were primed with normal distilled water and kept in rotary shaker along with the treated seeds[17]. After priming, the seeds were washed with distilled water thrice and placed in the seed germinator for 3 days.

Preparation of extract

The fenugreek sprouts of 3 different stages: 1, 2 and 3 day were crushed in mortar-pestle and processed with methanol through soxhlet extraction apparatus. The refluxed samples were filtered through Whatman No. 1 filter paper and separated from the residues and then after the extract

was concentrated to a definite concentration (1g/ml) using a vacuum rotary evaporator. The obtained methanolic extracts were stored in refrigerator for further experimental analysis.

DPPH based free radical scavenging activity (DPPH)

The DPPH radical scavenging activity of the sprout extracts were evaluated according to method reported by Blois (1958)[18]. The assay was performed by reacting 1.8 ml of 0.1mM DPPH and 0.2 ml of methanolic extracts. The absorbance was measured at 517 nm after 20 min of incubation.

Reducing antioxidant power (FRAP) assay

The reducing power of the extracts was measured by the standard method[19]. The methanolic extracts were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was kept for incubation at 50^{o}C for 20 min. After incubation of 20 min, 2.5 ml of 10% trichloroacetic acid was added to mixture. Then 2.5 ml of solution was mixed with distilled water (2.5 ml) and 0.5 ml of $FeCl_3$ (0.1%). The OD value was measured at 700 nm against a blank.

Metal chelating activity (MC)

The chelating property of the extracts for ferrous ions was determined according to the method of Dinis *et al.*,[20] (1994) with slight modifications. To 400 µl of methanol extract, 1600 µl of methanol was added and reacted with 0.04 ml of ferrous chloride (2 mM). After incubation of 30s, 0.8 ml of ferrozine (5mM) was added. After 10 min of incubation, the absorbance of the Fe^{2+}–ferrozine complex was measured at 562 nm.

ABTS+ radical cation(s) decolorization assay

The $ABTS^{+}$ radical cation(s) scavenging activity was determined according to Re *et al.*[21] (1999) method with some modifications. 2 ml of $ABTS^{+}$ solution was added to 1 ml of extract. After 30 min of incubation the absorbance was recorded at 734 nm.

Total phenolic content (TPC)

Total phenolic contents of sprout extracts were evaluated according to the standard protocol[22]. 1 ml of the methanolic extract was added to a mixture containing 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of 50% Folin-Ciocalteau reagent. After 5 min incubation 1 ml of 5% Na_2CO_3 was added. It was mixed thoroughly and further kept for 1h of incubation. Finally the absorbance of coloured reaction mixture was measured at 765 nm against the reagent blank. The total phenolic content was determined as mg of Gallic acid equivalent per gram fresh weight.

Total flavonoid content (TFC)

The flavonoid content was evaluated by performing a standard spectrophotometric method[23]. 1 ml of extracts was diluted with distilled 4 ml water in a volumetric flask. Initially, 0.3 ml $NaNO_2$ solution (5%) was added to each volumetric flask; after 5 min, 0.3ml $AlCl_3$ (10%) was added; and then again after 6 min, 2 ml NaOH (1M) was added to the mixture. Absorbance of the reaction mixture was taken after adding 2.4ml of distilled water at 510 nm. The total flavonoid content in different extracts was expressed as quercetin equivalent (QE) per gm fresh weight.

In situ ROS detection

The histochemical detection of lipid peroxidation was performed according to Pompella *et al.*[24] (1987). Schiff's reagent

was used to stain the freshly harvested roots; the roots were stained until the red colour developed on the roots. The extra stain imparted by Schiff's reagent was removed by rinsing the stained roots with potassium sulphite solution (0.5% w/v, in 0.05 M HCl). Plasma membrane integrity of the seedlings was detected by the method suggested by Yamamoto *et al*.[25] (2001). Firstly, roots were stained with Evans blue solution (0.025% w/v, in 100 mM $CaCl_2$) for 30 min, then after the roots were rinsed thrice in distilled water to remove extra stain. For detection of H_2O_2 localisation in the roots, the fenugreek seedlings were stained for about 40 to 45 min in potassium iodide/starch reagent (4% w/v starch and 0.1M potassium iodide solution) as reported by Olson and Varner,[26] (1993). Stained roots were photographed under a Nikon SLR camera (Model: D3200).

RESULTS AND DISCUSSION

The determination of antioxidant potential is essential for assessing the nutritional values of the given extracts[27]. Antioxidant property of the sprouts was measured in terms of their free radicals such as DPPH, and $ABTS^+$ scavenging potential; metal chelation and reducing power activity. The antioxidant potential in elicited sprouts of fenugreek was significantly improved over control. The elicitation showed a gradual increase in free-radical scavenging activity for DPPH and $ABTS^+$ from 24h to 48h stage. In comparison to control set, much lower IC_{50} values were exhibited by potassium ferricyanide, indicating higher antioxidant activity (Figure: 1). In case of $ABTS^+$ assay, sodium nitroprusside exhibited the highest scavenging activity among all other treatments in all 3 stages (Figure: 2).

The antioxidant compounds which are efficient chelator of ferrous ion helps in mobilization of tissue iron by forming soluble, stable complexes that are excreted along with the excretory products[28,29]. Consequently chelation therapy has been applied in reducing iron-related disorders such as thalassemia which is characterized by overloaded iron in body organs[30,31]. Here the sprouts treated with SNP exhibited the best metal chelating activity (Figure: 3) among all the treatments as well as control on 2nd day of germination (48h).

The reducing power of the sprouts was measured with ascorbic acid equivalent. Higher ascorbic acid equivalent value exhibited higher reducing capacity of samples. It was observed (Figure: 4) that the sprouts treated with potassium ferricyanide showed enhanced reducing activity with ascorbic acid equivalent value 768.30 mg/g fresh weight (fw) when compared to that of control (689.80 mg/g fw).

For phytochemical analysis, total phenol and flavonol content were evaluated. The phenol and flavonol content of fenugreek sprouts was calculated for 3 days of germination. The highest stimulation was detected at 72h stage for control as well as the elicitors used in this study. The enhancement was significant and detected at different concentration for the different elicitors. Only those elicitors showing the maximal stimulation of phenolics were discussed here. Among the different concentrations applied, 10 mM potassium ferricyanide and 40 mM sodium nitrite exhibited the maximum phenolic content which was found to be 28 % and 29% elevated than the control sprouts on day 3 of germination respectively (Figure: 5). From estimation of flavonol content it was observed that potassium ferricyanide treated samples exhibited the maximum flavonol content (Figure: 6). For all treatments the total phenol and flavonol contents were higher compared to that of control on all 3 days (*i.e*. day 1, 2 and 3).

After detailed analysis of antioxidant properties and their attributes, histochemical study was performed for investigating specific localization of different free-radicals and their pattern of accumulation with germination time.

The Evans blue stain was used to determine the loss of plasma membrane integrity of the seedlings. At 24h stage much variation was observed in the membrane integrity status of the seedlings (Figure: 7). The membrane integrity remained intact for the control set; the roots pre-treated with sodium nitrite exhibited darker stain at low concentration; the same pre-treated with another NO donor *i.e.* sodium nitroprusside stained darker at much higher concentration and the membrane integrity remained more or less stable at higher dosage of priming with potassium ferricyanide, showing less negative effects on the roots in comparison to other elicitors. At 48h stage, plasma membrane of almost all the treated as well as untreated roots were found to be sensitive and stained much darker (Figure: 8). At 72h stage, the membrane integrity of control roots were found to be sensitive and hence damaged (Figure: 9); interestingly sodium nitrite priming enhanced plasma membrane integrity as evidenced from mild stained patches of roots; however sodium nitroprusside greatly affected the membrane integrity of the roots and those treated with potassium ferricyanide were found to be more or less sensitive and not exactly dose-dependent.

The Schiff's reagent was utilized for the measurement of the degree of membrane lipids peroxidation of the seedlings. At 24h stage, accumulation of malonaldehyde took place at the growing region of the control roots (Figure: 10); the roots treated with potassium ferricyanide and sodium nitrite exhibited lighter stain at lower concentration and darker stain at higher concentration thus proving the protective effect of potassium ferricyanide against peroxidation of membrane lipids at lower concentration; in case of roots treated with sodium nitroprusside, at lower concentration the growing region were affected and at higher concentration the root tips and meristem region was affected. At 48h stage, roots of almost all the treated as well as untreated seeds were found to be sensitive and stained much darker towards higher concentration (Figure: 11). At 72h stage, the accumulation of malonaldehyde occurred at the root tip region (Figure: 12) of hydroprimed set and for the roots treated with different elicitors, the accumulation of malonaldehyde was dose dependent *i.e.* higher the treatment concentration darker the stains appeared and those roots treated with sodium nitrite were found to be much more sensitive than others.

The potassium iodide-starch reagent was used to determine the H_2O_2 localisation in the roots of fenugreek seedlings. At 24h stage no H_2O_2 localisation occurred in the roots of treated as well as control set (Figure: 13). At 48h stage, no accumulation of H_2O_2 was observed in the control roots (Figure: 14); the seedlings treated with potassium ferricyanide were found to be resistant but dose dependent H_2O_2 localisation was observed at the growing region and those treated with sodium nitroprusside and sodium nitrite were very much sensitive and again the accumulation of H_2O_2 at the growing region of the roots was found to be dose dependent. At 72h stage, strong accumulation of H_2O_2 was observed at the roots of control set (Figure: 15); the roots treated with sodium nitrite were much sensitive and stained darker even at lower concentration but those treated with sodium nitroprusside and potassium ferricyanide at lower concentration showed no H_2O_2 localisation in the roots signifying the defensive effect against H_2O_2 radicals.

It was earlier reported that plant cells accumulate a measurable quantity of H_2O_2

during biotic and abiotic stresses[32]. This H_2O_2 production was actually stimulated by the action of plasma membrane bound NADPH oxidases, which was class-III plant peroxidases and utilized $NADP^+$ as electron donor[33]. But excess generation of H_2O_2 has proved to be toxic for plant cells and H_2O_2 mediated death of cells during pathogen infection is a part of hypersensitive response. In contrast during abiotic stress situation moderate level of H_2O_2 accumulation might play a role as secondary messenger of stress signalling and establish tolerance mechanism[34]. During oxidative stress, NO also serves as a part of signalling cascade and exhibits antioxidant activity by directly quenching the ROS including peroxides and superoxides and protecting cell against oxidative injury[35]. This is in agreement with the responses obtained in present study where application of lower concentration of NO donors *viz.,* SNP and FCN showed protective action against H_2O_2 radicals. However dose-dependent localisation of H_2O_2 after treatment with higher concentration of NO donors might execute the pro-oxidant activity of NO signal, as explained by other workers[36].

Malonaldehyde accumulation might create injury to biological membrane particularly at growing region of the roots by forming free radicals for peroxidation of poly-unsaturated fatty acids[37]. During heavy metal and metalloid toxicity, NO supply might partially prevent lipid peroxidation and inhibit ion leakage from tissues[38]. In our study, protective action of nitric oxide against malonaldehyde accumulation was found after priming with mild doses of FCN and SNP during early stages of germination. It was revealed from earlier studies that NO partially alleviates UV-induced damage[39] or iron-deficiency induced oxidative stress by directly quenching with superoxide and peroxide[40]. Similar findings were also obtained by Rubbo *et al.*,[41] (2000), who claimed that NO is a potent inhibitor of malonaldehyde accumulation. Higher anti-lipid peroxidation activity was observed with the increasing level of NO upon addition of SNP in the roots of rice seedlings under arsenic phytotoxicity[42]. NO signalling in plants display equivalent response like mammalian system and kinetically terminates lipid peroxyl radical via LOO^- intermediate[43]. But same NO beyond optimized dose utilise catalytic metal centres of enzymes for propagating lipid peroxidation[43]. This is in agreement with our results where dose-dependent accumulation of malonaldehyde was observed particularly at root tip of fenugreek when elicited with stronger concentration of NO donors.

Accumulation of malonaldehyde is generally correlated with injury to biological membranes due to peroxidation of poly-unsaturated fatty acids which acts like free radicals[37]. Therefore membrane fluidity is altered due to lipid peroxidation and the ultimate fate is serious leakage from plant tissue. By mitigating lipid peroxidation, NO upholds the integrity of plasma membrane and hinders ion leakage from plant tissues[38]. Membrane function is important for all physiological activity and NO supply at higher concentration suggests a negative impact on membrane integrity, as determined from our experiments. In contrast, exogenous supply of NO at lower dose has an ameliorating effect on several heavy metal induced toxicity[44,45] and enhances membrane integrity by reducing oxidative stress mediated injury through elicitation of antioxidant enzymes. When fenugreek seeds were primed with FCN, membrane integrity of roots remained more or less stable but the same concentration of SNP deteriorated membrane functioning seriously as demonstrated by the uptake of non-permeable Evans blue dye into the damaged root cells. This phenomenon suggests differential response of two

different NO donors in fenugreek. Overall, at lower concentration NO displays a wide range of beneficial impact on growing seedlings and act as an antioxidant signal, whereas the same at higher concentration might act like pro-oxidants[35].

Overall it was observed that among all treatments, sodium nitroprusside and potassium ferricyanide treated sprouts exhibited consistency in their antioxidant potential but sodium nitrite treated sprouts was found to exhibit relatively inconsistent antioxidant behaviour. It is reported that the efficacy of plant phenolics in protection against oxidative damage depends on their reactivity towards the free radicals. The reduction of phenoxyl radicals by the cellular reducing agents is well-known to reprocess the phenolic antioxidants, thus enhancing the antioxidant potential in the system[46]. Sprouting significantly enhanced the antioxidant property of control and other treatments. The highest antioxidant property of sprouts during 48h stage indicates that the components responsible for free-radical scavenging activity were elicited appropriately at this stage. Another reason might be that, during early germination phase there is a higher oxygen demand and consequently the phenolic compounds might be involved in the process of protecting the cells from probable oxidative stress-induced damage[47]. High phenolics content but low antioxidant activity observed in treated sprouts compared to control during later stages of germination indicates that most phenolics under natural conditions are diverted towards lignification with growth which was also observed during elicitor induced germination of corn[48]. Whereas in case of treatments with nitric oxide elicitor, possibly the stress imposed by these elicitors reduces the amount of phenolics being partitioned for lignification because some are utilized for antioxidant function against the ROS generated in the system. It might also be same phenomenon for the sprouts of 72h that exhibited considerably high phenolic content but low antioxidant activity compared to 48h sprouts; it may be due to minimal utilization of phenolics against oxidative stress.

CONCLUSION

The present research work was an endeavour to study the effect of exogenous nitric oxide on ROS-induced stress and variation in antioxidant activities. Effects of NO donors were studied on the free-radical scavenging potential of the germinating fenugreek seedlings at different developmental phases (24h, 48h & 72h). When examining the time course of germination the stimulatory effect of nitric oxide donors was found to be most evident at the initial germination phase i.e. from 24h to 48h and after that the action was declined. The impact of these elicitors of NO was also studied by *in situ* detection of lipid peroxidation, loss of membrane permeability and H_2O_2 accumulation in the roots of fenugreek. As a result it was observed that NO at lower concentration exhibited protective role against these factors in the roots whereas loss of membrane integrity along with H_2O_2 and malonaldehyde accumulation was enhanced at higher doses. In a nut shell, it can be concluded that NO induces free-radical scavenging activity and also exhibits beneficial role during germination of fenugreek seedlings however priming with higher doses of NO donors leads to cellular damage and phytotoxicity resulting in inhibition of growth along with deterioration of free-radical scavenging properties. The work supports the hypothesis that NO acts as oxidant as it offered significant role in enhancement of phenolic compounds and antioxidant property during the germination process of fenugreek as well as pro-oxidant as it exhibited inhibitory role at higher doses.

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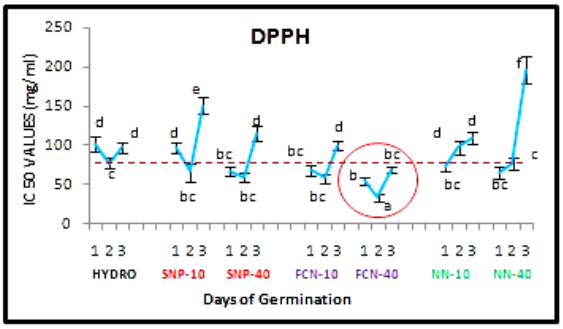

Figure:1- DPPH free radical scavenging activity of fenugreek sprouts primed with nitric oxide donors

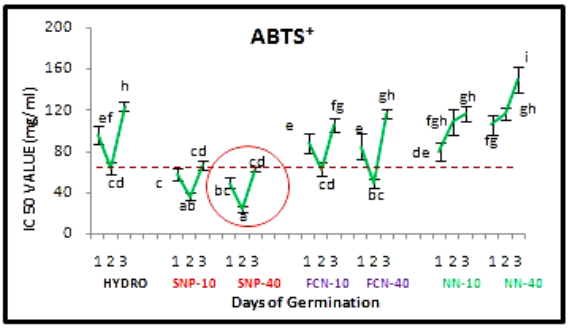

Figure:2- ABTS^{+} radical scavenging activity of fenugreek sprouts primed with nitric oxide donors

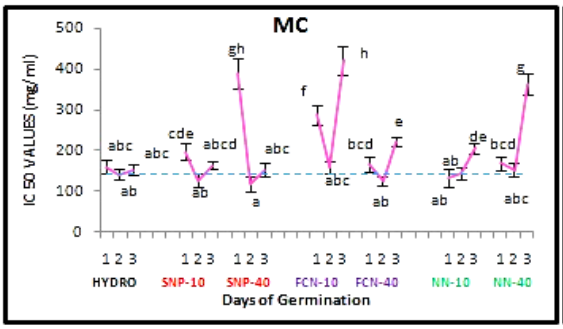

Figure:3- Metal chelating activity of fenugreek sprouts primed with nitric oxide donors

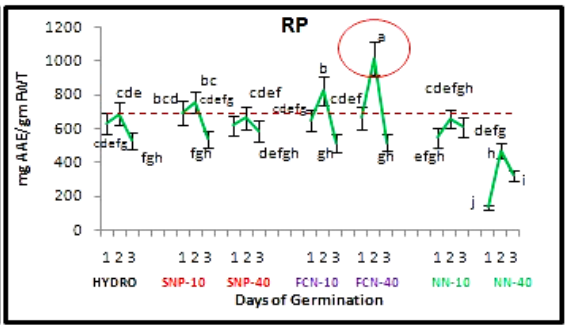

Figure:4- Reducing power activity of fenugreek sprouts primed with nitric oxide donors

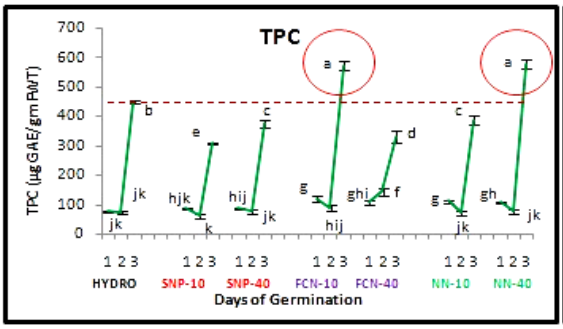

Figure:5- Total phenol content of fenugreek sprouts primed with nitric oxide donors

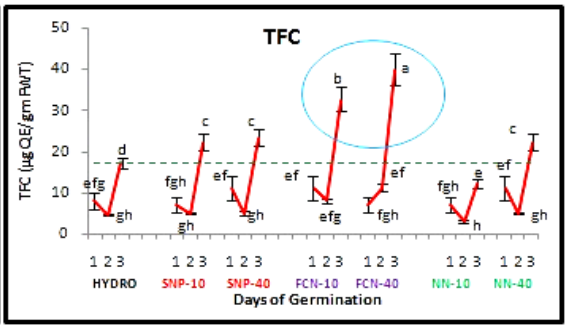

Figure:6- Total flavonol content of fenugreek sprouts primed with nitric oxide donors

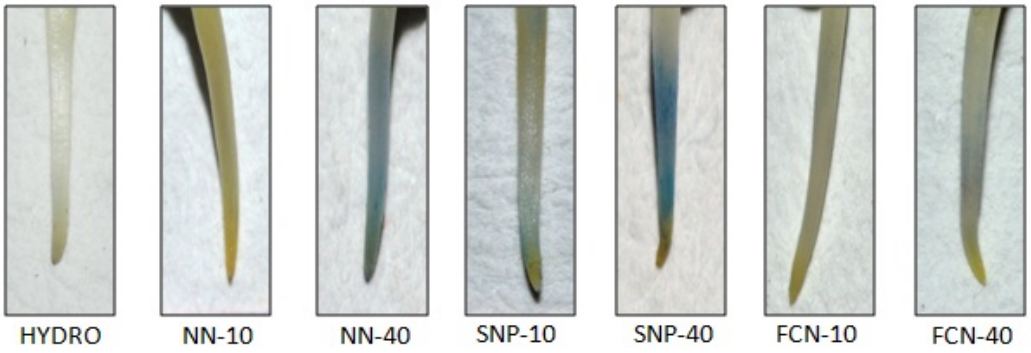

Figure.7- Histochemical studies showing the effect of different nitric oxide donors upon plasma membrane integrity in roots of 24 hours old fenugreek seedlings.

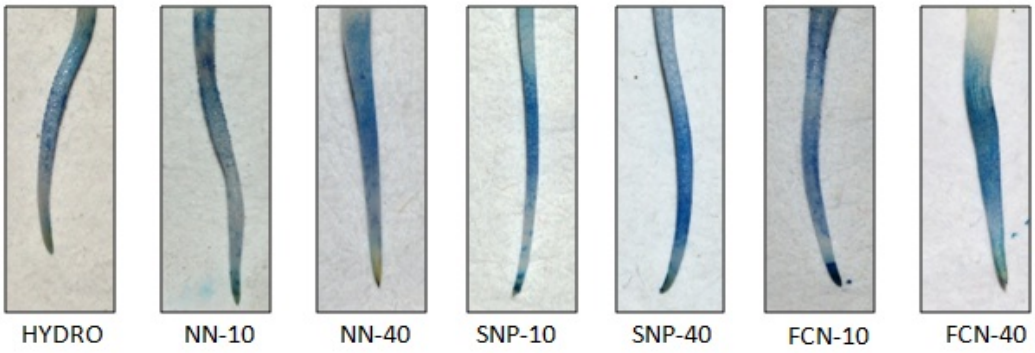

Figure.8- Histochemical studies showing the effect of different nitric oxide donors upon plasma membrane integrity in roots of 48 hours old fenugreek seedlings.

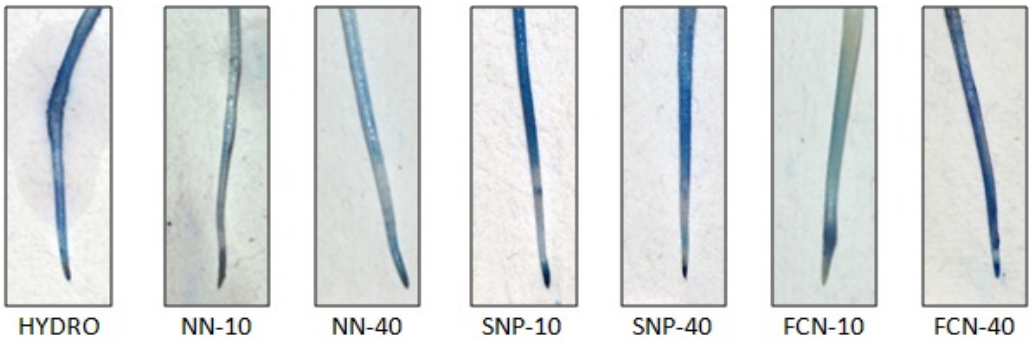

Figure.9- Histochemical studies showing the effect of different nitric oxide donors upon plasma membrane integrity in roots of 72 hours old fenugreek seedlings.

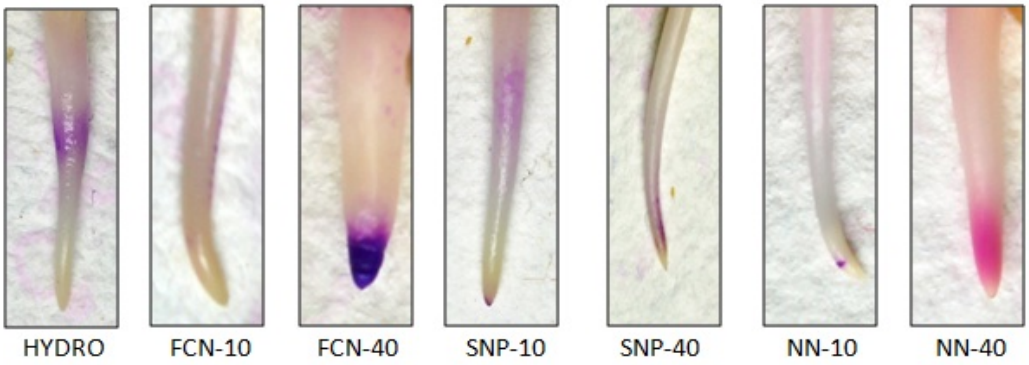

Figure.10- Histochemical studies showing the effect of different nitric oxide donors upon lipid peroxidation in roots of 24 hours old fenugreek seedlings.

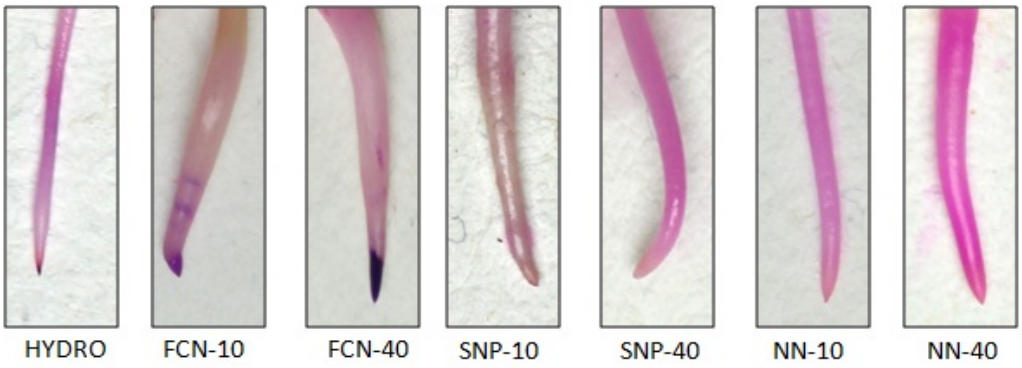

Figure.11- Histochemical studies showing the effect of different nitric oxide donors upon lipid peroxidation in roots of 48 hours old fenugreek seedlings.

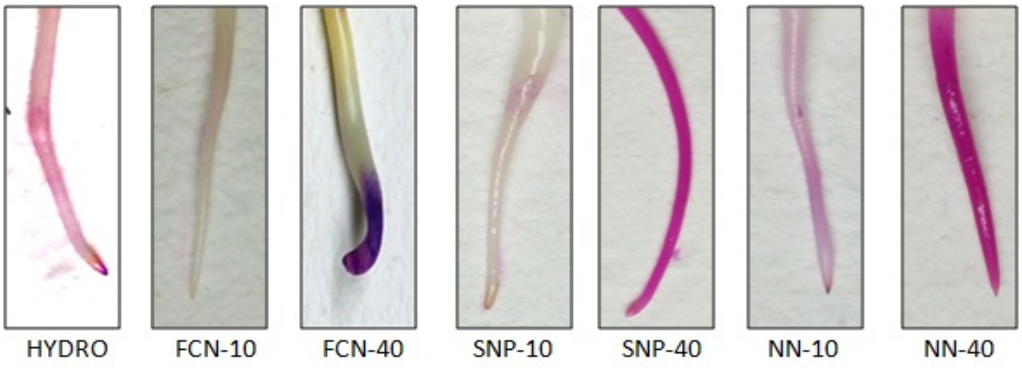

Figure.12- Histochemical studies showing the effect of different nitric oxide donors upon lipid peroxidation in roots of 72 hours old fenugreek seedlings.

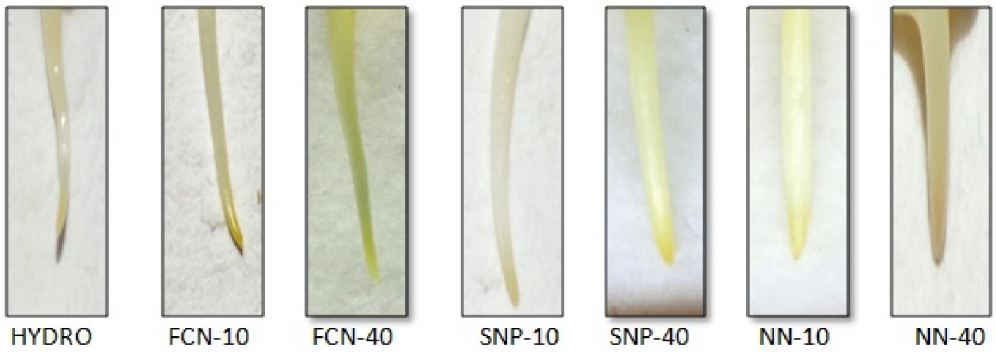

Figure.13 - Histochemical studies showing the effect of different nitric oxide donors upon H_2O_2 localization in roots of 24 hours old fenugreek seedlings.

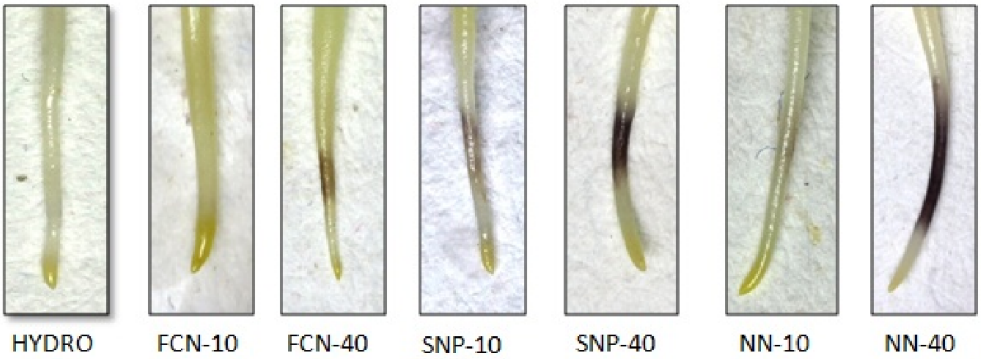

Figure.14 - Histochemical studies showing the effect of different nitric oxide donors upon H_2O_2 localization in roots of 48 hours old fenugreek seedlings.

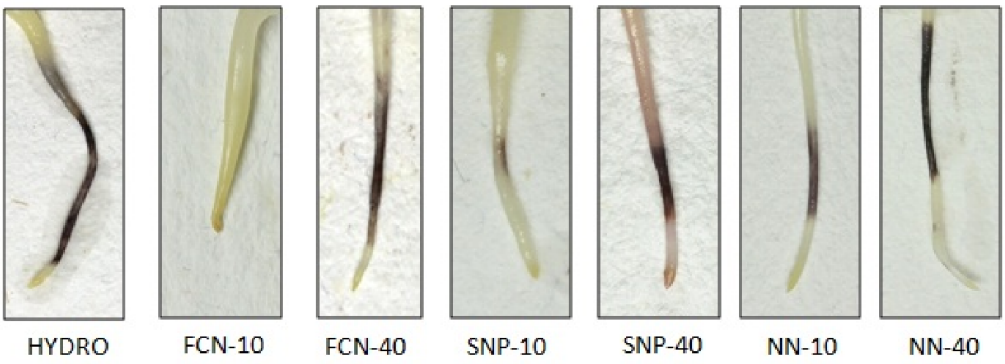

Figure.15 - Histochemical studies showing the effect of different nitric oxide donors upon H_2O_2 localization in roots of 72 hours old fenugreek seedlings.