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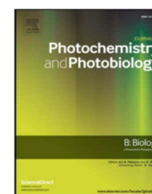

Magneto-priming promotes nitric oxide *via* nitric oxide synthase to ameliorate the UV-B stress during germination of soybean seedlings

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ABSTRACT

We have evaluated the contribution of nitric oxide (NO) in static magnetic field (SMF-200 mT for 1h) induced tolerance towards UV-B stress in soybean seedlings using various NO modulators like sodium nitroprusside (SNP), inhibitor of nitrate reductase (NR) sodium tungstate (ST), NO synthase (NOS) inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) and diphenylene iodonium (DPI) a NADPH oxidase inhibitor. The UV-B exposure significantly reduced germination, seedling growth together with activities of total amylase, NOS and NR in seedlings from un-primed seeds whereas SMF-primed seedlings showed significant enhancement in all these parameters along with higher level of NO/ROS. The supply of NO donor, SNP further improved all the seedlings parameters in un-primed and SMF-primed seeds after UV-B exposure. While ST, L-NAME and DPI significantly reduced the SMF-induced seedling performance after UV-B exposure. The gene expression study also showed significant up-regulation of α-amylase (*GmAMY1*, *GmAMY2*), nitric oxide synthase (*GmNOS2*) and nitrate reductase (*GmNR2*) encoding genes in UV-B exposed SMF-primed seedlings over un-primed seedlings. In particular, SNP+UV-B treatment enhanced the *GmNOS2* expression in both unprimed (31.9-fold) and SMF-primed (93.2-fold) seedlings in comparison to their respective controls of CK+UV-B. In contrast, L-NAME+UV-B treatment reduced the SMF-induced *GmNOS2* expression (4.8-fold) and NOS activity (76%). It confirmed that NO may be the key signaling molecule in SMF stimulated tolerance towards UV-B stress during early seedling growth and NOS may possibly be accountable for SMF-triggered NO production in soybean seedlings exposed to UV-B irradiations.

1. Introduction

Plants are frequently exposed to different abiotic stresses like drought, heat, salinity, floods, heavy metal toxicity which adversely affect their growth and development [1]. In agriculture, these stresses are matter of concern due to unpredictable and substantial loss in crop productivity [2]. Ultraviolet radiation (UV) is a part of the spectrum of electromagnetic rays emitted by the sun, and it is arbitrarily divided into three bands of wavelengths that are UV-C (290–200 nm), UV-B (320–290 nm) and UV-A (400–320 nm). The UV-C is totally absorbed *via* stratospheric ozone layer, while UV-B and UV-A can reach at the surface of the Earth [3]. The amount of solar UV-B radiation at the Earth surface is enhanced owing to considerable reduction of the stratospheric ozone (O_3) layer [3,4]. UV-B radiation is currently near to its highest level, and is predictable to revert to pre-1980s [5]. Though, numerous factors together with rising concentrations of greenhouse gases, may possibly delay this return [3,6]. The probable damaging consequences of enhanced UV-B intensity on biological and ecological system have been paid attention globally [3,4]. UV-B radiations can cause rigorous inhibition in biomass allocation, photosynthesis, transpiration, and eventually reduced the growth and yield of plants [3]. This is a consequence of damage caused by UV-B to macromolecules through protein degradation, lipids peroxidation and breakage of double helix DNA [3,7,8]. The reactive oxygen species (ROS) like superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) are potentially produced in plants by UV-B radiation [7–9]. On the other hand, various defense reactions are induced in plants to achieve the tolerance towards UV-B radiation [8,9].

A variety of seed priming methods have been used in several species of crops, to get better rate and uniformity of emergence along with seed

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viability in normal and stressed conditions [10,11]. Amongst physical priming, magneto-priming is widely used recently in which seeds are treated through magnetic field (MF) and it is found to enhance the seed germination, germination speed and seedling vigor in number of plant species [12,13]. In addition, several previous reports suggested that SMF-priming supports the plants to fight with the unfavorable consequences caused by abiotic stresses without affecting the environment adversely [12,14].

Nitric oxide (NO) is an extremely diffusible bioactive signaling molecule. It is recognized to regulate a broad variety of physiological development like seed germination, mitochondrial functionality, iron homeostasis, floral regulation, and fruit ripening in plants [15–17]. Furthermore, NO concentration also regulates the plant performance under different biotic and abiotic stresses like high and low temperature, UV-B, heavy metal, drought and salinity [18–23]. In particular to UV-B stress, various mechanisms have been proposed for NO mediated plant adaptation. For instance, UV-B exposure increases the abscisic acid (ABA) concentration which consequently activates the H_2O_2 and NO accumulation to recover the UV-B incited cell damage in maize leaves [24]. Similarly, UV-B exposure leads to increased H_2O_2 content which further up-regulates the expression of NOS gene to enhance NO content in kidney bean and Arabidopsis [25,26]. Also, NO provide shield from oxidative injure caused with UV-B radiation in soybean and lettuce [27,28].

The NO is generated by two pathways in plants; non-enzymatic and enzymatic. Enzymes participated in enzymatic pathway are nitrate (NO_3^-) or nitrite (NO_2^-) reliant nitrate reductase (NR), nitric oxide synthase (NOS, arginine dependent), nitrite-NO reductase (Ni-NOR) [18]. The NR enzyme has been proved to be involved in the NO production upon UV-B exposure in *Betula pendula* [29], upon toxicity of Cu in barley seedlings [30]. Recently, Kataria et al. [23] also reported the participation of NR regulated NO production in SMF provoked tolerance against salt stress using NO and ROS modulators such as SNP, 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), L-NAME or ST and DPI.

The consequences of UV-B radiation upon growth, photosynthesis, nitrogen metabolism and productivity has been broadly investigated earlier in soybean [31–33]. However, the adverse impact of enhanced UV-B radiation upon seed germination and seedling growth parameters of soybean has not been studied yet. Moreover, a little information is available about SMF-priming based attenuation of damaging effects of ambient UV-B stress in soybean plants under field conditions [31,32]. Hence, it is imperative to identify the physiological, biochemical and molecular markers along with regulatory mechanisms involved in the SMF-priming based tolerance towards UV-B stress. Therefore, present study was envisaged to see the contribution of NO in SMF-priming based stimulation of tolerance against UV-B stress during seed germination and early seedling growth of soybean.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The soybean seeds (*Glycine* max [L.] variety JS-335) were procured from ICAR-Indian Institute of Soybean Research, Indore, Madhya Pradesh, India. Seeds were first surface sterilized with 0.01% $HgCl_2$ for 2 min, after that all the seeds were washed 4–5 times with distilled water and 15 seeds were kept in 15 cm diameter Petri plates lined with Whatman No.1 filter discs moistened with distilled water or different modulators of NO and ROS. After keeping the seeds in Petri plates, these just imbibed seeds were exposed to UV-B. Subsequently, all the Petri plates were placed in the dark for 5 days at 25 ± 1 °C. After 5 days all the parameters were measured.

2.2. Treatments of Static Magnetic Field (SMF), UV-B and NO and ROS Modulators to Soybean Seeds

The SMF-treatment to soybean seeds was given by electromagnetic field generator "AETec" (Academy of Embedded Technology, Delhi, India) following the method mentioned in Kataria et al. [32]. The unprimed (UP) and SMF-primed (MP) soybean seeds were surface sterilized and were allowed to germinate in Petri plates with exogenously supplied modulators of NO and ROS like NO donor, sodium nitroprusside (SNP), sodium tungstate (ST) as nitrate reductase (NR) inhibitor, N (G)-nitro-L-arginine methyl ester (L-NAME) as nitric oxide synthase (NOS) inhibitor, and diphenylene iodonium (DPI) as NADPH oxidase inhibitor. Fig. 1 illustrates how the treatment of NO/ROS modulators and UV-B exposure were given to the soybean seeds. Different concentrations of modulators used for treatment in the present investigation were as follows: 200 μM SNP, 2 mM ST, 0.2 mM L-NAME and 0.1 mM DPI. The Whatman's filter paper number 1 was lined in Petri plates and seeds were imbibed in 10 ml of each modulators of NO/ROS or 10 ml of distilled water (used as control, CK) for without UV-B (CK–UV-B) and with UV-B exposed (CK+UV-B). On the basis of fluence response obtained for different time period exposure of UV-B (0 min to 120 min) irradiation on just imbibed soybean seeds (data not shown), 1 h of UV-B exposure time period was selected and used for the experiments to examine the participation of NO in SMF incite tolerance towards UV-B stress of soybean seedlings. The Petri plates containing just imbibed seeds with different modulators of NO/ROS were then transferred to UV-B radiation chamber and exposed for 1 h to fluorescent UV-B tubes. After UV-B exposure, these Petri plates were set aside in to the incubator at 25 ± 1 °C for 5 days in dark. For safety reasons the radiation chamber was enclosed by wooden cover. Two UV-B fluorescent tube (TL40W/12, Philips, Eindhoven, The Netherlands) was fitted in the chamber which showed its emission >280 nm to a maximum at 312 nm. The intensity of UV-B radiation was 2.9 $mW/cm^2/s$ measured by radiometer, Solar light Co. Inc. (PMA 2100), Glenside, PA, USA.

2.3. Germination Percentage and Seedling Growth Characteristics

After 3-days of treatment, seeds were observed for germination and abnormal dead seeds were excluded from the study. The germination percentage was determined as the relation of the germinated seeds number to the total number of seeds on the basis of normal seedlings. After 5-days of treatment, ten normal seedlings in triplicates (n = 3) were randomly selected to measure seedling length and dry weight. To

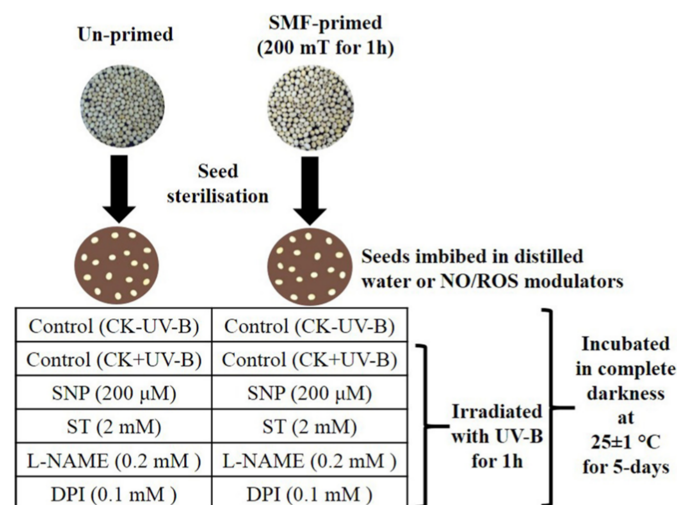

Fig. 1. The methodology used to treat the soybean seeds (both un-primed and SMF-primed) with different modulators of NO and ROS and UV-B irradiation.

obtain the seedling dry weight, seedlings were placed in an oven set at 70 °C for 72 h and dry weight was taken using an analytical balance.

Seedling vigor was measured as suggested by Abdul-Baki and Anderson [34].

$$\text{Vigor index I} = \text{Percentage germination} \times \text{Seedling length (Root + Shoot)}$$

$$\text{Vigor index II} = \text{Percentage germination} \times \text{Seedling dry weight (Root + Shoot)}$$

The germination stress tolerance index (GSTI) of unprimed and SMF-primed seeds was determined by using the formula suggested by Ashraf et al. [35]. Germination stress tolerance index (GSTI) = (PI of UV-B irradiated unprimed or SMF-primed seeds / PI of controls from unprimed or SMF-primed seeds which are not exposed to UV-B) x 100.

$$PI = nd1 + nd2 + nd3 + nd4$$

where, PI is promptness index, nd is the number of seeds germinated on the day of observation [36] and nd1, nd2, nd3, nd4 are the number of germinated seeds on the 1st, 2nd, 3rd and 4th day, respectively.

2.4. Reactive Oxygen Species (ROS) and Nitric Oxide (NO) Content Estimation

2.4.1. Superoxide Radical ($O_2^{\bullet -}$) Content

The $O_2^{\bullet -}$content in 5-days old germinated soybean seedlings was determined by the method of Chaitanya and Naithani [37]. The 100 mg seedlings were crushed with cold 0.2 M sodium phosphate buffer (pH 7.2) comprising of diethyldithiocarbamate (10–3 M) to inhibit the activity of super oxide dismutase (SOD) [38]. The homogenate was centrifuged at 10,000 rpm at 4 °C for 15 min. The $O_2^{\bullet -}$ content was estimated in the supernatant by its capability to reduce nitro blue tetrazolium (NBT, 2.5×10^{-4} M). The absorbance was recorded at 540 nm wavelength using UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan). The $O_2^{\bullet -}$ content was estimated using a molar absorption coefficient of 12.8 mM^{-1} cm^{-1} and represented as μmole $O_2^{\bullet -}$/g fresh weight (FW) of seedlings.

2.4.2. Hydrogen Peroxide (H_2O_2)

To estimate the H_2O_2 content, 500 mg seedlings were ground in the 5 ml of 100% acetone and then homogenate was filtered through Whatman No. 1 filter paper. After that, in the filtrate a titanium reagent (2 ml) and ammonium hydroxide solution (2.5 ml) was added for the formation of titanium-hydroperoxide complex [39]. After that complete reaction mixture was centrifuged at 12,000 rpm for 15 min at 4 °C. The pelleted precipitate was dissolved in the 2 ml of 2 M H_2SO_4 and re-centrifuged at 10,000 rpm for 10 min at 4 °C. The absorbance of supernatant was taken at 415 nm using UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan) and H_2O_2 content was expressed as μmole H_2O_2/g fresh weight of seedlings.

2.4.3. Nitric Oxide (NO) Content

The NO content was estimated by the method of Zhou et al. [40]. The soybean seedlings (500 mg) were homogenized in 50 mM acetic acid buffer (pH 3.6) containing 4% zinc diacetate (pH 3.6) and then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant extract was assorted with Greiss reagent (1% sulphanilamide +0.1% N-1-napthylethylenediaminedihydrochloride in 5% H_2PO_4 solution) and incubated for 30 min at room temperature. After incubation, absorbance of the reaction mixture was recorded at 540 nm using UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan) and NO content was expressed in nmole/g fresh weight of seedlings.

2.5. Assay of Enzymes

2.5.1. Total-Amylase Activity

The total-amylase activity was determined by the method of Sawhney et al. [41]. Soybean seedlings (100 mg) were ground in 5 ml of chilled 80% acetone by mortar and pestle and homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. After discarding the supernatant the pellet was resuspended in 10 ml of 20 mM phosphate buffer (pH 6.4) and centrifuged at 15,000 rpm for 20 min at 4 °C. The supernatant was considered as enzyme extract which was further used to hydrolyze the starch. The total-amylase activity was expressed as mg starch hydrolyzed/ h/g fresh weight of seedlings.

2.5.2. Nitric Oxide Synthase (NOS) and Nitrate Reductase (NR) Activity

The extraction of NOS was done *via* the method described by Lin et al. [42]. NOS activity was measured according to Gonzalez et al. [43] and calculated using the extinction coefficient of NADPH ($\epsilon = 6.22$ mM^{-1} cm^{-1}) and expressed as nmole NO/g fresh weight of seedlings. The NR activity was determined by using intact tissue procedure suggested by Jaworski [44]. The chopped seedlings (250 mg) were mixed in the reaction mixture containing 100 mM phosphate buffer (pH 7.2), 25% isopropanol and 200 mM potassium nitrate and were kept at 30 °C for 2 h. The nitrite formed in the reaction mixture during incubation was measured at 540 nm following azocoupling through naphthalenediaminedihydrochloride and sulphanilamide. The NR activity was represented as nmole NO_2/g FW/h.

2.6. Identification of Soybean α-Amylase (GmAMY), Nitric Oxide Synthase (GmNOS) and Nitrate Reductase (GmNR) Genes

There are three genes named *AT4G25000* (*AtAMY1*), *AT1G76130* (*AtAMY2*) and *AT1G69830* (*AtAMY3*) encodes for α-amylase genes in model plant *Arabidopsis thaliana* [45]. Similarly, one gene *AT3G47450* (*AtNOS1*) encodes for nitric oxide synthase [46] and two nitrate reductase encoding genes; *AT1G77760* (*AtNR1*), *AT1G37130* (*AtNR2*) are present in Arabidopsis genome [47]. The protein sequences of these Arabidopsis genes were downloaded from the Arabidopsis Information Resource (TAIR 10 genome release) and were used to perform blastp analysis against *Glycine* max Wm82.a2.v1 genome available at Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html) [48].

As per our pblast analysis, soybean genome contains three putative α-amylase encoding genes; *Glyma.17G242400* (*GmAMY1*), *Glyma.14G222600* (*GmAMY2*) and *Glyma.14G082700* (*GmAMY3*). Similarly, there are two putative nitric oxide synthase (*GmNOS*) genes encoded by *Glyma.09G224600* (*GmNOS1*) and *Glyma.12G012400* (*GmNOS2*)are present in the soybean genome. Also, two Nitrate reductase (*GmNR*) encoding genes are present in soybean genome which are encoded by *Glyma.13G083800* (*GmNR1*) and *Glyma.06G109200* (*GmNR2*). The identified putative orthologs of α-amylase (*GmAMY1, GmAMY2*), nitric oxide synthase (*GmNOS2*) and nitrate reductase (*GmNR2*) genes in soybean were considered for the expression studies (Table 1).

Table 1
List of soybean genes considered in the present study.

Gene name	Arabidopsis	Soybean	Reference
1. Alpha amylase	AT4G25000 (*AtAMY1*) AT1G76130 (*AtAMY2*)	Glyma.17G242400 (*GmAMY1*) Glyma.14G222600 (*GmAMY2*)	[45]
2. Nitric oxide synthase	AT3G47450 (*AtNOS*)	Glyma.12G012400 (*GmNOS2*)	[46]
3. Nitrate reductase	AT1G77760 (*AtNR1*) AT1G37130 (*AtNR2*)	Glyma.06G109200 (*GmNR2*)	[47]
4. Elongation factor-1-alpha	AT5G60390 (*AtEF1*)	Glyma.19G052400 (*GmEF1*)	[50]
5. Tubulin	AT1G50010 (*AtTUA2*)	Glyma.20G136000 (*GmTUB*)	[50]

2.7. RNA Extraction and cDNA Synthesis

Total RNA was extracted from 5-day old dark grown soybean seedlings of unprimed and SMF-primed seeds grown in different NO/ROS modulators and exposed to UV-B (1 h) using RNAiso Plus reagent (TaKaRa, Shiga, Japan). The genomic DNA was removed from the each RNA sample by treating it with TURBO DNA-freeTM kit (Thermo Fisher Scientific, Invitrogen, USA) according to manufacturer's protocol. Next, RNA integrity was checked on 1.2% (*w/v*) denaturing agarose gel. The complete removal of genomic DNA from all samples was confirmed by a PCR reaction using RNA as template. After that, all samples were quantified using NanoDrop™ 2000 (Thermo Scientific, USA). The cDNA was synthesized with ~2 μg of total RNA template and oligo (dT)18 primer by using SuperScript® III first-strand synthesis kit (Thermo Fisher Scientific, Invitrogen USA) as per manufacturer's protocol.

2.8. Primer Designing and Relative Gene Expression Analysis Using qPCR

Gene specific intron flanking primers were designed using IDT PrimerQuest tool (http://eu.idtdna.com/scitools/Applications/RealTimePCR/) (Supplementary Table. S1). The qRT-PCR reaction (10 μl) was performed in Step One Plus™Real-time PCR system (Thermo Fisher Scientific, Applied Biosystem, USA) using PowerUp™ SYBR™green master mix (Thermo Fisher Scientific, Applied Biosystem, USA). For each sample, two biological replicates each having three technical replicates were considered. In each reaction, ~100 ng cDNA was used as template. The PCR amplification was attempted using initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s. Further, melting curve analysis was done from 60 °C to 95 °C with an increment of 0.3 °C/s for data acquisition. Relative expression level and fold change were calculated using $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen [49]. The transcript abundance in terms of Ct value of Elongation factor-1-alpha (*Glyma.19G052400*) and Tubulin alpha-2 (*Glyma.20 g27280*) genes were used to normalize the expression of target genes [50]. The specificity of amplicons generated by each primer pair was confirmed by melting curve analysis.

2.9. Statistical Analysis

The data presented are in triplicates ($n = 3$); for germination related parameters ten seedlings and for biochemical analysis, three seedlings from each replica were taken for the sampling of all the studied parameters. The data are represented as means ± SE and scrutinize by Prism 4 software for Windows, GrafPad Software, La Jolla, California using the analysis of variance (ANOVA) followed by *post hoc* Newman–Keuls Multiple Comparison Test.

3. Results

3.1. Effect of SMF-Priming on Germination Stress Tolerance Index (GSTI) of Soybean Seedlings after UV-B Irradiation in Presence of Different Modulators of NO/ROS

The NO modulators such as ST and L-NAME and SNP or ROS inhibitor (DPI) were used to study the possible contribution of NO in SMF induced tolerance of soybean seedlings towards UV-B irradiation (1 h). After UV-B exposure for 1 h, the maximum difference was noticed in GSTI amongst SMF-primed (98) and un-primed (74) seedlings in control ones (CK+UV-B) (Fig. 2). Under UV-B irradiation, the SMF induced GSTI values were significantly reduced by ST (29%), L-NAME (25%) and DPI (32%) while in the unprimed seeds values of GSTI was enhanced by SNP (60%) treatment as compared to unprimed seeds of CK + UV-B (Fig. 2). While in case of SMF-treated seeds of SNP + UV-B treatment did not show further enhancement in GSTI values as compared to SMF-primed seeds of CK + UV-B (Fig. 2).

3.2. Effect of SMF-Priming on Growth of Soybean Seedlings after UV-B Irradiation in Presence of Different Modulators of NO/ROS

We have observed that SMF-induced seedling length of soybean under UV-B irradiation was significantly reduced by all of the inhibitors used (ST, L-NAME and DPI) in comparison to SMF-primed seedlings of CK + UV-B (Fig. 3A). The SMF stimulated seedling length after UV-B (1 h) exposure was maximally decreased by DPI (48%), ST (39%) and after that by L-NAME (36%); However, the inducible effect of SNP was found on seedling length of SMF-primed and unprimed seeds exposed to UV-B (1 h) irradiation (Fig. 3A).

The vigor index I and II was significantly decreased by inhibitors of NO and ROS in seedlings obtained from SMF-primed seeds after UV-B irradiation, whereas NO donor-SNP caused substantial enhancement in vigor index-II (Fig. 3B, C). There was 36% reduction observed in vigor index II of soybean seedlings obtained from unprimed seeds after 1 h of UV-B exposure as compared (CK–UV-B) conditions (Fig. 3C). The vigor index II was significantly improved in SMF-primed seeds by 66% in control conditions (CK–UV-B) and by 137% after UV-B (1 h) exposure in comparison to the seedlings from unprimed seeds (Fig. 3C). The maximum reduction in SMF induced vigor index-II after UV-B irradiation was found in DPI (58%), L-NAME (45%) and then in ST (35%) (Fig. 3C).

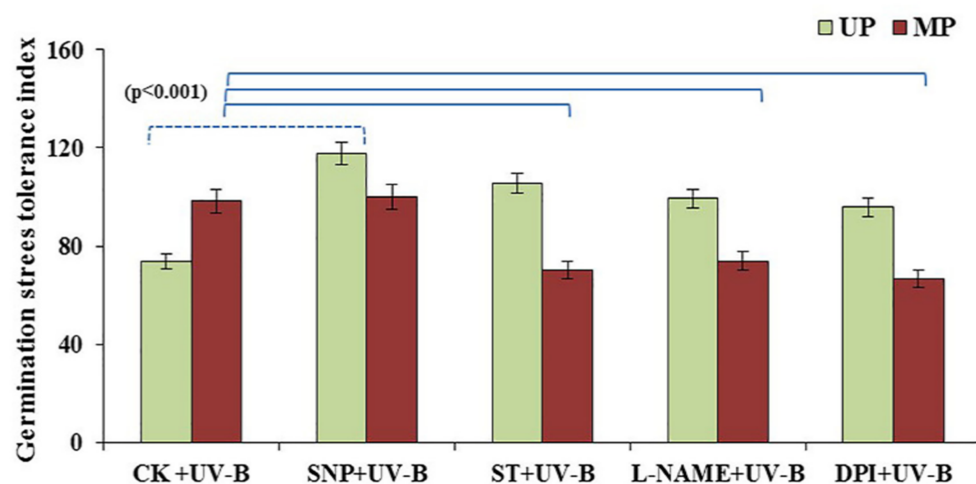

Fig. 2. Effect of SMF-priming on germination stress tolerance index (GSTI) of soybean seedlings after UV-B (1 h) exposure in presence of different NO/ROS modulators. Control+UV-B (CK + UV-B) = seedlings grown in distilled water and exposed to UV-B (1 h). The data are expressed as means ±SE of triplicates ($n = 3$). The dotted line bracket signify the difference ($P < 0.001$) amongst the seedlings from un-primed seeds grown in different modulators of NO/ROS after UV-B exposure with the control seedlings of unprimed seeds after UV-B exposure (CK + UV-B); The solid line bracket signify the difference ($P < 0.001$) amongst the seedlings of SMF-primed seeds grown in different modulators of NO/ROS after UV-B exposure with control seedlings exposed to UV-B (CK + UV-B) from SMF-primed seeds.

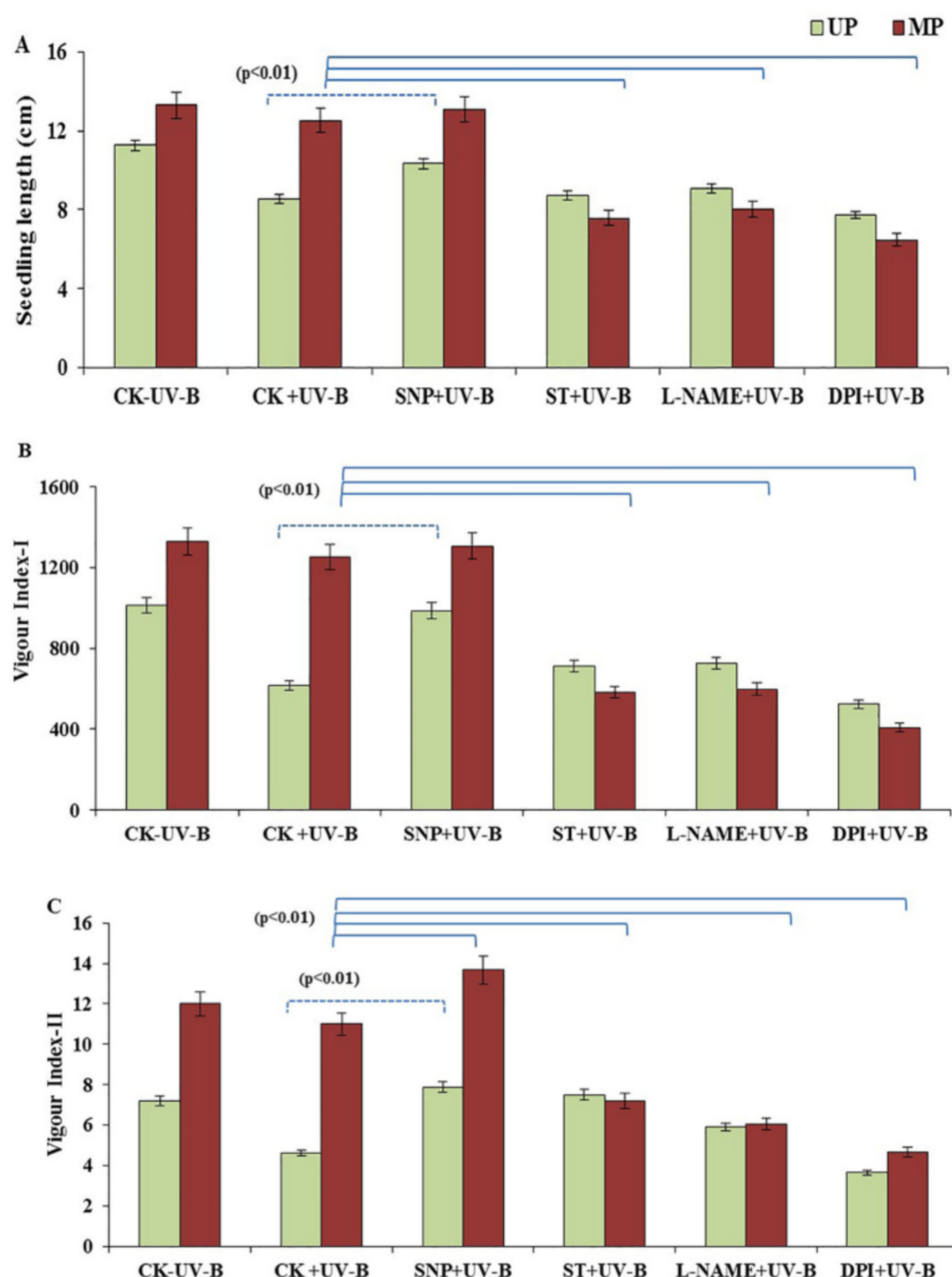

Fig. 3. Effect of SMF-priming on the seedling length (a) vigor index-I (b) and vigor index-II (c) of soybean seedlings after UV-B (1 h) exposure in presence of different NO/ROS modulators. Control-UV-B (CK-UV-B) = seedlings grown in distilled water and not exposed to UV-B; Control+UV-B (CK + UV-B) = seedlings grown in distilled water and exposed to UV-B (1 h). The data are expressed as means ±SE of triplicates (n = 3). The dotted line bracket signify the difference ($P < 0.01$) amongst the seedlings from un-primed seeds grown in different modulators of NO/ROS after UV-B exposure with the control seedlings of un-primed seeds after UV-B exposure (CK + UV-B); The solid line bracket signify the difference ($P < 0.01$) amongst the seedlings of SMF-primed seeds grown in different modulators of NO/ROS after UV-B exposure with control seedlings exposed to UV-B (CK + UV-B) from SMF-primed seeds.

3.3. Effect of SMF-Priming on Content of ROS and NO in Soybean Seedlings after UV-B Irradiation in Presence of Different Modulators of NO/ROS

UV-B (1 h) irradiation caused 15% increase in superoxide ($O_2^{\bullet-}$) content in seedlings commencing from unprimed seeds in contrast to seedlings of unprimed seeds not exposed to the UV-B. SMF caused 35% enhancement in content of superoxide in control (CK-UV-B) conditions (Fig. 4A) as well as 15% increase was found after UV-B (1 h) exposure (CK + UV-B) in comparison to their respective seedlings emerged from unprimed seeds (Fig. 4A). The SNP slightly enhanced the $O_2^{\bullet-}$ content in both the seedlings obtained from unprimed and SMF-primed seeds (Fig. 4A). The NO and ROS inhibitors ST, L-NAME and DPI caused significant reduction in the SMF induced superoxide content after 1 h of UV-B exposure but the level of reduction was more with ST (43%), followed by DPI (35%) and L-NAME (28%) (Fig. 4A). The NO inhibitor L-NAME did not show noteworthy difference in $O_2^{\bullet-}$ content in the seedlings obtained from unprimed seeds exposed to UV-B (1 h) but it was reduced by 28% in DPI + UV-B and 16% in ST + UV-B treatment as compared to the unprimed seeds of CK + UV-B (Fig. 4A).

UV-B (1 h) caused 53% increase in H_2O_2 content of unprimed seedlings when compared to seedlings from unprimed seeds which were not exposed to UV-B irradiation (Fig. 4B). SMF pre-treatment significantly increased H_2O_2 content by 104% in control condition (CK-UV-B) and 31% increase was observed after UV-B exposure (CK + UV-B) (Fig. 4B) in comparison to their respective seedlings emerged from unprimed seeds (Fig. 4B). However, when seedlings were treated with the ST, L-NAME and DPI, then the H_2O_2 content was reduced significantly in the seedlings obtained from unprimed and SMF primed seeds after 1 h UV-B exposure; though the amount of decrease was higher in SMF primed seedlings. The highest decline in SMF induced H_2O_2 content after UV-B (1 h) exposure was caused by L-NAME (80%), ST (69%)

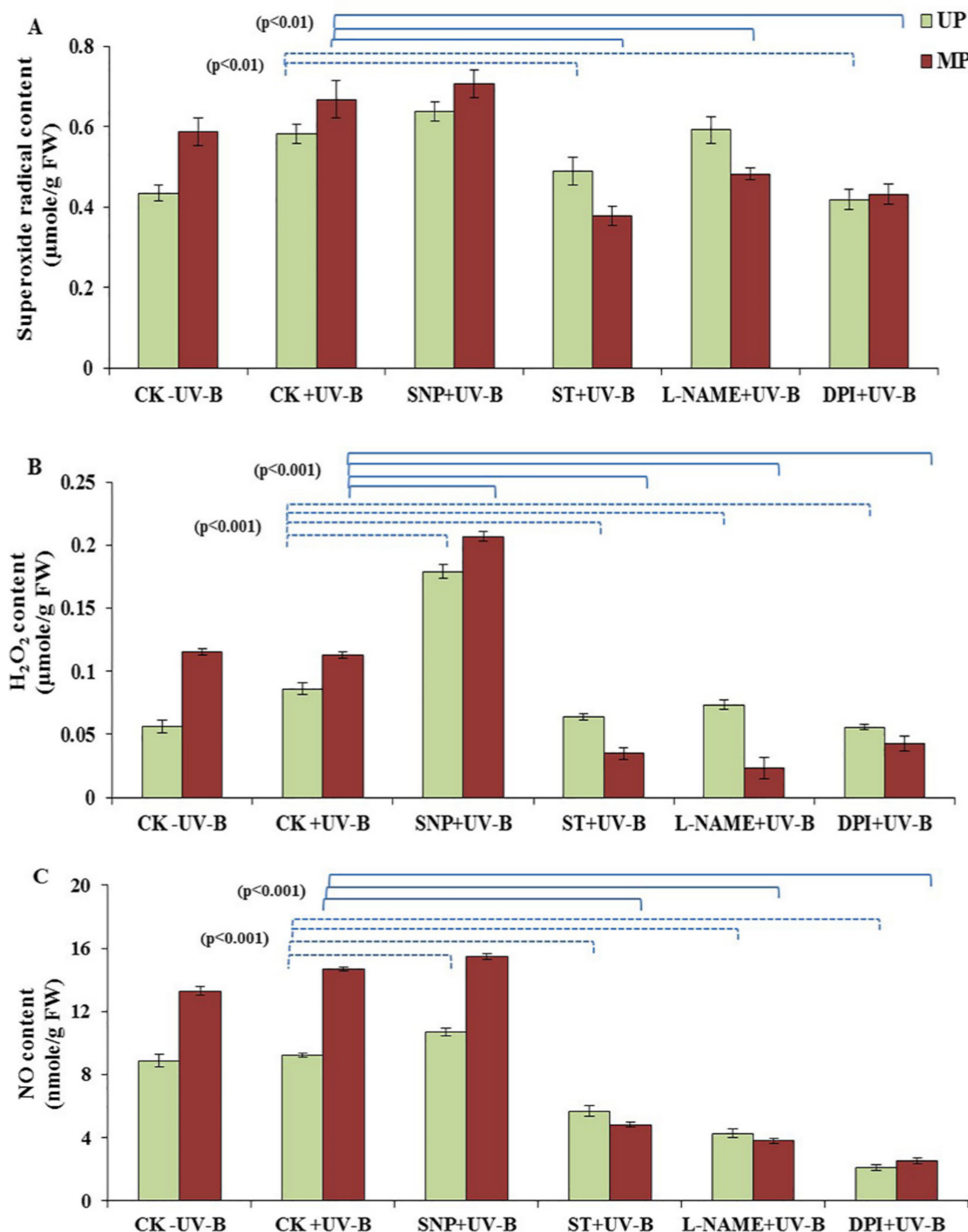

Fig. 4. Effect of SMF-priming on the superoxide (a), hydrogen peroxide (b) and nitric oxide (c) content in soybean seedlings after UV-B (1 h) exposure in presence of different NO/ROS modulators. Control-UV-B (CK-UV-B) = seedlings grown in distilled water and not exposed to UV-B; Control+UV-B (CK + UV-B) = seedlings grown in distilled water and exposed to UV-B (1 h). The data are expressed as means ±SE of triplicates (n = 3). The dotted line bracket signify the difference ($P < 0.001$; $P < 0.01$) amongst the seedlings from un-primed seeds grown in different modulators of NO/ROS after UV-B exposure with the control seedlings of un-primed seeds after UV-B exposure (CK + UV-B); The solid line bracket signify the difference ($P < 0.001$; $P < 0.01$) amongst the seedlings of SMF-primed seeds grown in different modulators of NO/ROS after UV-B exposure with control seedlings exposed to UV-B (CK + UV-B) from SMF-primed seeds.

followed by DPI (62%) (Fig. 4B). On the other hand, DPI caused 35%, ST (26%) and L-NAME (15%) reduction in H_2O_2 content of the seedlings from unprimed seeds after UV-B (1 h) exposure as compared to the control seedlings of unprimed seeds exposed to 1 h UV-B irradiation (Fig. 4B). However, SNP + UV-B caused further enhancement in H_2O_2 content of seedlings from both unprimed and SMF-primed seeds (Fig. 4B).

Nitric oxide content was found to increase slightly by UV-B exposure in seedlings from unprimed seeds, however significant increase was observed in NO content by SMF pretreatment *i.e.* 50% in control (CK-UV-B) and also 60% after UV-B exposure (CK + UV-B) (Fig. 4C). SNP caused further promotion in NO content of unprimed as well as SMF-primed seeds after UV-B irradiation (Fig. 4C). The inhibitors of NO and ROS caused reduction in NO content in seedlings emerged from unprimed and SMF-primed seeds after UV-B irradiation. The seedlings from unprimed seeds showed reduction of 77% (DPI), 54% (L-NAME) and 38% (ST) in NO content upon UV-B irradiation in comparison to their respective unprimed controls (CK + UV-B) (Fig. 4C). On the other hand,

these inhibitors of ROS and NO severely reduced SMF induced NO content after UV-B irradiation; DPI caused (83%), L-NAME (74%) and ST (67%) reduction in SMF induced NO production (Fig. 4C).

3.4. Effect of SMF Priming on Activity of Enzymes in Soybean Seedlings after UV-B Irradiation in Presence of Different Modulators of NO/ROS

When unprimed soybean seeds were exposed to UV-B (1 h), then the total-amylase activity decreased substantially (28%) as compared to control dark grown seedlings not irradiated with UV-B (CK-UV-B) (Fig. 5A). SMF pre-treatment increased the total amylase activity after UV-B exposure by 128% and 150% compared to their respective unprimed controls of CK-UV-B and CK + UV-B (Fig. 5A). NO donor, SNP enhanced the total-amylase activity by 188% and 30% respectively in the seedlings from unprimed and SMF-primed seeds after UV-B (1 h) irradiation (Fig. 5A) as compared to their respective controls (CK + UV-B). While, DPI caused 73%, L-NAME (67%) and ST (64%) reduction in SMF induced total-amylase activity after UV-B (1 h) exposure as compared to SMF-primed seedlings of (CK + UV-B) (Fig. 5A).

UV-B irradiation (1 h) caused slight increment (10%) in NOS activity of unprimed seedlings as compared to the seedlings of (CK-UV-B) (Fig. 5B). Activity of NOS was significantly increased *i.e.* 75% and 114%

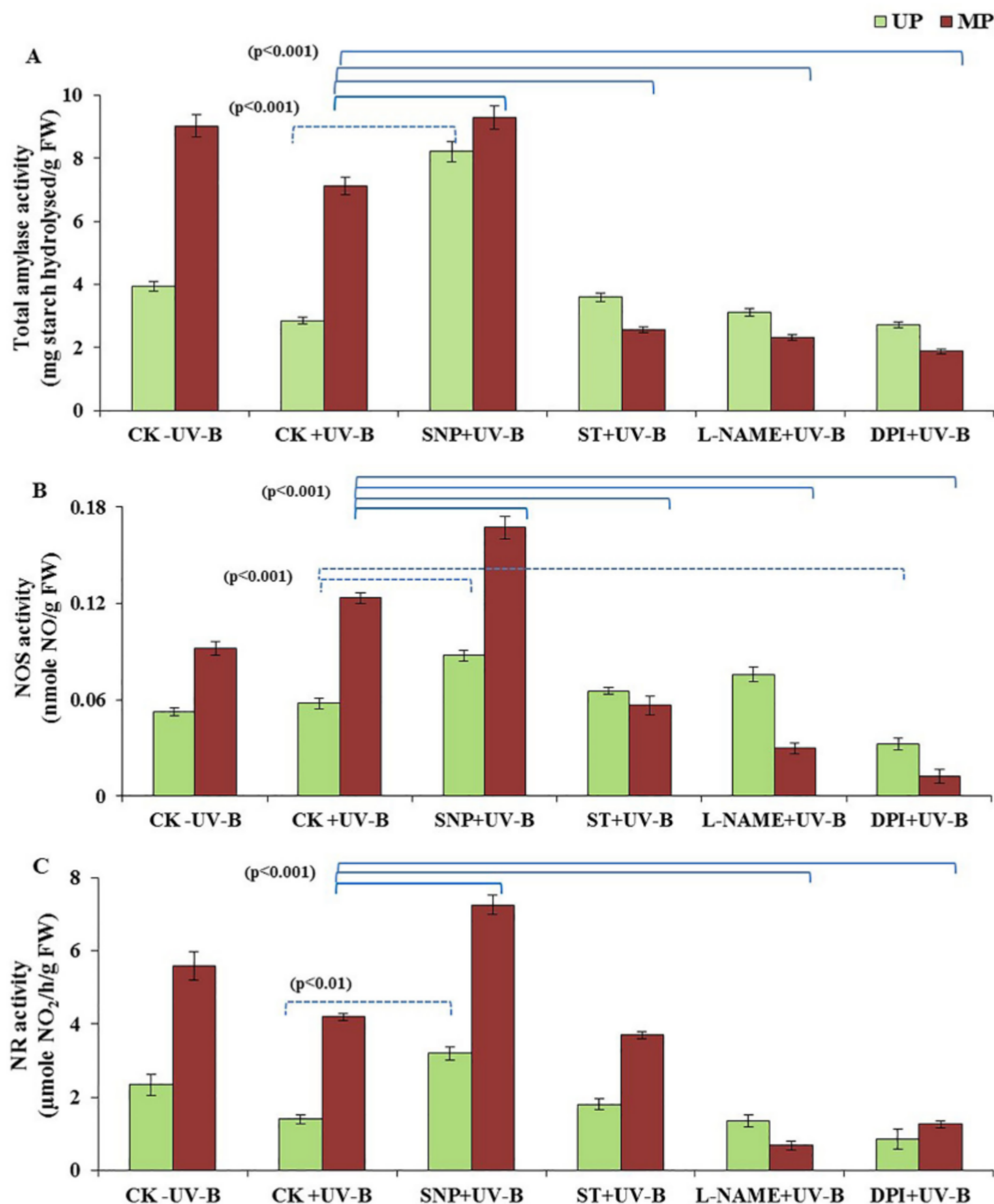

Fig. 5. Effect of SMF-priming on the total-amylase (a) NOS (b) and NR (c) activities in soybean seedlings after UV-B (1 h) exposure in presence of different NO/ROS modulators. Control-UV-B (CK-UV-B) = seedlings grown in distilled water and not exposed to UV-B; Control+UV-B (CK + UV-B) = seedlings grown in distilled water and exposed to UV-B (1 h). The data are expressed as means ±SE of triplicates (n = 3). The dotted line bracket signify the difference ($P < 0.001$; $P < 0.01$) amongst the seedlings from un-primed seeds grown in different modulators of NO/ROS after UV-B exposure with the control seedlings of un-primed seeds after UV-B exposure (CK + UV-B); The solid line bracket signify the difference ($P < 0.001$) amongst the seedlings of SMF-primed seeds grown in different modulators of NO/ROS after UV-B exposure with control seedlings exposed to UV-B (CK + UV-B) from SMF-primed seeds.