

## Research Article

# Redox regulation of adventitious root formation through downstream changes in hormonal system in mung bean [*Vigna radiata* (L.) R. Wilczek]

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## Abstract

Redox regulation on plant morphogenetic process of Adventitious Rooting (AR) can be vouched from the experimental data of redox manipulated salicylic acid treated hypocotyl explants of mung bean. In our previous work, application of pro-oxidant  $H_2O_2$  (500  $\mu M$ ) followed by salicylic acid (SA, 600  $\mu M$ ) have been shown to significantly augment Adventitious Root Formation (ARF), whereas  $10 \times 10^{-4} M$  DPI (Diphenyleneiodonium, an inhibitor of NADPH-oxidase) - 600  $\mu M$  SA and  $10 \times 10^{-4} M$  (Dimethylthiourea, a free radical scavenger) - 600  $\mu M$  SA combination reduced the formation of ARF in hypocotyl explants of mung bean. In the present study, we have examined the impact of such treating conditions or redox manipulation on changes in endogenous titer of bonafide hormone system (gibberelic acid, abscisic acid, jasmonic acid) to document ROS- hormone interaction during adventitious rooting. The work suggests significant impact of redox manipulation on changes in endogenous titer of these hormonal system associated with AR. 500  $\mu M$   $H_2O_2$  - 600  $\mu M$  SA treatment combination, which has augmented formation of AR, has also shown higher accumulation of these hormones as compared to the untreated control. Further, DPI with or without combination of SA which inhibited the ARF, has also reduced the endogenous concentration of these Plant Growth Regulators (PGRs), suggesting role of these growth regulators in low concentration on adventitious root formation. Role of the SA signalling with prooxidant  $H_2O_2$  as second messenger during AR is suggested.

## Introduction

ROS acts downstream of many signaling pathway initiated by plant growth hormones leading to the genesis of adventitious root formation [1-6]. ROS are generated in response to any physical damage or wound [7], acting as a second messenger in response [8,9]. Removal of primary roots may induce a wound signal including ROS that initiates ARF for water and nutrient uptake from the surroundings [3,4,10-13]. Removal of primary roots increased the endogenous  $H_2O_2$  in water treated cucumber explants in compared to the DPI (Diphenyleneiodonium, an inhibitor of NADPH-oxidase) treated explants of cucumber where the primary root removal increased the endogenous  $H_2O_2$  but it got lowered after few hours and remained at lower

concentration unlike in control treatment where it reached its higher peak after 3 hr then declined to its lowest concentration after 11 h, increasing again attaining second peak after 17 h. exogenous application of 20-40 mM  $H_2O_2$  or 10 mM Indole Acetic acid (IAA) significantly increased the number of AR in cucumber seedling explants and 10-50 mM  $H_2O_2$  significantly increased the fresh weight of adventitious roots. 2 mM ascorbic acid, 100 U CAT (Catalase) or 1 mM DPI eliminated the root promoting effect and the effects of IAA were eliminated by 4 mM ascorbic acid, 100 U CAT or 5 mM DPI which were partly rescued by exogenous  $H_2O_2$  clearly indicating the role of  $H_2O_2$  in ARF [14]. Recent study in cucumber, ROS, auxin and ethylene accumulated during water logged condition. Auxin and ethylene both induced the ROS generation. Both ethylene and auxin



induced AR were inhibited by NADPH oxidase inhibitor, DPI, suggesting a significant role of ROS in AR morphogenesis [15]. The Plant growth hormones like auxin, ethylene, NO etc. make use of ROS and other PGRs like Gibberelic acid, Abscisic acid, Jasmonic acid (GA, ABA and JA) etc. in initiating ARF. ABA, GA and JA play an important role in plant responses to biotic and abiotic stress. There is less study in the direct cross talk of ROS with ABA, GA and JA during ARF.

SA, an endogenous plant growth regulator which is found to have both stimulatory and inhibitory effects on ARF [16–21]. It works synergistically with auxin in IAA-induced ARF [22]. In a recent work, it has been shown that SA induces ARF in cucumber through competitive inhibition of the IAA-Asp synthetase activity of CsGH3.5 (*Gretchen Hagen3.5*) leading to the increase in free IAA content. Application of 50  $\mu$ M SA could lead to activation of cyclin and CDK gene expression indicating its effect on adventitious rooting during induction phase [23]. Unlike methyljasmonate, SA increased the scopolamine in adventitious root cultures of *Scopolia parviflora* but did not show any negative effect on root growth, infact SA exposure for relative short time and during active growth phase, all the concentration studied slightly increased the root growth index and did not stimulate root browning [24]. In a study of Tomato stem rooting, it was found that in addition to auxin, ABA, zeatin and SA may play a complementary role in the induction, initiation and emergence of the developing AR and hormone homeostasis is important during all the stages of ARF [25]. However, some workers have found that SA inhibited adventitious root formation. It decreased the weights of roots in mung bean hypocotyls cuttings [20]. It is reported to promote IAA decarboxylation and reduces rooting when applied during the initial days when auxin enhances rooting. During the later phase, when auxin inhibits rooting, it promotes outgrowth of root primordia [21].

ROS supposed to act downstream of SA and application of ROS scavenger DMTU and NADPH Oxidase inhibitor DPI both led to the reduction of ARF in Mung bean hypocotyl [5,16]. Mutants in the alleles *eds5-1* and *eds5-2* (SA induction deficient mutant), unable to synthesize SA produces significantly lower number of ARF as compared to the wild type [16]. In most of the cases, SA found to induce ARF via up-regulation of  $H_2O_2$  formation [16] indicating the implication of ROS [16,18]. Salicylic acid increases the production of ROS via changing the antioxidative defence system like inhibiting the activity of Catalase enzymes and increasing the activity of  $H_2O_2$  formation enzymes [16,26].

GA, a tetracyclic di-terpenoid compound, is a plant hormone involved in plant growth and development. It stimulates Dormancy, seed germination, flowering and sex expression, embryo development, trigger transitions from meristem to shoot growth, juvenile to adult leaf stage, grain development and also interacts with different environmental conditions like Light, temperature etc [29–34]. GA also has a stimulatory effect on root formation [35–37]. Gibberellic acid at low concentration ( $10^{-9}$ – $10^{-6}$  M) promotes rooting, might because of mobilization of low- molecular weight carbohydrates from shoot to lower part. In low irradiance, light may modify the rooting response

of  $GA_3$  treated cuttings of stock plants [38]. Plant cuttings of *Pisum sativum* formed higher number of roots at an irradiance of 16  $W\ m^{-2}$  but lower rooting in higher irradiance than this [39].  $GA_3$  dissolved in water promoted rooting significantly at  $10^{-7}$  M and  $10^{-8}$  M [40]. It is also suggested that root initiation required an appropriate combination of  $GA_3$  and auxin [41]. Higher  $GA_3$  or the lower IAA concentration resulted in more roots in *Persicaria perfoliata* [42].  $GA_3$  increased the number of roots and the effect increased with concentration (from 10 and with 100 mg/L) in stem cuttings of *Ipomoea fistulosa* [39]. However many work have also reported that  $GA_3$  inhibits ARF [39,43–47]. Gibberellic acid in high concentration inhibited root formation [39] and may probably inhibits the outgrowth of root primordia [40]. In some cases, GA treatment reduced the cell division in established primordia and also blocks the action of IAA in some process subsequent to the primordia initiation [48]. This inhibition might be due to a competitive diversion of mobilized reserve food materials towards the shoot than to the root meristems [47]. GA-treated barley (*Hordeum vulgare*) aleurone layers causes rapid death of all cells when incubated with  $H_2O_2$  but ABA- treated did not cause cell death upon incubation with same. Also there was decrease in the amounts and activities of ROS scavenging enzymes, like catalase (CAT), Ascorbate Peroxidase (APX), and superoxide dismutase (SOD) in aleurone layers treated with GA. on the other hand, the amount and activity of CAT and *Cat2* mRNA was found to be increased in the ABA-treated layers which also maintains high amounts of ascorbate peroxidase and superoxide dismutase, whereas GA causes rapid reduction in the amounts of these enzymes resulting in oxidative stress leading to cell death [49]. Also, it has been reported that GA-treated aleurone protoplasts are less able to tolerate endogenous or exogenously applied  $H_2O_2$  than ABA-treated protoplasts [50]. These findings suggest that  $GA_3$  might suppress the antioxidative defence system resulting in oxidative stress and may promote ARF at low concentration by encouraging the ROS formation with downregulating effect on ROS scavenging enzymes. Gibberellins also have a negative effect on AR formation in *poplar*. Here the mutants *gibberellins insensitive (gai)* and *repressor of GA1-like1 (rgl1)*, affected in gibberellins perception and responses to gibberellins as well, AR formation remains unaffected [51].

ABA, also called stress hormone, is an isoprenoid phytohormone [52]. It regulates various physiological roles such as stomatal closer, leaf senescence, Bud dormancy, seed germination, osmotic regulation, cuticular wax accumulation, modulation of root architecture and also it controls various downstream processes related to abiotic and biotic stresses [53,54]. Li, *et al.* [55] have showed that ABA promotes adventitious rooting in mung bean by reducing the activities of antioxidative enzymes (CAT, APX) and antioxidant levels (GSH, AsA) similar to the work of Fath, *et al.* [49]. ABA also acts as a second messenger in the signaling pathway of adventitious rooting. ABA is found to up-regulate *CsHO1* cucumber Heme Oxygenase (HO) gene that forms carbon monoxide that helps in the adventitious rooting [56]. Endogenous  $H_2O_2$  was suggested to be involved in ABA-induced adventitious root development under drought stress in cucumber [57]. Later on comparative proteomic analysis of key proteins have revealed



that H<sub>2</sub>O<sub>2</sub> might be involved in ABA-induced adventitious root development by regulating photosynthesis-related proteins, stress defense-related proteins, folding-, modification- and degradation-related proteins under drought stress in cucumber [58]. ABA at a concentration of 5 × 10<sup>-5</sup> M promoted adventitious root development, and the root number increased when it is applied along with IAA [59]. In a complete opposite story, ABA is not only found to be a complete inhibitor of GA activity, also a complete inhibitor of AR formation [59]. ABA always remains highest in the non-rooting tissues [60]. But application of IAA attenuates AB and helps in AR formation [61]. Death of epidermal cells overlying the root primordia are important for the emergence of novel adventitious roots which is done by the Programmed Cell Death (PCD). PCD is induced by the ethylene with the help of ROS and also found to be promoted by GA but is blocked by the ABA [60]. It has also been reported that root emergence is dependent on GA<sub>3</sub> activity and concentration while the growth rate of the root depends largely on GA concentration. ABA interferes with the ethylene signaling of root elongation and GA signaling inhibiting root elongation and root emergence [60]. ABA inhibits PCD and that's what may be the reason its concentration got decreased in the first few hours of submergence [60,62-64] and by ten-fold in growth responsive region of deep water rice [60,62,63]. It is also a negative regulator of AR development in tomatoes [65] However, it is also reported that H<sub>2</sub>O<sub>2</sub> mediate the inhibition of root growth in Arabidopsis Columbia by ABA [66].

JA (3-oxo-2-2'-cis-pentenyl-cyclopentane-1-acetic acid), its methyl ester MeJA and isoleucine conjugate (JA-Ile) are considered to be plant stress hormones or endogenous plant growth regulator which comes into play in various stress responses, growth and developmental process [67,68]. They happen to induce stomatal opening, inhibits rubisco biosynthesis, affects nitrogen and phosphorus uptake and transport of glucose and other organic matter [28]. Jasmonate and its methylester, methyljasmonate (MeJA) was found to accumulate rapidly in wounded stems of Soybean and remained elevated up to 24 h and also addition of MeJA up regulated the expression of wound response genes (chalcone synthase, vegetative storage protein, and proline-rich cell wall protein) indicating its major role in wound response [69] and along with ABA, they are suggested to be mediators of wound responses in plants [69]. In contrary finding, it has been proposed that auxin promotes adventitious rooting by inducing expression of *Gretchen Hagen3* (GH3) gene family, GH3.3, GH3.5 and GH3.6, via the positive regulators auxin response factors, ARF6 and ARF8, resulting in increased conjugated JA and thus the concentration of free JA level gets reduced. JA negatively regulates adventitious rooting through the activation of the CORONATINE INSENSITIVE1 (COI1) signaling pathway [70-73]. JA acts as a positive regulator of AR formation in Petunia cuttings [74]. The gene CbNN1 was identified in a study in Catalpa spp, which showed high expression levels in accessions with greater adventitious rooting ability [75]. CbNN1 encodes a WRKY transcription factor and its ortholog in Arabidopsis modulates the crosstalk between the salicylic acid and jasmonic acid (JA) pathways in response to a wide range of biotic and abiotic stimuli [75-77].

So, there are many works that supports both the stimulatory and inhibitory effect of ABA, GA, JA and SA in adventitious root formation. But there had been no study in the ROS- PGR interaction. In this study we have tried to explore the effect of redox modulation in SA treated hypocotyl explants on endogenous PGRs GA, ABA and JA and also endogenous ROS-hormone interaction during the formation of ARF.

## Material and method

Fresh, viable and healthy mung bean [*Vigna radiata* (L.) R. Wilczek] seeds, collected from local harvest, were surface sterilized (in 0.1% HgCl<sub>2</sub> for 2 min) and given five wash in MiliQ distilled water. The seeds were imbibed in distilled water in dark for the period of 12 h at 25 ± 2 °C. After 12 h, the seeds were placed in petriplates for germination and maintained at 25 °C for 4 d with 14 h photoperiod having 200 μM m<sup>-2</sup>s<sup>-1</sup> light intensity in a seed germinator. Healthy seedlings with terminal buds, two primary leaves and approximately 3 to 4 cm hypocotyls were selected. The primary roots were removed and the explants were put in 50 mL beakers containing distilled water and following standardized pretreating and treating solutions [comprising of Hydrgen peroxide (H<sub>2</sub>O<sub>2</sub>), Salicylic acid (SA), Diphenyleneiodonium (DPI), Dimethylthiourea (DMTU)] for 4 and 24 h respectively and maintained at 25 ± 2 °C with 14 h photoperiod and 200 μM m<sup>-2</sup>s<sup>-1</sup> light intensity in a growth chamber.

Experimental Set	Redox-manipulating conditions of hypocotyl explant	Treatment Conditions
a	Dist. H <sub>2</sub> O	Dist. H <sub>2</sub> O
b	500 μM H <sub>2</sub> O <sub>2</sub> (4 h)	600 μM SA (24 h)
c	1 mM DMTU (4 h)	600 μM SA (24 h)
d	1 mM DPI (4 h)v	600 μM SA (24 h)
e	500 μM H <sub>2</sub> O <sub>2</sub> (4 h)	Dist. H <sub>2</sub> O
f	Dist. H <sub>2</sub> O (4 h)	600 μM SA (24 h)
g	1 mM DMTU (4 h)	Dist. H <sub>2</sub> O
h	1 mM DPI (4 h)	Dist. H <sub>2</sub> O

### Average number (no.) of ARF

Average no. of ARF were taken from 5 days old post treatment-raised tissues.

### RP-HPLC coupled photodiode assay of GA, ABA, JA

To monitor the changes in Abscisic acid, Gibberelic acid and Jasmonate in the different treatment conditions, the differently treated explants were collected and crushed in LN<sub>2</sub>. Then, 400 mg crushed powder was homogenized with 2 mL of cold acetonitrile and homogenates were kept at 4 °C for 12 h. Then homogenate were centrifuge at 20000 rpm for 10 min at 4 °C. The supernatants were collected. The supernatant were transferred to a new centrifuge tube and mix it with 1.5 mL 0.1 M phosphate buffer (pH 7.1) and kept at -80 °C for 30 min. After that, the supernatants were thawed adequately at 4 °C. The mixture is then extracted with 2.5 mL of ethyl acetate thrice after the addition of 1 mL of HCl. The mixture is then centrifuged at 15,000 g for 10 min. at 4 °C and the ethyl acetate phase is collected. The samples are then dried in Rotary-evaporator and dissolved in 1 mL of mobile phase (consisting

of methanol and H<sub>2</sub>O, 1:1). The extracts were filtered through 0.2 mm membrane filter paper for HPLC from RP-HPLC in C<sub>18</sub> column and UV-detection. GA<sub>3</sub> and ABA and JA peak can be detected by using absorbance at 280 nm. The mobile phase consisted of methanol and 0.2 % phosphoric acid solution has flow rate of 0.8 mL/ min. Standard GA<sub>3</sub>, ABA, JA are used for the development of standard curve.

### Spectrofluorometric estimation of total ROS and asperotrophometric estimation of prooxidant (H<sub>2</sub>O<sub>2</sub>)

For the extraction and estimation of total ROS and endogenous H<sub>2</sub>O<sub>2</sub> the process of Kora and Bhattacharjee (2020) [5] was followed.

### Statistical analysis

For Average no. of ARF, Results has been calculated as mean of five independent experiments with 15 explants per treatment ± standard error from 5 days old post treatment-raised tissues. For estimation of Total ROS and endogenous H<sub>2</sub>O<sub>2</sub>, RP-HPLC assay of GA, ABA & JA the experiments was carried after the end of pretreating and treating conditions i.e. at 28 hr from the primary root removal. Total ROS and endogenous H<sub>2</sub>O<sub>2</sub> studies was carried thrice and results has been calculated as mean of three independent experiments ± standard error. Statistical analysis of the data for significance, the t-test paired two samples for means was done using Microsoft Excel 2010.

## Results

Application of pro-oxidant H<sub>2</sub>O<sub>2</sub> (500 μM) followed by SA (600 μM SA) have been shown to significantly augment Adventitious Root Formation (ARF) by 197.2% as compared to untreated control, whereas 10 ×10<sup>-4</sup> M DPI (an inhibitor of NADPH-oxidase) - 600 μM SA and 10×10<sup>-4</sup> M DMTU (a free radical scavenger) - 600 μM SA combination reduced the formation of ARF in hypocotyl explants of mung bean by 53.5 % and 52.1% (Figure 1,2A,3A). The individual treatment of H<sub>2</sub>O<sub>2</sub> and SA have also found to increase the no. of ARF in hypocotyl explants of mung bean. DMTU and DPI also when treated individually further decreased the no. of AR [5].

The concentration of GA found to be increased in all treatment conditions as compared to the control one except in the individual treatment of NADPH-oxidase inhibitor (DPI), where it got decreased by 30.4%. The concentration of GA in 500 μM H<sub>2</sub>O<sub>2</sub> - 600 μM SA combinations increased by 162.4 %, by 157.8 % in 1 mM DMTU - 600 μM SA and by 131.6% in 1 mM DPI - 600 μM SA combination. Individual treatment of H<sub>2</sub>O<sub>2</sub> and SA showed increment in GA concentration by 107.82 % and 219 %. The individual treatment of ROS scavenger (DMTU) showed surprised maximum increase by 1355 % (Figure 2B). When plotted against the Endogenous H<sub>2</sub>O<sub>2</sub> data and DCFDA oxidation data, it is clear that endogenous GA, endogenous H<sub>2</sub>O<sub>2</sub> and total ROS increased in the 500 μM H<sub>2</sub>O<sub>2</sub> - 600 μM SA combination and both get lowered when the NADPH-oxidase gets inhibited by DPI (Figure 2B,3B).



Figure 1: Micrograph showing impact of different redox manipulation on adventitious root formation in mung bean (Detail in the text).

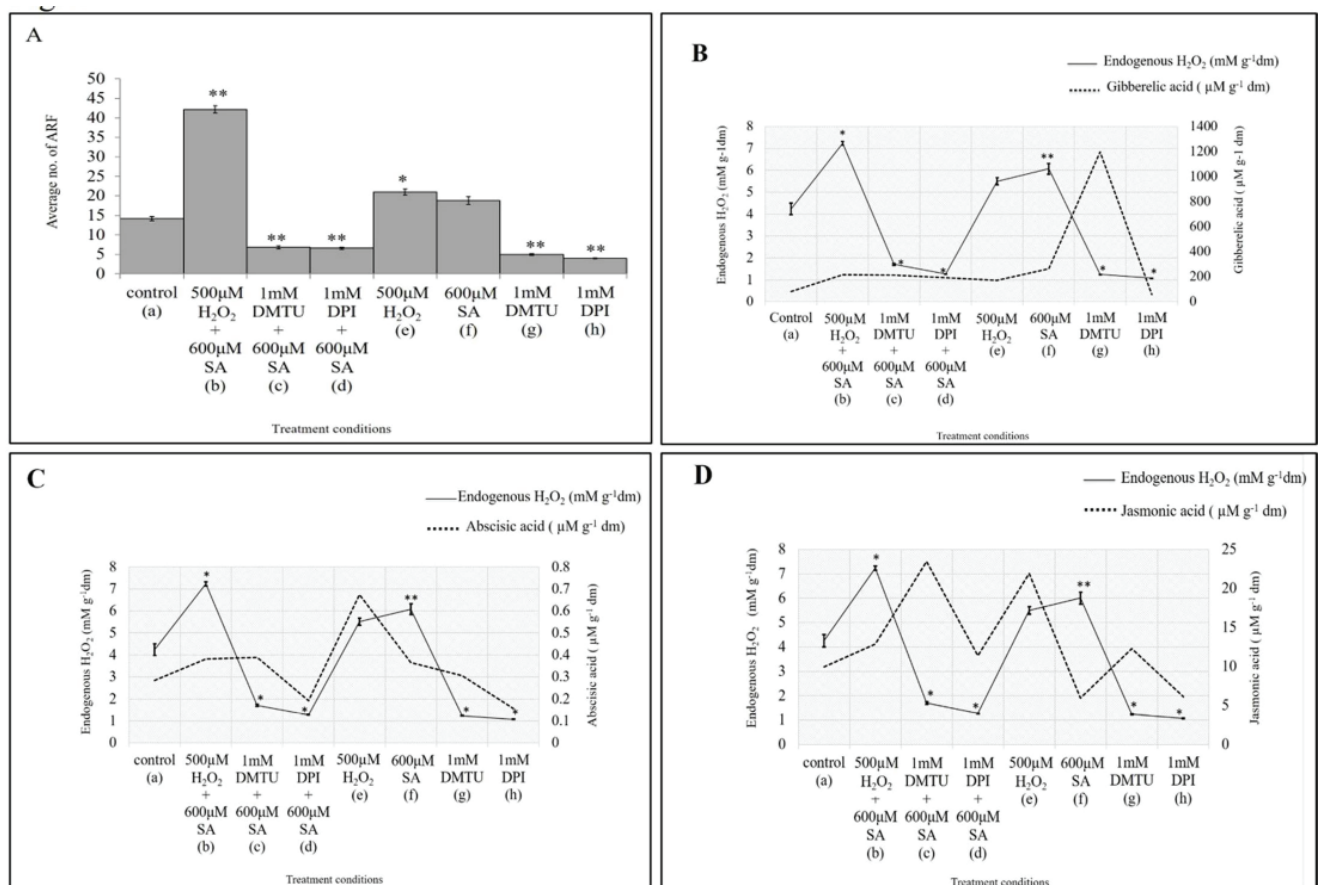
The Abscisic acid concentration found to be increased in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  - 600  $\mu\text{M}$  SA as well as in individual treatments of 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 600  $\mu\text{M}$  SA by 34.5 %, 137.7 % and 28.5 %. The ABA concentration gets decreased in both 1 mM DMTU - 600  $\mu\text{M}$  SA combination and 1 mM DPI - 600  $\mu\text{M}$  SA combination by 36.97 % and 32.8 %. Further, lone treatment of 1 mM DMTU and 1 mM DPI also decreases ABA concentration by 7.7 % and 45.4 %. When plotted against Endogenous  $\text{H}_2\text{O}_2$  and DCFDA oxidation data, the ABA data corroborates well with later two and shows increase in the treatment conditions of 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 600  $\mu\text{M}$  SA and their combination treatment condition in compared to the control. The result clearly suggests the enhancement of the phytohormone ABA and endogenous ROS accumulation in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 600  $\mu\text{M}$  SA and their combination treatment condition (Figure 2C,3C) and lower accumulation in DPI treated seedlings (Figure 2C, 3C).

In case of jasmonic acid, it has not shown any clear trend in the results. It has been found to increase in the 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  - 600  $\mu\text{M}$  SA by 29.8 %, in 1 mM DMTU - 600  $\mu\text{M}$  SA by 136.4 %, in 1 mM DPI - 600  $\mu\text{M}$  SA by 14.9 %, in 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  individual treatment by 121.1 %, in 1 mM DMTU individual treatment by 23.7 % and got decreased in the 600  $\mu\text{M}$  SA and 1 mM DPI individual treatments by 39.9 % and 38.3 %. When the trends of Endogenous  $\text{H}_2\text{O}_2$  and total ROS was compared with the

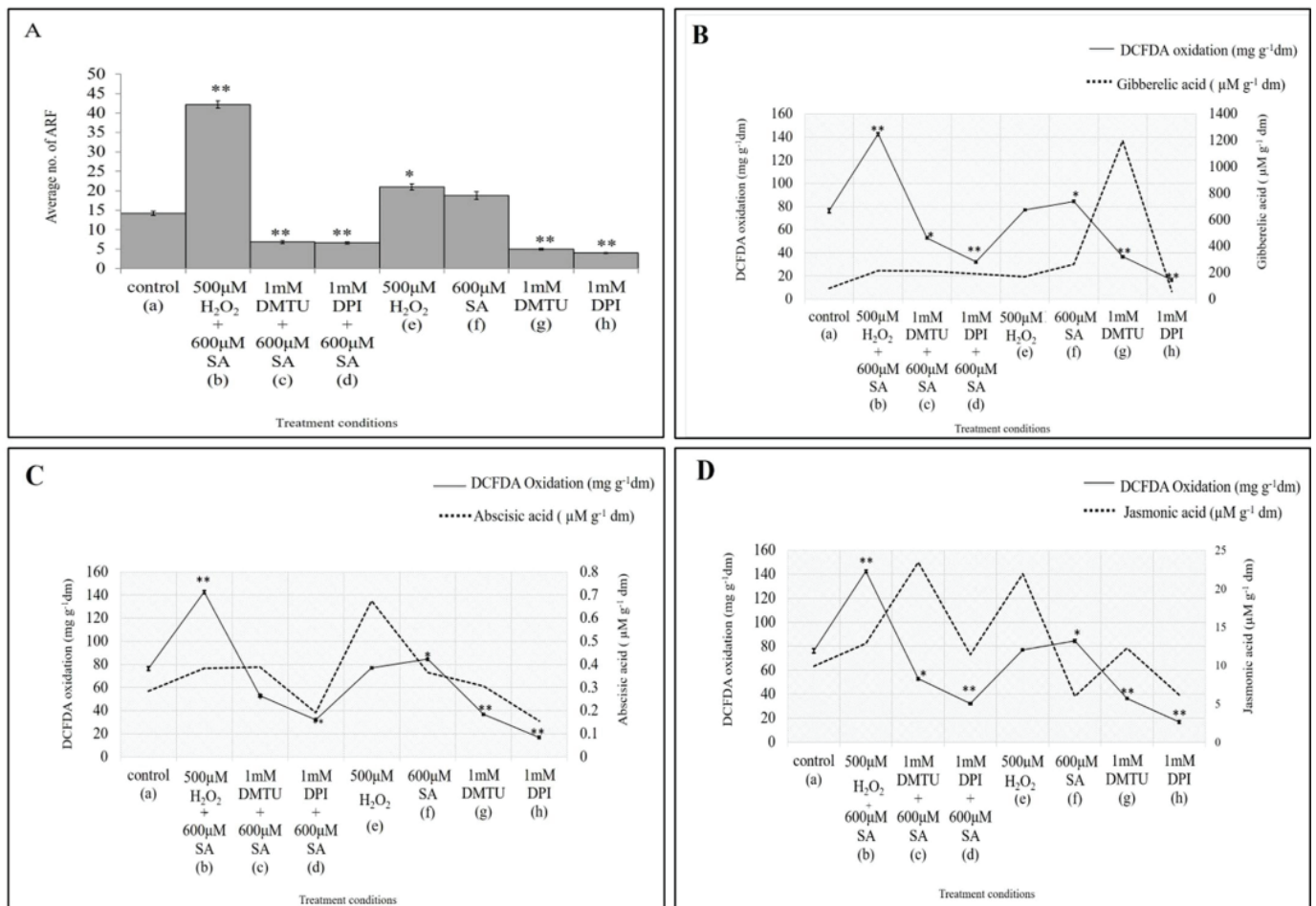
trend of ABA accumulation in different treatment conditions, It is found to be opposite in relation to these (Figure 2D,3D).

## Discussion

We have earlier reported that  $\text{H}_2\text{O}_2$  has significant role in the adventitious root formation in SA treated hypocotyl explants of mung bean [5]. The 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  - 600  $\mu\text{M}$  SA combination has showed greater accumulation of  $\text{H}_2\text{O}_2$  and total ROS in compared to the control one. Also where the DPI and DMTU has been used with or without SA, both  $\text{H}_2\text{O}_2$  and total ROS found to be greatly reduced suggesting that mung bean explants require ROS signature with a distinct role of redox regulation through antioxidant buffering [5]. The interaction of ROS - PGRs like GA, ABA and JA during ARF has been less studied. There is little direct evidences of the cross talk of ROS and these PGR in ARF. However, many work have suggested the involvement of ROS, ABA, JA and GA in the signaling pathway initiated by other plant growth hormones like auxin, ethylene etc. [40,48]. In this work, when redox status of SA treated hypocotyl explants of mung bean is manipulated by using pro-oxidant  $\text{H}_2\text{O}_2$ , NADPH-oxidase inhibitor, DPI and ROS scavenger, DMTU, no clear trend has been found in the endogenous accumulation of these PGRs in different treatment conditions. However, 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  - 600  $\mu\text{M}$  SA combination which has shown significantly higher number of AR have also showed comparatively higher



**Figure 2:** Figure showing ARF (A) and changes in the endogenous titer of GA (B), ABA (C) JA (D) along with fluctuations of pro-oxidant ( $\text{H}_2\text{O}_2$ ) level under redox manipulation in mung bean. Results are mean of five and three replicates  $\pm$  standard error for ARF and endogenous  $\text{H}_2\text{O}_2$  estimation respectively. \*\* represents significant changes from control at 0.01 level (t-test) and \* represents significant changes from control at 0.05 level (t-test).



**Figure 3:** Figure showing ARF (A) and changes in endogenous titer of GA (B), ABA(C) JA (D) along with fluctuations of the level of total ROS (assessed spectrofluorometrically through DCFDA oxidation) under redox manipulation in mung bean. Results are mean of five and three replicates  $\pm$  standard error for ARF and Total ROS estimation respectively. \*\* represents significant changes from control at 0.01 level (t-test) and \* represents significant changes from control at 0.05 level (t-test).

accumulation of GA, ABA and JA as well indicating their requirement in low concentration as suggested by previous work [5,35]. It is also evident from the results that DPI which inhibits the NADPH-oxidase that gives rise to H<sub>2</sub>O<sub>2</sub> decreased the formation of necessary ROS and hence decreased the ARF. In the DPI treatment conditions, all of the three PGRs were found to be in lowest concentration indicating that ROS might be necessary for their up-regulation. The results may suggest that ROS, SA and PGRs like ABA, JA and GA acting downstream of other bonafide plant growth hormones and abiotic stress creating a large complex, sensitive signalling pathway that possess stimulatory and inhibitory effect on morphogenesis of ARF depending on their concentration and time.

## Conclusion

Taken as a whole, we can conclude that there lies a complex sensitive hormonal network system where GA, ABA and JA cross talk with ROS in the morphogenesis of AR.

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