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Research Article

Auxins Increase the Efficiency of 24-Epibrassinolide to Promote Growth, Photosynthesis and Antioxidant System in *Vigna radiata*

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Abstract

A pot experiment was conducted to observe the response of *Vigna radiata* plants to auxins (IAA and/or 4-Cl-IAA) and their interaction with 24-epibrassinolide (EBL). At 30-day stage of growth, foliar spray of 10^{-8} M IAA, 4-Cl-IAA, and/or EBL were given. The plants were then allowed to grow for 45 days and were finally harvested to evaluate plant growth, leaf gas exchange traits and selected biochemical parameters. The plants gave positive response to the hormones (IAA, 4-Cl IAA and EBL) applied alone or in various possible combinations. Combined dose of all the three (IAA + 4-Cl IAA + EBL) generated maximum values for all the parameters observed. The lowest readings were recorded in the plants applied with water solely.

ABBREVIATIONS

Aux: Auxin; BR: Brassinosteoids; CA: Carbonic Anhydrase; CAT: Catalase; EBL: 24-Epibrassinolide; POX: Peroxidase

INTRODUCTION

Occurrence of auxin (Aux) biosynthesis, signalling and transport apparatus in single-celled green algae suggests an evolutionary role of auxin in the acclimatization of plants to varying environmental conditions [1]. Auxin binds to TIR1/ AFB nuclear receptors, which are F-box subunits of the SCF ubiquitin ligase complex to co-ordinate plant development. Aux controls growth, phototropism and gravitropism, apical dominance, initiation of lateral root, differentiation of vascular tissues, embryogenesis, fruit setting, ripening and the process of senescence [2]. Some plants also haverelated auxins,like indole-3-butyric acid, phenyl acetic acid and 4-chloroindole-3acetic acid (4-Cl-IAA) [3,4]. Out of these, 4-Cl-IAA was discovered from the species Pinus sylvestris [5], and the members of the family fabaceae [6]. Some extraordinary properties of 4-Cl-IAA (high auxin activity proved via different bioassays such as pea stem curvature and straight growth, oat and wheat coleoptile growth and mung bean hypocotyls growth etc.) distinguishedit from other auxins [7,8]. Increased enzymes activity and/or their inducibility are other impacts of the application of 4-Cl-IAA [9], which in turn enhanced the photosynthetic rate and finally produced more seed in*Brassica juncea*, at harvest [10].

Brassinosteroids (BRs) is a group of steroidal compounds that are dispersed in all over the plant kingdom.Brassinolide, 24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) are the three analogues of BRs that possess maximum activity out of all the known BRs and have asignificantinfluence on plant growth and metabolism, and also high stabile under field conditions [11]. BRs regulate/coordinate varied functions like, protein synthesis, reproductive development, gene expression, seed germination, division of cell and growth of pollen tube etc. [12]. Moreover, they also provide resistance to plantsfrom numerous biotic and abiotic stresses, such as, drought, heat and chilling [13,14]. Exogenously applied BRs promote yield and stress tolerance of wheat, by stimulating nucleic acid and protein synthesis, activating ATPase and antioxidant activity, and osmo-protectants accumulation, inducing phytohormone responses, regulating stress-responsive genes expression and inducing photosynthetic efficiency and the translocation of photosynthates to the sink [15-20]. However, the mechanism through which they act on plants is poorly understood [12].

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This study was conducted to investigate the efficacy of 24-epibrassinolide in the presence or absence of two auxin analogues (IAA and 4-Cl-IAA) to explore the possible relationship in their impact by selecting certain biomarkers of *Vigna radiata*.

MATERIALS AND METHODS

Hormone preparation

EBL was procured from Sigma-Aldrich Chemicals Pvt. Ltd. India. Required quantity of hormone was dissolved in ethanol to make a stock solution of 10⁻⁴ M. Final volume was made up to 100 ml by using double distill water (DDW). The stock solution was diluted to 10⁻⁸ M. EBL concentration was fixed on the report of Fariduddin et al (2013).Indole acetic acid (IAA) was obtained from Sigma Chemicals Ltd., USA whereas 4-Cl-IAA was gifted by Prof. K.C. Engvild, RISO National Laboratory, Copenhagen, Denmark. The required quantities of the hormones were dissolved in 0.5 ml of ethanol, separately in a volumetric flasks and the volume was made up to 100 ml using DDW. 0.5 ml of surfactant 'Tween-20' was added to each flask prior to the treatment.

Biological materials

The seeds of *Vigna radiata* var. PDM Sarat were acquired from National Seed Corporation Ltd., New Delhi, India. Surface of the seeds were using sodium hypochlorite solution (1%) for 5 min, followed by repeated washings with DDW.

Experimental design and Treatment pattern

The experiment was conducted in net house of Department of Botany, Aligarh Muslim University, Aligarh, India under natural environment conditions. 40 earthen pots of 6 inch in diameter were used and filled with soil and farmyard manure in the ratio 3:1. Sterilized seeds were sown in pots and allowed to germinate. Each treatment was repeated 5 times (replicate). At 30 day stage, foliage of each plant was sprayed by adjusting the nozzle so as to pump out 1 ml (approx) in a single spray. Sampling was done at 45-day stage of growth to assess growth parameters and leaf gas exchange traits as well as biochemical markers. The treatment patterns are as follows:

Set I: DDW spray on foliage (Control)

Set II: EBL (10⁻⁸ M) spray on foliage.

Set III: IAA (10⁻⁸ M) spray on foliage.

Set IV: 4-Cl-IAA (10⁻⁸ M) spray on foliage.

Set V: EBL (10⁻⁸ M) and IAA (10⁻⁸ M) spray on foliage.

Set VI: EBL (10⁻⁸ M) and 4-Cl-IAA (10⁻⁸ M) spray on foliage.

Set VII: IAA (10^{-8} M) and 4-Cl-IAA (10^{-8} M) spray on foliage.

Set VIII: EBL (10 8 M), IAA (10 8 M) and 4-Cl-IAA (10 8 M) spray on foliage.

Morphological traits

The plants were removed from the pots along with adhering soil and dipped in a water filled bucket and moved gently to remove the adhering soil from the roots. The length of the tap root and that of the shoot were measured on the meter scale. The plants were blotted to record their fresh mass and subsequently placed in an incubator at 70° C for 3 days to record their dry mass.

The area of leaf was measured using a portable leaf area meter (ADC Bioscientific, UK).

Chlorophyll content (SPAD level) and gas exchange parameters

The value of SPAD chlorophyll in the leaf was calculated, using the SPAD chlorophyll meter (SPAD-502; Konica, Minolta sensing, Inc., Japan).

Gas exchange parameters were recorded on the third fully expanded leaf between 11:00 and 12:00 h with the help of infrared gas analyzer (IRGA) portable photosynthetic system (LI-COR 6400,LI-COR, and Lincoln, NE, USA). Net photosynthetic rate (P_N) and its related attributes [stomatal conductance (gs), internal CO₂concentration (Ci), water use efficiency (WUE)] were calculated by maintaining air temperature, relative humidity, CO_2 concentration and PPFD at 25°C, 85%, 600 ppm and 800 µmol mol⁻²s⁻¹, respectively.

Biochemical analysis

The level of carbonic anhydrase (CA), catalase (CAT), superoxide dismutase (SOD), peroxidase (POX) enzymes and that proline, along with H_2O_2 were determined, as per earlier observations [21].

Assay for leaf protein content

The amount of protein in fresh leaves was demonstrated by the method given by Bradford [22]. Bradford reagent (2 ml) was added in to the 100 μl supernatant and mixed smoothly. The samples were incubated at 25°C for 5-10 min. for their absorbance and read absorbance at 595nm by spectrophotometer.

Statistical analysis

Data were analyzed statistically and the standard error (SE) was calculated. Analysisof variance (ANOVA) was performed on the data using SPSS (ver. 10.0 Inc., USA) to determine the least significant difference (LSD). The treatment means were separated by LSD test. Data are presented as mean \pm SE (n = 5).

RESULTS AND DISCUSSION

In a natural system, the ratio of various phyto-hormones is maintained to a required level by monitoring their synthesis, transport, metabolism and/or destruction to ensure coordinated growth of various tissues/organs along a defined pattern of growth and development, in the life span of the plant. A limited desired deviation in this set pattern of growth and development may, however, be possible by enhancing the level of any of these regulators by their exogenous application to intact plants or their parts. Observations reveal that length of shoot and root as well as fresh and dry mass of plants increased upon phyto-hormones application, over control plants (Figure 1A-D). In the present study, application of the Aux (IAA and 4-Cl-IAA) or EBL alone to the foliage considerably enhanced the values of the growth parameter (root and shoot length, fresh and dry mass of root and shoot and leaf area; Figure 1A-E) whereas, Aux analogues generated comparable response. This could have been feasible because Aux have a distinctive role in cell division and elongation as well as in cellular differentiation [23]. The combination of EBL+IAA+4-Cl-IAA proved best among the treatments (Figure 1A-C) increasing the root (54.4%) and shoot length (73.4%) and fresh and dry mass (42.5% and 69.6%) of plants to a maximum,

over the control plants. The earliest auxin action is to shift the electrical properties of the plasma membrane by influencing the proton pump that activates the shift of protons into cell wall, formulating "acid growth theory" favoring cell wall loosening [24]. It also has an impact on the functioning of the ionic channels thereby affecting the direction of the movement of ions and solutes and the turgor of the cells [2]. Moreover, the expression of genes is also encouraged by auxin by altering the type, activity and level of proteins [23]. All these modified process have a cumulative impact favoring a shift in plant growth biomarkers. Similarly, Mangus et al. [25], noted auxin (4-Cl-IAA) induced positive shift in the fresh and dry mass of the *Pisum sativum;* Ali

et al. [26] in *Vigna radiata* by IAA or 4-Cl-IAA. The synergistic effects of auxins and BRs were also shown by Choudhary et al. [27].

It is proposed that, competent photosynthetic machinery and higher production of chlorophyll is the result of healthy growth and cell water relation. In present study, photosynthetic attributes along with net photosynthetic rate (Figure 2B-E), SPAD chlorophyll values increased extensively in the presence of EBL and/or Aux (IAA and 4-Cl-IAA). EBL (10⁻⁸ M) alone generated a considerable increase in the chlorophyll content (SPAD values) which was 48% more than water treated plants (Figure 2A). Moreover, synergistic impacts of EBL with Aux



Figure 1 Effect of 24-epibrassinolide (EBL; 10⁻⁸ M) in the presence and/or absence of auxin (IAA) or 4-Cl-IAA on (A) root length, (B) shoot length, (C) shoot dry mass, (D) root dry mass, and (E) leaf area of *Vigna radiata* at 45 days stage of growth.

improved the values further (Figure 2A-E) favoring Aux induced shift in transcriptional and translational process [28] thus, improving the level of enzyme proteins involved in the process. A reason to defend the enhancement of the level of chlorophyll pigments and finally the photosynthesis rate [29] coupled with higher rate of phosphorylation [30] that finally culminated into an improvement of the values for other related attributes (Figure 2B).

Figures 2B-E revealed that EBL, IAA and 4-Cl-IAA alone, significantly enhanced the activity of photosynthetic attributes ($P_{N'}$, gs, Ci, and WUE). However, the combined effects of EBL+IAA+4-Cl-IAA induced a maximum increase in P_{N} (60%), gs (50%), Ci (54.73%) and WUE (72%) in comparison to control plants. Our study is strengthened from that of Ali et al. [26], in

which Aux enhanced the level of chlorophyll content and the rate of photosynthesis in *Vigna radiata*. Moreover, Ahmad et al. [10] established a positive correlation between higher CA levels with photosynthesis and our observations proves that activity of CA was notably increased by EBL (10⁻⁸ M) by 29 % in comparison to control plants whose were at par with those treated with IAA and/or 4-Cl-IAA.Moreover, EBL, IAA and 4-Cl-IAA, in combination induced highest enzyme activity, 43% more than the control plants (Figure 2F). Auxin and BRs-induced increase in CA activity (Figure 2F) and/or that of the ribulose 1, 5-bisphosphatecarboxylase [31]; may be the reason to explain an increase in photosynthetic rate (Figure 2B). According to Fariduddin et al. [14], BRs ameliorate photosynthetic machinery, together with PSII quantum yield which gives strength to our findings.



Figure 2 Effect of 24-epibrassinolide (EBL; 10^{-8} M) in the presence and/or absence of auxin (IAA) or 4-Cl-IAA on (A) SPAD chlorophyll content, (B) net photosynthetic rate, (C) stomatal conductance, (D) internal CO₂ concentration, (E) water use efficiency, and (F) Carbonic anhydrase activity of *Vigna radiata* at 45 days stage of growth.

According to Asada [32], antioxidants are those molecules which keep plants in an equilibrium state to overcome the effect of reactive oxygen species (ROS). Antioxidants are of two types, one which have low molecular weight i.e. glutathione (GSH), ascorbate (AsA) and tocopherol. The second type includes the examples such as, SOD, POX, CAT and glutathione reductase (GR) [32]. The level of SOD was also increased in the leaves, treated with either of the hormones (EBL, IAA and 4-Cl-IAA) alone or in various concentrations, compared with that of the control (Figure 3C). Moreover, the most significant increase of 81% was found in plants treated with EBL + IAA + 4-Cl-IAA. Catalase activity improved by the exogenous application of EBL (10^{-8} M) which is 29.6% more in comparison to the control plants. On the other hand, the maximum increase of 74% in CAT activity

was found in plants treated with EBL + IAA + 4-Cl-IAA, over the control. Phytohormones (EBL, IAA and 4-Cl-IAA) alone increased the activity of POX respectively by 50%, 60% and 61% over the control (Figure 3D) and the values increased further if used in various concentrations. Therefore, the plants exposed to EBL + IAA + 4-Cl-IAA exhibited maximum activity of POX which was 85% more than the control. These antioxidants minimize the production of ROS under specific conditions, directly or indirectly however, in control conditions ROS produce signals for stress response activation [33]. In our study treatment of normal plants with Aux and EBL alone or in varied combinations, considerably enhanced the antioxidant enzymes (catalase, peroxidase and superoxide dismutase) (Figure 3 B-D) to give further strength to the plants with higher metabolic state. This elevation in the level



Figure 3 Effect of 24-epibrassinolide (EBL; 10⁻⁸ M) in the presence and/or absence of auxin (IAA) or 4-CI-IAA on (A) protein content, (B) catalase activity, (C) superoxide dismutase activity, (D) peroxidase activity, and (E) proline content, of *Vigna radiata* at 45 days stage of growth.

of antioxidant enzymes by EBL is proposed to be the consequence of enhanced expression of det2 gene, which counters the excess ROS in *Arabidopsis* [34]. Application of EBL, like ours is known to alter the antioxidant enzymes activity, under several abiotic stresses [14] and also in stress free conditions [12]. Contents of the proline (an non-enzymatic antioxidant) in the treated plants increased through the application of either of the phytohormones (Figure 3E), compared with the control. Highest leaf proline content, 80% more than the control, was found in the plants treated with EBL+IAA+4-Cl-IAA combination.

This suggests that Aux along with other plant growth regulators control the activity of key antioxidant enzymes, and some of their isoforms that are involved in the regulation of plant growth [35,36]. Moreover, BRs also bring about additional power in the plants by elevating their proline content (Figure 3E) through the involvement of genes [18]. The expression of both regulatory genes, such as RBOH (respiratory burst oxidase homologue), MAPK1 (mitogen-activated protein kinase 1) and MAPK3 (mitogen activated protein kinase 3) and the genes involved in defence is under the control of BRs [18]. According to Gill and Tuteja [37], proline is a influential inhibitor of PCD and it also acts as non-enzymatic antioxidant that stabilizes subcellular structures like, proteins and cell membrane, scavenging free radicals and buffering redox potential, and also has the ability as a molecular chaperon that protects the integrity of proteins and enhances the activity of different enzymes [38]. Phyto-hormones treatment enhanced the total protein, though combination of IAA + 4-Cl-IAA excelled over their individual effects but, maximum impacts on protein content (50% more than the control) was reported in EBL + IAA + 4-Cl-IAA treated plants. Similarly, an increase in the activity of antioxidant enzymes and the contents of proline and protein by phytohormones is reported in sorghum and wheat [12,39].

CONCLUSION

It may be derived from the above study in mung bean that the Aux boost selective aspects of the plant system that are expressed as improvement in general growth, photosynthetic traits and antioxidant system. Moreover, IAA and 4-Cl-IAA generate a comparable response even though the latter is recognized as an active Aux. This impact was further boosted in the presence of EBL suggesting a cumulative response by the plants of *Vigna radiata* to the two groups (Aux and BR) of phytohormones.

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