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Effect of 24-Epibrassinolide on Growth, Metabolite content and Antioxidant activities in Ashwagandha (*Withania somnifera* l. Dunal).

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ABSTRACT

Effect of 24-epibrassinolide (EBL) on the growth of ashwagandha (*Withania somnifera*) was studied. 24-EBL stimulated the growth of ashwagandha plants which was reflected in increase in plant height, fresh weight and dry weight of plants. The growth promotion was associated with elevated levels of chlorophyll pigments, fractions of carbohydrates and content of soluble proteins. 24-EBL also improved the antioxidative enzyme activities. The results obtained in the study clearly demonstrated the positive impact of brassinosteroids on the growth performance of *Withania somnifera*.

Keywords: Ashwagandha, 24-epibrassinolide, Growth, Metabolites.

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INTRODUCTION

Ashwagandha [*Withania somnifera* L. Dunal (*Solanaceae*)] is one of the most noteworthy herbs of traditional system of medicine in India and used for its ample ranging vigour remuneration. The word ashwagandha in sanskrit literally translates to odour of a horse; the ground root has an aroma that is reminiscent of horse [1]. Ashwagandha grows naturally all around the Indian subcontinent. Ashwagandha is indigenous to the Middle East and North Africa, but it also grown in North America and other temperate climates. It is essentially the roots and seeds of *Withania* are used in medicine of ayurveda. The roots and leaves of ashwagandha contain various alkaloids *viz.,* withanolodes [2], [3] and withaferins [4]. The leaves contain the steroidal lactones, withanolides and witahferin-A. Withaferin-A known to have significant antistress activity aligned with acute models of experimental stress [5].

Brassinosteroids are new group of phytohormones, naturally occurring as polyhydroxy steroids. BRs are possibly omnipresent in plant kingdom [6]. BRs are plant growth regulators with pleotrophic effect and they influence diverse developmental events such as growth, germination, flowering abscission and senescence [7]. The positive impact of exogenously applied brassnosteroids on several crop plants has been reported [8]. In the present study, the influence of 24-epibrassinolide, a biologically active brassinosteroid, on the growth, metabolite content and activities of certain antioxidants enzymes in *Withania somnifera* has been investigated.

MATERIALS AND METHODS

24- EBL was obtained from M/s CID tech Research Inc., Mississauga, Ontario, Canada. Seeds of ashwagandha (*Withania somnifera* L. Dunal) Poshita variety were obtained from Central Institute of Medicinal and Aromatic Plants (CIMAP), Resource centre, Boduppal, Hyderabad. The field experiments were conducted in Botanical Garden of the Department of Botany at Osmania University, Hyderabad.

Withania somnifera seeds were sowed by broadcasting method in the nursery beds. The nursery beds were watered as recommended. On 20th day thinning was made and uniform sized plants at specified distance were retained and the plot was demarcated into blocks for different treatments. On 45th, 60th and 75th day, foliar spray of 24- EBL was given at three concentrations *viz.*, 0.5µM, 1µM, 2µM and water spray was given to control plants. The plants were watered regularly and maintained up to 130 days.

Growth

On 130th day growth parameters were recorded .The field was flooded with water and the plants were gently removed from the soil without causing any damage to root system. Plant growth was recorded in terms of height of the plant, fresh weight and dry weight of the plants. For recording dry weight the plants were dried in the oven at 110º C for 24 hours. Root growth parameters (number of roots/plant, total root length, surface area of the root) were recorded

with WhinRhizo Root Scanner (XLRHIZO 2012a, Regent Instruments Inc, and Canada). Leaf area of the plant was recorded employing Leaf Area Meter (CI-203, CID Inc. Vancouver, Washington-USA).

The leaf material from different treatments was homogenized in 70% (v/v) ethyl alcohol and the homogenate was stored in deep freezer (-20˚C) for further biochemical analysis. However, fresh leaf material was employed for the extraction of photosynthetic pigments and antioxidant enzymes.

Chlorophylls

The chlorophyll pigments were extracted from the leaves and estimated adopting the procedure described by Arnon [9].

Soluble Proteins

Soluble proteins in the ethanol homogenate were precipitated by addition 20% (w/v) trichloroacetic acid (TCA) and the precipitate was dissolved in 1% (w/v) sodium hydroxide and protein was estimated following the method of Lowry et al. [10].

Carbohydrates

The alcohol homogenate was heated and centrifuged. The supernatant was used for the estimation of total sugars [11] as well as reducing sugars [12]. Non-reducing sugars were calculated by the formulae given by Loomis and Shull [13]. The residue was used for the estimation of starch [14].

Antioxidative enzymes

Fresh leaf material was homogenized in chilled phosphate buffer (pH=7). The homogenate was centrifuged and the supernatant was used for assaying enzyme activities.
Catalase (EC 1.11.1.6)

CAT activity was assayed by Barber [15] method. The reaction mixture contained enzyme extract, hydrogen peroxide and phosphate buffer (pH=7). The reaction was stopped after incubation by adding conc. sulphuric acid and the residual hydrogen peroxide was titrated with potassium permanganate and catalase is expressed as unit g^{-1} fw.
Peroxidase (EC1.11.1.7)

Peroxidase (POD) activity was assayed by adopting the method of Kar and Mishra [16]. The assay mixture for POD activity contained phosphate buffer (pH=7), pyrogallol, H_2O_2 and enzyme extract. After incubation, the reaction was stopped by adding conc.suphuric acid. The quantity of purpurogallin formed was estimated by measuring the absorbance at 420 nm. The peroxidase activity is expressed in absorbance units.

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Statistical analysis

The experiments were repeated twice with adequate replicates. Standard error (SE) of the mean values (n=5) in figures of sample variability was given. The data was analyzed by One way analysis of variance (ANOVAs), followed by Post Hoc Test (Multiple Comparison). The differences were considered significant if P was at least ≤0.05. SPSS version 13.0 software is used for all statically analysis.

RESULTS

Plant growth

Exogenous application of 24-epibrassinolide improved the growth of ashwagandha plants. There was progressive enhancement in plant height, fresh mass and dry mass in dose dependent manner and maximum increase was seen at 2µM 24-EBL (Table 1). Application of 24-EBL significantly improved root growth as reflected in total number of roots per plant, total root length and surface area of the plant (Table 1). Among the three concentrations of 24- EBL employed in the study, the impact of 2 µM concentration was found more effective in stimulating root growth. Exogenous applications of 24-EBL also enhanced the foliage growth as reflected in increase of total leaf area (Table 1). The plants treated with 2 µM concentration exhibited maximum leaf area.

Table 1: Effect of 24-epibrassinolide on the growth of Ashwagandha plants

Treatment	Plant height (cm)	Shoot FW (g)	Shoot DW (g)	No. of Roots/ Plant	Root Area (cm^2)	Root FW (g)	Root DW (g)	Leaf Area (cm^2)
Control	21.02±1.0d	38.05±3.1d	5.31±0.8d	57±0.98d	14.06±1.32d	7.81±0.9 d	1.08±0.4 d	49.81±33d
0.5 µM EBL	23.21±0.9c	44.16±3.2c	5.92±0.9c	66±1.03c	16.31±1.65c	8.52±1.2 c	1.43±0.6 c	55.12±24c
1 µM EBL	28.06±1.1b	51.25±5.4b	6.38±0.7b	75±1.55b	21.04±2.08b	9.81±2.1 b	1.75±0.7 b	69.84±32b
2 µM EBL	31.24±2.1a	69.01±7.3a	8.14±0.9a	84±2.11a	23.25±3.12a	10.7±2.3 a	1.91±0.8 a	74.35±14a

The data presented above are Mean ± S.E. (n=5). Mean followed by the same alphabet in column is not significantly different at *P*=0.05 level

Chlorophyll

Exogenous applications of 24-EBL resulted in a significant increase in chlorophyll levels. 2µM concentration of 24-EBL was found to be most effective in increasing the contents of chlorophyll pigments (Table 2).

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Table 2: Effect of 24- Epibrassinolide on chlorophyll content of Ashwagandha

Treatment	Chl-a mg.g^{-1} fresh weight	Chl-b mg.g^{-1} fresh weight	Total Chlorophylls mg.g^{-1} fresh weight
Control	0.843±0.01d	0.346±0.03d	1.189±0.04d
0.5 µM EBL	0.861±0.024c	0.381±0.21c	1.242±0.45c
1 µM EBL	0.962±0.31b	0.396±0.05b	1.358±0.36b
2 µM EBL	0.984±0.38a	0.399±0.08a	1.383±0.46a

The data presented above are Mean ± S.E. (n=5). Mean followed by the same alphabet in column is not significantly different at *P*=0.05 level

Carbohydrates

Foliar application of EBL caused sharp increase in the levels of carbohydrate fractions *viz*. reducing sugars, non-reducing sugars and starch in ashwagandha plants (Table 3). With increasing concentration of EBL supplementation to ashwagandha plants compared to the control, there was gradual and significant increase in all carbohydrate fractions. Among the three concentrations of treatments to plants, there was maximum increase in the carbohydrate content at 2µM- EBL.

Table 3: Effect of 24- Epibrassinolide on the carbohydrate content of Ashwagandha

Treatment	Reducing Sugars mg.g^{-1} fresh weight	Non-Reducing sugars mg.g^{-1} fresh weight	Total sugars mg.g^{-1} fresh weight	Starch mg.g^{-1} fresh weight
Control	18.1±1.02c	21.2±2.04c	39.3±3.06c	29.1±0.35d
0.5 µM EBL	18.8±1.03b	21.3±2.08c	40.1±3.11c	36.2±0.46c
1 µM EBL	18.9±1.04b	24.6±2.13b	43.1±3.17b	39.6±0.65b
2 µM EBL	21.1±2.06a	27.8±2.24a	48.9±4.30a	41.8±0.89a

The data presented above are Mean ± S.E. (n=5). Mean followed by the same alphabet in column is not significantly different at *P*=0.05 level.

Soluble protein

Compared to the control, soluble protein levels in ashwagandha plants significantly increased. Due to supplementation of 3 concentrations of EBL to aswagandha plants, protein levels enhanced progressively and maximum increase was being at 2 µM concentration (Table 4).

Antioxidant enzyme activities

Catalase activity significantly increased in EBL treated ashwagandha plants when compared with untreated control plants. Plants treated with 2 µM EBL exhibited the maximum catalase activity in comparison to other treatments. (**Table 4**). Similarly the activity of peroxidase enzyme was found increased due to brassinosteroid supplementation. Among all

the treatments 2 µM concentration proved to be more effective in accounting increase in peroxidase activity (**Table 4**).

Table 4: Effect of 24- Epibrassinolide on the soluble protein content and activities of Catalase and Peroxidase of Ashwagandha

Treatment	Soluble Proteins mg g^{-1}fw	Catalase*	Peroxidase**
Control	2.8±0.05d	39.1±2.43c	0.61±0.01b
0.5 µM EBL	3.5±0.12c	40.1±2.51c	0.62±0.04b
1 µM EBL	4.3±0.34b	46.3±3.16b	0.64±0.03a
2 µM EBL	5.12±0.46a	51.2±3.65a	0.64±0.01a

*Catalase activity is expressed in terms of enzyme units.
**Peroxidase activity is expressed in absorbance units which indicates the amounts of purpurogallin formed.

The data presented above are Mean ± S.E. (n=5). Mean followed by the same alphabet in column is not significantly different at *P*=0.05 level

DISCUSSION

The present study clearly revealed the beneficial impact of 24-EBL on the growth of ashwagandha. Exogenous application of 24-EBL significantly stimulated the growth of the plants as evidenced by increase in all vegetative parameters in comparison to untreated control plants. The results obtained in the present study are consistent with the observation of Houimli et al., [17] who reported increase in height and leaf length in field grown maize plants treated with 24-epibrassinolide.Similarly increase in biomass production and leaf area in wheat was found in response to foliar application of 24-epibrassinolide [18]. BR application accounted substantial improvement in plant growth resulting 49% and 40% increases in leaf area and shoot biomass in cucumber [19]. Transgenic rice plants over expressing a sterol C-22 hydroxylase that catalyzes a key step in BR biosynthesis had higher rates of CO_2 assimilation, more tillers, and increased biomass and seed yields [20].

Roots are the prime source of alkaloid content in *Withania somnifera*. Supplementation of 24-EBL to ashwagandha plants showed significant improvement in root yield. The ability of brassinosteroids in increasing the root formation and growth in Coleus was observed [21]. It was found that lateral roots in BR-deficient mutants are fewer than in the wild-type of and *DR5, GUS* expression in root tips of BR-deficient mutants was reduced compared to wild type of *Arabidopsis* [22], [23]. 50% increase in root growth of wild type of *Arabidopsis thaliana* and up to 150% enhancement in BR-deficient mutant dwf-6 and cbb-3 by the application of 24-epicastarine and 24-epibrassinolide [24].

Foliar application of 24-EBL to plants resulted in significant increase in chlorophyll levels. Such an increase chlorophyll levels was also observed in green gram plants treated with brassinosteriods [25]. The cadmium-stress alleviation in tomato by 24-epibrassinolide was found associated with improved pigment content and photosynthetic activity [26].

Feeding of ashwagndha plants with 24-EBL caused enhancement in the levels of carbohydrate fractions such as reducing sugars, non-reducing sugars and starch. The increase might be due to enhanced photosynthetic capacity of the plants as influenced by the EBL. Brassinosteroid mediated increase in carbon dioxide fixation was observed in case of coleus [27]. Similarly, Vardhini et al. [28] and Mahid and Ali [29] reported that EBL increased the carbohydrate fractions in radish and *Satureja* respectively.

Exogenous application of 24-EBL resulted in elevation in the levels of soluble proteins in ashwagandha. It has been suggested that brassinosteroids stimulate the synthesis of particular proteins associated with growth [30]. Brassinosteroids increased protein content in radish under drought stress [31]. Dalio et al. [32] and Fedina [33] reported increase in the content of soluble proteins in pigeon pea and pea respectively due to brassinosteroid application.

In the present study significant increases in activities of ROS scavenging enzymes (catalase and peroxidase) were found by EBL treatment. Catalase and peroxidase enzyme constitutes the major part of the antioxidative system of the plants that scavenge the ROS (reactive oxygen species). These enzymes detoxify the harmful H_2O_2 formed during the metabolism, which is otherwise lethal to the plants. Elevated antioxidant activity enhanced due to brassinosteroid application might have resulted in increased H_2O_2 scavenging which further translated into enhanced growth. Higher antioxidant activity was observed in pepper due to the application of DI-31, DI-100, and two brassinosteriods analogue [34]. The counteractive effect of brassinosteriods on zinc toxicity induced growth inhibition in radish was reflected in elevated activities of antioxidative enzymes [35].

CONCLUSION

The present study demonstrated the effectiveness of 24-EBL, a bioactive brassinosteroid in improving the growth of *Withania*. The impact of 24-EBL is particularly spectacular on root and foliage growth, which are medicinally useful parts of the plant. Exogenous application of 24-EBL improved the contents of chlorophylls, carbohydrates and proteins which have significant bearing on growth of the plants. 24-EBL treated ashwagandha plants showed good antioxidant activity as reflected in elevated activities of catalase and peroxidase enzymes.

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