EFFECT OF EPIBRASSINOLIDE ON PIGMENT CONTENT, TOTAL PROTEIN AMOUNT AND PEROXIDASE ACTIVITY IN EXCISED CUCUMBER COTYLEDONS

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ABSTRACT
Brassinosteroids (BRs) are a group of plant steroids that are known to elicit remarkable growth responses such as growth, seed germination, rhizogenesis, senescence, flowering, abscission, maturation, and also confer resistance on plants against various abiotic stresses. Brassinosteroids increase chlorophyll breakdown and inhibit anthocyanin biosynthesis. In this study the effect of epibrassinolide (epi-BL) on pigment content, soluble protein content, and peroxidase (POD) activity was investigated in excised cotyledons of cucumber seedlings. The cucumber seedlings were grown in dark conditions for 9 days and then their etiolated cotyledons were harvested. After that, they were transferred into Petri dishes containing 10^{-7} \text{mol/L}, 10^{-9} \text{mol/L} and 10^{-11} \text{mol/L} epi-BL. Cotyledons were incubated for 14 h in the dark at room temperature, then they were incubated in light for 3 h. Results showed that epi-BL affected the chlorophyll and carotenoid content, but no significant changes were observed when compared with the control.

Introduction
Brassinosteroids (BRs) are a new group of steroid hormones which play an effective role in plant growth and development (19). They promote plant growth under both normal and stress conditions (23). BRs are natural growth-promoting compounds found at very low concentrations in pollen, seeds and young plant tissues. BRs are essential for plant growth and development and are actively involved in many physiological processes (6, 25, 32). These hormones promote elongation, bending, cell division, vascular differentiation, reproductive development and noduleation of stress processes in plants (2). They play a significant role in the amelioration of various biotic and abiotic stresses (13). The exogenous application of BRs increased the tolerance to low and/or high temperature stress, drought stress and moisture stress. Similarly, the BR treatment countered NaCl stress (7, 22) and also enhanced the antioxidant system in plants subjected to salinity stress (17). Brassinosteroids increase chlorophyll breakdown (28), accelerate senescence (9, 20, 31, 34), and inhibit anthocyanin biosynthesis (4).

The molecular mechanisms and signal transduction pathways of BRs have been intensively studied in recent years (29, 30). Recently, several BR-regulated genes associated with diverse physiological responses, such as cell division and expansion, differentiation, programmed cell death, stomatal development and functions, homeostasis and gene expression have been isolated by genome-wide microarray analysis (8, 27). Mazorra et al. (15) reported the ability of brassinosteroids in imparting drought tolerance in groundnut.

Yu et al. (32) observed that 24-epibrassinolide (EBL) increased the Rubisco activity in Cucumis sativus. The chilling injury alleviation in cucumber seedlings by EBL was found to be associated with a sharp increase in the net photosynthetic rate (33). Another study showed that exogenous application of EBL increased the rate of photosynthesis and growth promotion was also associated with increased chlorophyll content in geranium leaves (26).

The available data about BR action on photosynthetic pigments, chlorophyll a, chlorophyll b and carotenoids, still leave many questions which can be answered only by additional detailed analysis. Almost all authors who described an increase in the amounts of these photosynthetic pigments worked with homobrassinolide, whereas those that did not observe such an effect worked with epibrassinolide (10).

The aim of the present study was to examine the influence of epibrassinolide (epi-BL) on chlorophyll and carotenoid content, soluble protein content and POD activity in excised cucumber cotyledons.

Materials and Methods
Experimental design
Epibrassinolide (epi-BL) (Sigma-E 1641) [(22R,23R)-2α,3α,22,23-Tetrahydroxy-7-oxa-B-homo-5α-ergostan-6-one] 78821-43-9; 72962-43-7 (dissolved in ethanol) was used in this study. Cucumber (Cucumis sativus L.) seeds were planted in sawdust and were grown at 25 ± 2 °C in darkness to prevent...
any chlorophyll formation in the cotyledons. Under a dim green light two cotyledons were excised from several uniform seedlings. Three cotyledons pairs were placed in 5 cm diameter Petri dishes already containing 5 ml of experimental solution. The cotyledons were allowed to absorb the epi-Bl solution in various concentrations (10^{-7} \text{ mol/L}, 10^{-9} \text{ mol/L} and 10^{-11} \text{ mol/L} epi-Bl) in darkness for 14 h at room temperature and they were placed in the light for 3 h.

**Pigment content**

Pigments were extracted by grinding the cotyledons of cucumber in acetone (volume fraction of 90 %) and the total chlorophyll and carotenoid content was determined spectrophotometrically (Shimadzu 1601) (18).

**Protein content**

The cotyledon samples were homogenized with ice-cold 0.1 mmol/L Sodium phosphate buffer (pH 6.8). The homogenates were centrifuged at 13000 rpm for 30 min at 4 °C and the supernatants were used for determination of total soluble protein content and for the total peroxidase enzyme assay. The protein content of the extracts was determined according to Bradford (3), using bovine serum albumin as a standard.

**Peroxidase activity assay**

The reaction mixture consisted of guaiacol (volume fraction of 0.25 %) in 1 mL of 0.1 mol/L sodium phosphate buffer, pH 7.0, containing 0.1 % hydrogen peroxide. Crude enzyme extract (60 µL) was added to initiate the reaction, which was measured spectrophotometrically at 470 nm. The reaction is based on guaiacol oxidation and was recorded for 2 min., and defined quantitatively as ΔA/g·Fr·W·xMin (1).

**Statistical analysis**

Each treatment was analyzed with at least 3 replicate tissue samples; bulked at least 20 plants. The data presented here are the mean values ± SE of three independent experiments.

**Results and Discussion**

The changes in the chlorophyll content by epi-Bl concentrations (10^{-7} \text{ mol/L}, 10^{-9} \text{ mol/L} and 10^{-11} \text{ mol/L}) in the excised cucumber cotyledons incubated under light for 3 h in both control and treatment variants are presented in Fig. 1.

The comparison of the chlorophyll \( a \) (Chl. \( a \)) content in the experimental variants with that in the control showed that there was a decreases with 11 %, 48 %, and 64 % following treatment with 10^{-11} \text{ mol/L}, 10^{-9} \text{ mol/L}, and 10^{-7} \text{ mol/L} epi-Bl, respectively. For the chlorophyll \( a+b \) (Chl. \( a+b \)) content there was a decrease with 20 %, 49 %, and 58 % at 10^{-11} \text{ mol/L}, 10^{-9} \text{ mol/L}, and 10^{-7} \text{ mol/L} epi-Bl, respectively. The exogenous application of 10^{-9} \text{ mol/L} and 10^{-7} \text{ mol/L} solutions to excised cotyledons could affect chlorophyll biosynthesis.

**Fig. 2.** Effect of different concentrations of epibrassinolide (epi-Bl) on carotenoid content in excised cucumber cotyledons.

The effect of epi-Bl treatment on the carotenoid content in the cotyledons is presented in Fig. 2. The amount of carotenoids showed a decrease in comparison to the control, with 20 %, 28 %, and 19 % at 10^{-11} \text{ mol/L}, 10^{-9} \text{ mol/L}, and 10^{-7} \text{ mol/L} epi-Bl, respectively.

There were no significant changes in the total protein content when compared with the control group (Fig. 3). There were some variations in the peroxidase activity following treatment with the applied concentrations of epibrassinolide, most pronounced at 10^{-11} \text{ M} epi-Bl (Fig. 4).

**Fig. 3.** Effect of different concentrations of epibrassinolide (epi-Bl) on protein content in excised cucumber cotyledons.

Analyses of various morphological, physiological and developmental traits in BR-treated plants are often accompanied by the determination of chlorophyll content in leaves. Chls \( a \) and \( b \) are an important part of the photosynthetic apparatus (16). Elevated amounts of both total Chl and Chls \( a \) or \( b \) caused by BR application to plants have been reported in a number of studies. On the other hand however, not a negligible number of studies do not indicate any effect of BRs on these parameters at all or reveal a strong dependence on BR dosage in combination with plant species. Hence, there is still
controversial evidence about the possible influence BRs could have on these photosynthetic pigments (10).

**Fig. 4.** Effect of different concentrations of epibrassinolide (epi-BL) on peroxidase activity in excised cucumber cotyledons.

In the present study, chlorophyll biosynthesis of cotyledons incubated in different epi-BL concentrations were observed to decrease, depending on the increasing concentration of epi-BL. There was a marked decrease in the chlorophyll contents of cotyledons incubated in 10⁻⁷ mmol/L epi-BL compared to the control group. Vardhini and Rao (28) reported that BRs increase chlorophyll breakdown. In a previous study, we showed that 10 µmol/L epi-BL decreased the total chlorophyll content as compared to that in the control group (5). Treatment of tomato pericarp discs with brassinosteroids resulted in considerable decrease in total chlorophyll content (28). An incubation of segments cut from mature *Rumex* or *Xanthium* leaves for 4 d with BL in the dark actually promoted chlorophyll loss as compared to the control group (14).

Carotenoids, a second group of photosynthetic pigments, have been less frequently examined than chlorophylls in connection with BRs. No significant effect of the addition of EBL to the growth medium of winter rape plants on the content of total carotenoids was reported by Janeczko et al. (12) and the same was true when EBL was injected into the apoplast of cotyledons or primary leaves of spring rape (11). In the present study, the amount of carotenoids showed a decrease in comparison to that measured in the control.

The protein content, in this research, remained unchanged compared to the control group. In our previous studies, no significant differences on protein content were observed as compared to the control, when different concentrations of EBL were applied (5, 21). Another research showed that exogenous application of 24-epiBL did not alter the total soluble proteins content under saline or non-saline conditions (23).

There was no significant change in POD activity when compared with that in the control group. The lowest decrease in POD activity (14 %) was seen in cotyledons incubated in 10⁻¹¹ mol/L epi-BL as compared to the control group. Similarly, Sharma et al. (24) did not observe any effect on POD specific activity compared with the control group, when different concentrations of 24-EBL (10⁻¹¹ mol/L, 10⁻⁹ mol/L, 10⁻⁷ mol/L) were applied.

**Conclusions**

The results from this study demonstrated that the influence of epi-BL on the photosynthetic pigment content, protein amount and peroxidase activity of excised cucumber cotyledons was differently related to concentration. Epi-BL affected negatively the chlorophyll and carotenoid content. For protein amount and POD activity, epi-BL caused no significant effect on cotyledons.

**REFERENCES**