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S. Çağ, N. Gören-Sağlam, Ç. Çıngıl-Barış & E. Kaplan

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THE EFFECT OF DIFFERENT CONCENTRATION OF EPIBRASSINOLIDE ON CHLOROPHYLL, PROTEIN AND ANTHOCYANIN CONTENT AND PEROXIDASE ACTIVITY IN EXCISED RED CABBAGE (*BRASSICA OLERACEAE* L.) COTYLEDONS

S. Çağ[1], N. Gören-Sağlam[1], Ç. Çıngıl-Barış[2] and E. Kaplan[1]
Istanbul University, Faculty of Science, Department of Botany, Istanbul, Turkey[1]
Istanbul University, Faculty of Education, Department of Primary Education, Istanbul, Turkey[2]
Correspondence to: Nihal Gören-Sağlam
Email: gorenn@istanbul.edu.tr

ABSTRACT

Brassinosteroids are steroidal plant hormones that influence varied growth and development processes such as germination of seeds, flowering, senescence and abscission. This study aimed to explore the effect of cotyledon growth by different epibrassinolid (epiBL) concentrations in excised red cabbage cotyledons. As an experimental material, the cotyledons of 8-day-old Brassica oleraceae seedlings, excised from the petiole were incubated in 3 ml distilled water (control group) and in epiBL of various concentrations (0.001, 0.1 and 10 μM) for 3 days. Following incubation, peroxidase activity, total chlorophyll, soluble protein and anthocyanin contents were determined. The data indicate that the effect of epiBL on the growth of excised red cabbage cotyledons show difference related to concentration.

Keywords: Epibrassinolid, cotyledon growth, chlorophyll, protein, anthocyanin, peroxidase activity

Abbreviations: epiBL, epibrassinolid; BRs, brassinosteroids; POD, peroxidase

Introduction

Brassinosteroids (BRs) are a new group of plant hormones with significant growth-promoting activity. BRs have been demonstrated in almost all the aerial parts of plants, pollen, flowers, shoots, vascular cambium, leaves, fruits, and seeds (14, 18, 30). BRs as plant hormones with pleiotropic effects as they influence varied developmental processes such as growth, seed germination, rhizogenesis, senescence, flowering, abscission, maturation and also confer resistance to plant against varies abiotic stresses (29). BRs can also induce a broad spectrum of cellular responses such as stem elongation, pollen tube growth, leaf bending and epinasty, root inhibition, induction of ethylene biosynthesis, proton-pump activation, xylem differentiation, and regulation of gene expression (10, 22).

The most important role played by the brassinosteroids is their stimulation of the growth of coleoptile in monocotyl plants, and growth in the stem, petiole and flower stalks in dicotyl plants (31). The promotion of growth by BRs is due to both cell division and cell elongation. BRs have been shown to stimulate cell division (in the presence of auxin and cytokinin) in cultured parenchyma cells of *Helianthus tuberosus* (7). The treatment of Chinese cabbage protoplasts by BRs resulted in the activation of cell division (24). BRs promoted elongation of soybean (36) and pea epicotyls (8), sunflower and cucumber hypocotyls (17), *Arabidopsis* peduncles (9), and wheat coleoptiles (28). Brassinosteroids increase chlorophyll breakdown (33) but inhibit anthocyanin biosynthesis (6).

Potential abilities of BRs in agricultural applications as well as their characteristic physiological effect on growth and development of plants have started to be examined. Recent studies point out that BRs are natural substances suitable for application in plant protection and crop promotion in agriculture.

The purpose of this study is to investigate the effect of different epiBL concentrations on the growth, and consequently contribute to fill the knowledge gap in this field.

Materials and Methods

Plant Material: Epibrassinolide (Sigma-E 1641) [(22R, 23R)-2α,3α,22,23-Tetrahydroxy-7-oxa-B-homo-5α-ergostan-6-one] 78821-43-9; 72962-43-7 (solved in ethanol) was used in this study. Cotyledons of red cabbage (*Brassica oleraceae* L.) were used as an experimental material. Red cabbage seeds had been germinated in the incubator at 25°C. Cotyledons of red cabbage seedlings were harvested on the 8th day. Six harvested cotyledons were transferred into each petri dishes of 5 cm diameter filled with 3 ml of distilled water as control group and test solutions with different concentrations (0.001 μM, 0.1 μM, and 10 μM epiBL), selected after some treatments. They were incubated for 3 days in growth chamber (12-h photoperiod, 8000 lux light, 65-75% humidity, and 25±2˚C).

Chlorophyll determination: Pigment was extracted by grinding the cotyledons of red cabbage in 80% acetone (v/v) and the total chlorophyll content determined spectrophotometrically (Shimadzu 1601) (1).

Extraction of protein: The cotyledons of red cabbage seedlings were homogenized with ice-cold 0.1 mM sodium phosphate buffer (pH 6.8). The homogenates were then centrifuged at 13000 rpm for 30 min at 4 ºC and supernatants

were used for determination of total soluble protein content and total peroxidase enzyme assays. Protein content of the extracts were determined according to Bradford (4) using bovin serum albumin as standard.

Peroxidase activity assay: Peroxidase activity (POD) was determined by employing the method of Birecka et al. (3). With this method, by experimental materials were homogenized in 0.1 M pH 5.8 K-phosphate buffer, using the proportion of 100 mg fresh weight/ml, and then the extracts were centrifugated for 45 min at 13000 rpm. Supernatants were treated with 15 mM guaiacol (Merc) and 5 mM H_2O_2 (Merc) in 0.1 M phosphate buffer. The absorbance of the coloured product in the extract was recorded every 10 seconds for 2 minutes at 470 nm in spectrophotometer (Shimadzu UV 160), and the peroxidase activity was quantitatively provided as ΔA/g fresh weight/minutes with the spectral method.

Anthocyanin determination: After being thoroughly extracted in 3 ml methanol-HCl (1% HCl, v/v), the samples were left at 4 ºC in the refrigerator for 2 days. Later on, the extract were filtered and the total anthocyanin content was measured by an UV visible spectrophotometer as the difference between the absorbance at 530 and 657 nm wavelength and placed in the A530-A657 formula to eliminate the chlorophyll content in the extract, defined quantitatively as OD 530 g-1 fresh weight (21).

Statistics: All experiments were repeated 7 times, and the vertical bars in the histograms show the standard error of the mean. Student's *t*-test was employed to establish the importance of differences among the obtained data.

Results and Discussion

Following the incubation of cotyledons of 8-day-old red cabbages in the epiBL solutions at the concentrations of 0.001, 0.01, 10 μM, and distilled water as the control in the growth chamber for 3 days, the data of the fresh weight amounts are given in **Fig. 1.** Alterations in the fresh weight amounts showed that there are decreases among different epiBL solutions related to concentration. When the control group was compared to the experimental groups, the highest fresh weight accumulation in the cotyledons took place in 0.001 μM epiBL (%21) ($P<0.05$). Besides, fresh weights of cotyledons incubated in 0.1 μM epiBL increased by 9% ($P<0.05$) and fresh weights of cotyledons in 10 μM epiBL decreased by 6% ($P<0.05$) compared to the control group.

The changes in the total chlorophyll content in the cotyledons were determined after incubation. According to analyses, it was seen that a gradual increase was observed in the chlorophyll contents of cotyledons, depending on the decreasing concentrations of epiBL as changes of fresh weight amounts (**Fig. 2**). The total chlorophyll content in the cotyledons increased with 20% ($P<0.05$) in 0.001 μM epiBL compared to the control group, whereas decreased by 20% ($P<0.05$) in 10 μM epiBL. There was no change in the total chlorophyll contents of cotyledons incubated in 0.1 μM epiBL compared to the control group (1%) ($P<0.05$).

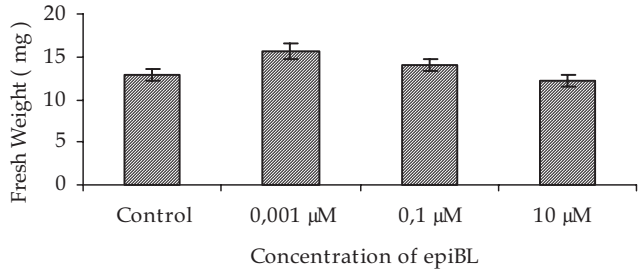

Fig. 1. Comparison of the fresh weights of cotyledons after incubation in epiBL concentrations

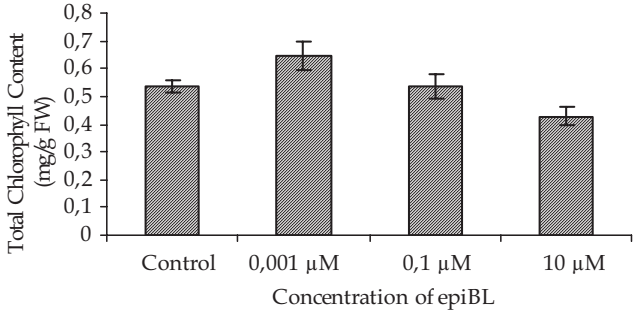

Fig. 2. Total chlorophyll contents of the cotyledons after incubation in epiBL concentrations

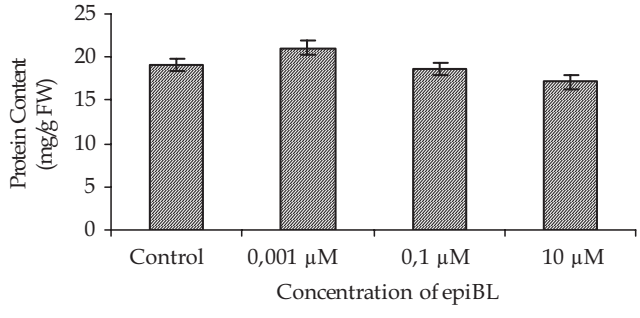

Fig. 3. Soluble protein contents of the cotyledons after incubation in epiBL concentrations

Fig. 3. represents histograms of total protein contents of the cotyledons incubated in the experimental concentrations of epiBL. When compared with the control group, it was determined that the total protein content in the cotyledons increased with 10% ($P<0.05$) in 0.001 μM epiBL and decreased with 11% ($P<0.05$) in 10 μM epiBL. The content of protein in 0.1 μM epiBL remained unchanged compared to the control group (2%) ($P<0.05$).

It is known that brassinosteroids inhibit anthocyanin biosynthesis (2). When the data related to the pigment contents of red cabbage cotyledons incubated in epiBL solution at different concentrations are compared to the control group, the anthocyanin contents of cotyledons incubated in 10 and 0.1 μM epiBL decreased by 52% ($P<0.05$) and 21% ($P<0.05$), respectively, but no difference was seen in cotyledons in 0.001 μM epiBL (2%) ($P<0.05$) (**Fig. 4**). In terms of the effect of epiBL on the anthocyanin contents of the cotyledons, the optimum concentration was found to be 10 μM epiBL.

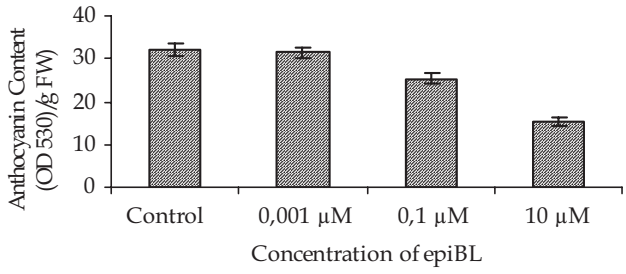

Fig. 4. Anthocyanin contents of the cotyledons after incubation in epiBL concentrations

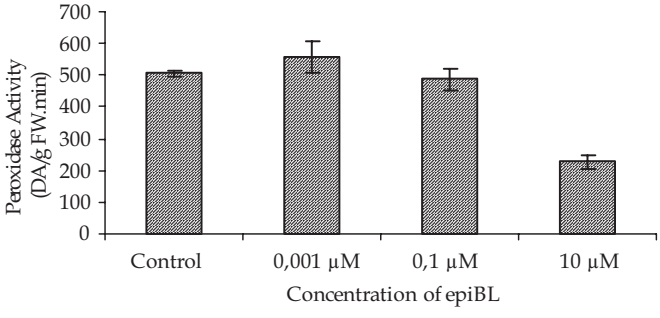

Fig. 5. Peroxidase activities of the cotyledons after incubation in epiBL concentrations

Fig. 5 shows the effect of epiBL concentrations of 0.001, 0.1, 10 µM on the peroxidase activity. As can be seen from the figure, the lowest elevation of POD activity was seen in cotyledons incubated in 10 µM epiBL and the decrease of the activity is 55% ($P>0.05$) compared to the control group. While an increase of 10% ($P<0.05$) was observed in 0.001 µM epiBL, POD activities of cotyledons incubated in 0.1 µM epiBL apeared unchanged compared the control group (4%) ($P<0.05$).

BRs were considered promising compounds for application in agriculture, because they showed various kinds of regulatory activity on growth and development in plants. The practical value of BRs in promoting yield was confirmed by studies. Some BRs have been tested in field trials to determine their influence on plant growth and development and on crop yield in natural conditions. The result obtained in field trials do not always coincide with those predicted from bioassays. Thus, we studied the effects of different epiBL concentration on the excised cotyledons of *Brassica oleraceae* and the obtained data about growth.

The best known characteristic of BRs is its effect on growth: it has been shown to stimulate cell elongation and division, in studies on intact plants or cuttings. In cultured cells, BRs, especially at higher concentrations, did not show significant enhanced growth effects (35). Conversely, in a tobacco callus culture, BR showed a broader inhibitory concentration (26).

The present study shows that fresh weight in cotyledons increased in 0.001 and 0.1 µM epiBL. Amounts of fresh weight in the control and in the concentration series suggested that 0.001 µM epiBL has a positive effect on the fresh weight. Braun and Wild (5) reported that growth of wheat and mustard showed a promotive effect of a brassinolid isomer on photosynthetic capacity and biomass production in their primary developmental stages. In mustard, fresh weights of the shoots increased, and both elongation and radial growth were stimulated. The study of Krishnan et al. (19), in which 0.1 µM BR increases fresh weight of grains in IR50 indica rice confirms our study.

In this study, the total chlorophyll contents of cotyledons incubated in epiBL concentrations were observed to increase, depending on the decreasing concentrations of epiBL. Particularly, there is a marked increase in chlorophyll contents of cotyledons incubated in 0.001 µM epiBL compared to the control group. Similarly, Sağlam-Çağ (27) has reported that exogenously applied epiBL (at 0.001 µM) reduced the loss of photosynthetic pigments of wheat leaves during the senescence. Vardhini and Rao (33) reported that application of different epiBL concentrations (0.5, 1.0, 3.0 µM epiBL) in the pericarp discs of tomatoes caused a decrease in the chlorophyll level. According to results of this study, the changes in the chlorophyll level may lead to decreased activity of many enzymes related with photosynthetic events. Besides, studies related to the relationship between BRs and senescence in whole plants revealed that 0.001 µM epiBL promoted loss of chlorophyll in sunflower (16) and soybean (12). Yu et al. (37) found in their study with *Cucumis sativus* that epiBL treatment (especially 0.1 mg l^{-1} epiBL) increased the capacity of CO_2 assimilation in the Calvin Cycle, and the soluble protein content but this concentration had little effect upon the chlorophyll or the protein content. Moreover, Kalinich et al. (15) explained that brassinolid treatment enhanced the levels of RNA, DNA, and protein and suggested that brassinolid responses are dependent on nucleic acid and protein synthesis. In this study it was demonstrated that the protein content in cotyledons incubated in 0.001 µM epiBL increased, and decreased in 10 µM epiBL. From the results of total protein, it is obvious that the chlorophyll content decreases in parallel to decreasing in the protein content.

Anthocyanin levels are higher early in a plant's life and then decrease as photosynthetic capacity increases (34). Anthocyanin contents of the cotyledons appeared to be decreased in epiBL concentrations compared to control group in this study. On the other hand, Brosa (6) and Clouse and Sasse (10) demonstrated the inhibitive effect of brassinosteroids on anthocyanin biosynthesis. Our findings are parallel to the results of Luccioni et al. (20) where they revealed the increased anthocyanin levels in *dwf1-101* mutants and to the Brosa (6) and Clouse and Sasse (10).

Mazorra and Nunez (23) emphasized that BR analogues effect the POD activity largely and the total protein content depends on the concentrations applied and on the type of analogue used in tomatoes. The results of POD activity levels of red cabbage cotyledons incubated in different concentrations of epiBL indicated that POD activities were seen to be increased as epiBL concentrations were decreased and especially the lowest elevation was found in cotyledons incubated in 10 µM

epiBL compared to the control group. From the literature, we know that there are two main mechanisms whereby POD can effect the differentiation of plant cells. Peroxidase activity can alter the concentration of phytohormones through enzymatic metabolism (32). Peroxidase activity can catalyze cross-linking reactions in the plant cell wall and, therefore, cell expansion (13). Likewise, POD activity may regulate endogenous levels of the main growth promoting factor indolacetic acid and the later stages of cellular growth resulting from the biophysical process of cell expansion in radish cotyledon (25). It is clear whether the enhanced activity is due to stimulation of synthesis, inhibition of degredation, or activation of the enzymes (11). These changes may depend on the incubation period, the concentration of the applied solution, and on experiment materials.

Conclusions

In conclusion, this study has demonstrated that the effect of epiBL on the growth of excised red cabbage is different related to concentration. The data presented in this study show that 0.001 µM epiBL promotes growth on cotyledons, whereas 10 µM epiBL inhibits, 0.01 µM epiBL causes no significant effect on cotyledon growth.

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