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THE EFFECTS OF BRASSINOSTEROIDS ON SEQUENTIAL LEAF SENESCENCE OCCURRING IN *GLYCINE MAX* L.

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ABSTRACT

Brassinosteroids (BRs) are steroidal plant hormones that influence varied growth and development processes such as germination of seeds, flowering, senescence and abscission. The effect of epibrassinolide (eBL), one of the most active brassinosteroids, on senescencing cotyledons of Glycine max L. seedlings, which represent epigeic germination, has been investigated in this study. The process of senescence was examined by spraying eBL solutions of 10^{-11} M and 10^{-9} M and 2,3,5-triiodobenzoic acid (TIBA), an inhibitor of auxin transport, to the 12 days-old seedlings. eBL (especially 10^{-9} M) was observed to have induced senescence, while it has delayed senescence when applied with TIBA together. These observations were confirmed with the measurements of total chlorophyll and protein amounts, and rate of peroxidase activity. One has discussed that combined brassinosteroid and TIBA, the former being known to work with auxin synergistically and the latter being an inhibitor of auxin transport, do not affect senescence but brassinosteroids accelerate senescence in presence of auxin. As a result, one may think that eBL and auxin together function as a senescence signal.

KEYWORDS: Brassinosteroids, Epibrassinolide, Sequential Leaf Senescence, TIBA, Glycine Max

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Original Article

INTRODUCTION

Brassinosteroids (BRs) are a new class of plant hormones with a polyoxygenated steroid structure showing pronounced plant growth regulatory activity (Mussig and Altmann, 1999; Bajguz, 2000; Rao *et al.*, 2002; Kim *et al.*, 2005; Vardhini and Anjum, 2015), especially when exogenously applied (Mandava, 1988; Fujioka and Sakurai, 1997; Latha and Vardhini, 2016). Brassinosteroids (BRs) are common plant-produced compounds structurally similar to animal steroid hormones that can function as growth regulators (Clouse, 2001; Clouse, 2002). In 1970, brassins were isolated from rape *(Brassica napus* L.) pollen by Mitchell and coworkers (Mitchell *et al.*, 1970).

Brassinolide (2α,3α,22α,23α-tetrahydroxy-24α-methyl-B-homo-7-oxa-5α-cholestan-6-one) was identified as a major biologically active component of brassins (Grove *et al.*, 1979; Clouse, 2002). Senescence in plants is usually viewed as an internally programmed degeneration leading to death (Lim *et al.*, 2003). It is a developmental process that occurs in many different tissues and serves different purposes (Noodén *et al.*, 1997; Oh *et al.*, 2015). Senescense is not a case of passive decay of structural and biochemical machinery of cells. Rather it is a highly regulated, ordered series of events in which organelles, membranes, and macromolecules are broken down and nutrients, i.e., amino acids, sugars and minerals, are reclaimed for export out of the senescing

leaf to be reused in other parts of the plant (Srivastava, 2002). Decreased photosynthetic activity vs. increased respiration rate is the most distinctive metabolic change occurring in course of senescence (Jiang *et al.*, 1993; Srivastava, 2002). The senescence that takes place in cell, tissue, organ and the whole plant during the normal growth process of the plant includes very complicated catabolic reactions at molecular level and is dependent on energy input (Taiz and Zeiger, 2002). The complex interaction of both endogenous and exogenous factors is seen in senescence process in the plants (Nam, 1997; Noodén *et al.*, 1997). While endogenous factors include plant growth regulators (phytohormones) and age, stress factors such as excess temperature changes, darkness, lack of minerals, high light, aridity and pathogen infections are exogenous factors (Hensel *et al.*, 1994). The genetic factors are also one of the most important internal factors. A lot of special genes (SAGs-senescence-associated genes) that play a role in the arrangement of senescence have been defined recently (Weaver *et al.*, 1972; Buchanan-Wollaston, 1997; Quirino *et al.*, 2000).

BRs are phytohormones with pleiotropic effects and are involved in several developmental processes such as germination, rhizogenesis, flowering, senescence, abscission and maturation. They also provide the plants with strength against varied abiotic stresses (Mussig and Altmann, 1999; Rao *et al.*, 2002; Michelini *et al.*, 2004; Jajic *et al.*, 2015). BRs play a role in physiological events including seed development, stem and root elongation, vascular differentiation and apical dominancy. The fact that these events are also regulated by auxin makes think an interaction between these two hormones (Halliday, 2004).

BRs are thought either to increase sensitivity of plant tissues to oxygen or to induce auxin synthesis. In addition, they act as regulators of several events involved in senescence process (Clouse and Sasse, 1998; Khripach *et al.*, 2000; He *et al.*, 2001; Rao *et al.*, 2002; Srivastava, 2002; Nemhauser and Chory, 2004). Studies are available, indicating that BRs are able to induce senescence in excised cotyledons of cucumber seedlings (Zhao *et al.*, 1990), while epibrassinolid (eBL) in leaves of bean seedlings (He *et al.*, 1996) and in excised leaves of *Arabidopsis* (He *et al.*, 2001), and that BL increases senescence in *Xanthium* and *Rumex* explants (Mandava *et al.*, 1981). He *et al.* (1996) reported 24-eBL accelerates senescence. Most of the BR mutants possess an increased lifespan, and show delayed senescence. A wild type of *Arabidopsis* undergoes senescence about 60 days later whereas BR mutants remain green and produce flowers after even 100 days (Choe *et al.*, 1999). Additionally, Srivasta (2002) claims that BRs are related to delayed senescence. In spite of extensive studies some of which are above mentioned, molecular mechanism(s) of effect of BRs on senescence is still unknown. The aim of the present study is to investigate effects of BRs on senescence, and to clarify whether BRs and IAA are synergistic, and IAA is a senescence signal.

METHODS

Plant Material and Hormone Treatment

Epibrassinolide (Sigma-E 1641) [(22R,23R)-2α,3α,22,23-Tetrahydroxy-7-oxa-B-homo-5α-ergostan-6-one] (dissolved in ethanol) was used in this study. *Glycine max* L. (Soybean) seedlings which belong to Fabaceae family were purchased from MAY seed company (A-3935) and used as experimental material. The seeds were germinated on moistened filter paper in petri dishes in incubator at 25ºC for 48 h in the dark and were transferred into pots containing 2 parts sand and 1 part soil. The pots were placed in a growth chamber (6000 lux, 12 h photoperiod, 26±2ºC). The grade of the senescence in the cotyledons was scored that it was adapted from Lindoo and Noodén (Plastocron Index) (1976). Just before senescence starts (12^{th} day), seedlings excluding cotyledons were applied with eBL (10^{-9} M and 10^{-11} M) and 2,3,5-tri-iodobenzoic acid (TIBA) (Sigma), which is an inhibitor of polar auxin transport. Exogenously TIBA solution was

prepared as 10 mg/L. Spraying was carried out every other day, by avoiding overlapped applications. The biochemical analyses were conducted on the day when the Average Green Areas of the cotyledons in the control group of plants reached 50%.

- **Measurement of Total Chlorophyll Amount**

Total chlorophyll amount was determined according to the Arnon (1949) in soybean (*Glycine max* L.). Cotyledons were homogenized in 80% acetone. Total chlorophyll amount determined spectrophotometrically (Shimadzu UV-1601) and were put in their places in the Arnon formula and total chlorophyll amount was calculated as mg chlorophyll per cotyledon.

- **Measurement of Soluble Protein Content**

The cotyledons of soybean seedlings were homogenized with ice-cold 0.1 mM phosphate buffer (pH 6.8). The homogenates were then centrifuged at 13.000 rpm for 30 min at 4ºC and supernatants were used for determination of total soluble protein content and total POD enzyme assays. Protein content of the extracts was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard. The amount of total protein was estimated as µg/cotyledon.

- **Determination of Peroxidase (POD) Activity**

Peroxidase (POD) activity was determined by employing the method of Birecka *et al.* (1973). The absorbance of the colored product in the extract was recorded every 10 seconds for 2 min. at 470 nm in spectrophotometer (Shimadzu UV-1601), and the peroxidase activity was quantitatively provided as ΔA/g fresh weight minutes with the spectral method.

- **Changes in the Permeability of the Membrane**

In order to measure the changes in the permeability of the membrane, fresh weights of all cotyledons under senescence which were Green (G)- 100% green, Green-Yellow (G-Y)- 50% green, and Yellow (Y)- 0% green (not dry), were harvested, and fresh weights of them were taken and green-health cotyledons of (control) seedlings, cotyledons were placed in petri dishes with filter paper and containing 8 ml of distilled water. Then they were incubated in an oven at 25ºC for 24, 48 and 72h. Absorption values of samples which were removed from the liquid medium in petri dishes at the end of these periods were measured with spectrophotometer at 280 nm (Poovaiah and Leopold, 1976).

- **Statistic Evaluation of the Results**

Standard error estimate was made to evaluate the results obtained from experiment and control groups statistically. In case of the number obtained when $\pm$ values of differences between the result's square's total sum's square root is multiplied with three is found to be smaller than the difference between values, the difference is decided to be significant statistically.

RESULTS

Changes in Membrane Permeability

The Figure 1 shows the spectrophotometrical values of soluble substance which leaked into the fluid in which cotyledons of different senescence degree were incubated in the course of 24, 48 and 72h. As seen from the accompanying

figure, amount of leaking substance is lower in 100% green cotyledons when compared to other cotyledons. In other words, as percentage of green area decreased amount of leaking substance was elevated.

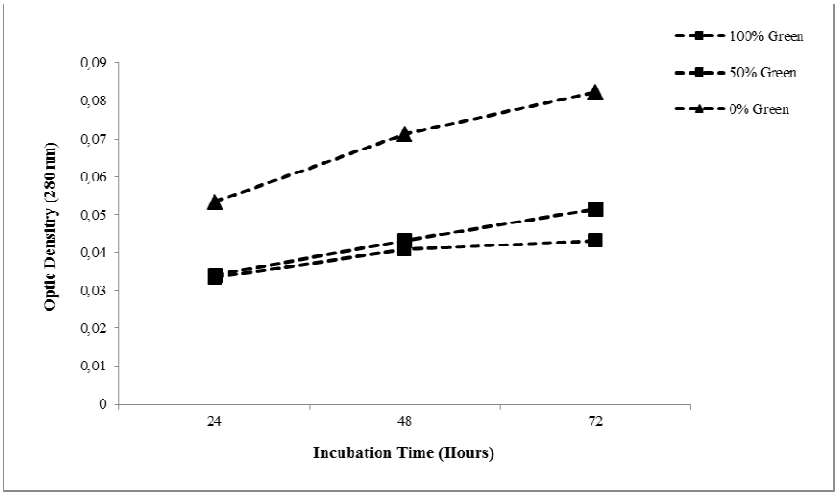

Figure 1: Comparison of the Substance Amount that Leaks to İncubation Medium from the Cotyledons of *Glycine Max* L. Seedlings which are in Different Senescence Stage

Changes in Fresh Weight and Chlorophyll Content

When the average green space (AGS) of the cotyledons of the control reached 50% (green-yellow), all plants were harvested and fresh weights were taken. Fresh weights of cotyledons of the seedlings treated with 10^{-9} M and 10^{-11} M eBL, 10^{-9} M eBL+TIBA and 10^{-11} M eBL+TIBA are given in Figure 2.

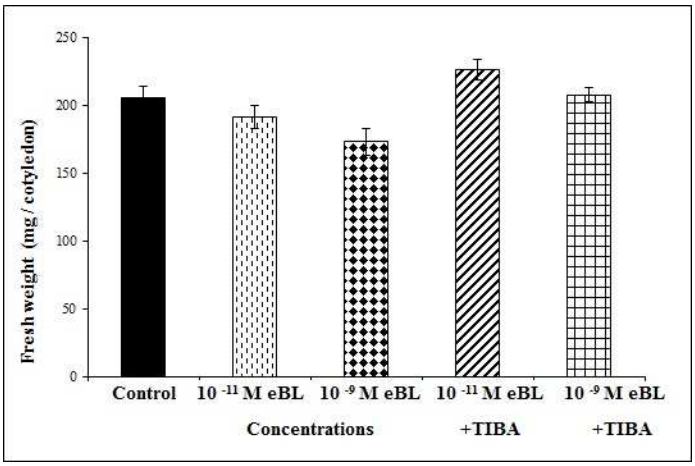

Figure 2: Comparison of the Fresh Weight of the Seedlings' Cotyledons which was Applied 10^{-9} M Ebl, 10^{-11} M Ebl, 10^{-9} M Ebl+TIBA Ve 10^{-11} M Ebl+TIBA to all its Parts Except Cotyledons

From the results, fresh weights of the cotyledons treated with 10^{-9} M eBL and 10^{-11} M eBL are seen to have decreased by %16 and %7, respectively. However, combined TIBA and eBL caused an increase in fresh weight of the test cotyledons in comparison with the controls, although increase being inconspicuous. 10^{-9} M eBL+TIBA application resulted in increased fresh weight of 6% while increment was 16% at 10^{-11} M concentration of eBL+TIBA. Figure 3 indicates chlorophyll contents of cotyledons of the seedlings treated with 10^{-9} M and 10^{-11} M eBL, 10^{-9} M eBL+TIBA and 10^{-11} M eBL+TIBA. eBL concentrations of 10^{-9} M and 10^{-11} gave rise to a decrease by 27% and 12%, respectively. Total chlorophyll content was elevated by 94% in cotyledons of the seedlings treated with 10^{-9} M eBL+TIBA, whereas elevation

was 204% with 10^{-11} M eBL+TIBA application.

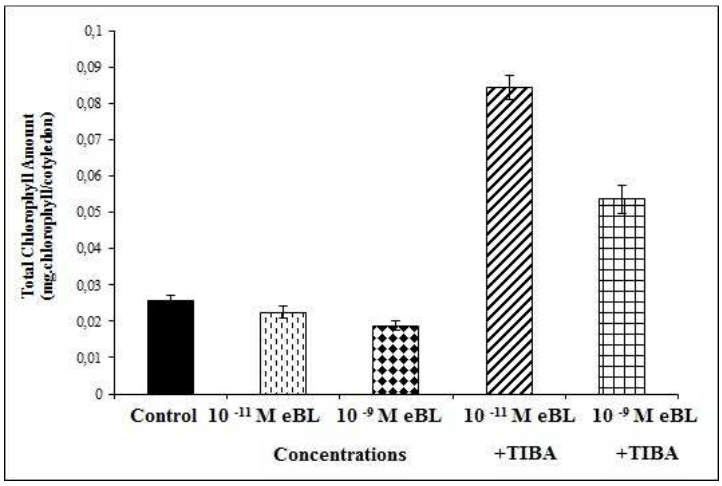

Figure 3: Comparison of the Chlorophyll Amounts in Cotyledons of the Harvested Seedlings Treated with 10^{-9} M Ebl, 10^{-11} M Ebl, 10^{-9} M Ebl+TIBA Ve 10^{-11} M Ebl+TIBA and the Average Green Space of the Control Plants' Cotyledons when they Reach to 50%

Protein Amount

Protein amount was lowered by 4% with 10^{-9} M eBL treatment. However, 10^{-11} M eBL resulted in 3% decrease (Figure 4). Combined applications showed striking results in that 10^{-9} M eBL+TIBA led to 72% increase, while 10^{-11} M eBL+TIBA to 85%.

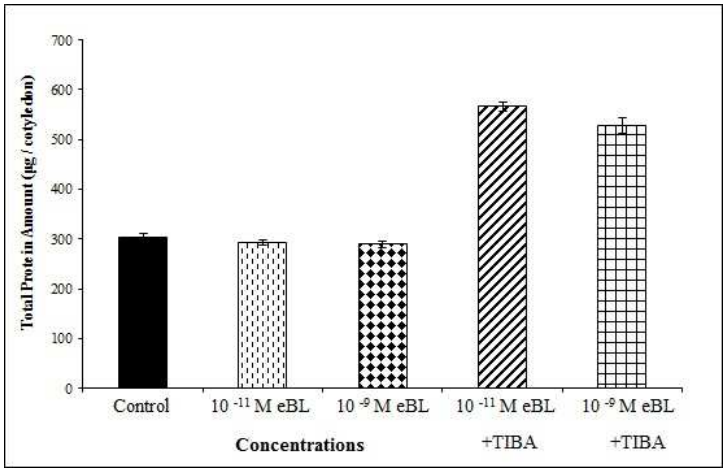

Figure 4: Comparison of the total Protein Amounts in Cotyledons of the Harvested Seedlings Treated with 10^{-9} M Ebl, 10^{-11} M Ebl, 10^{-9} M Ebl+TIBA Ve 10^{-11} M Ebl+TIBA and the Average Green Space of the Control Plants' Cotyledons when they Reach to 50%

Peroxidase Activity

An increase of 39% and 34% was found with eBL treatments of 10^{-9} M and 10^{-11} M, respectively (Figure 5). However, combined applications gave rise to decreased POD activities. The decrease was 14% for 10^{-9} M eBL+TIBA application, while 16% for 10^{-11} M eBL+TIBA.

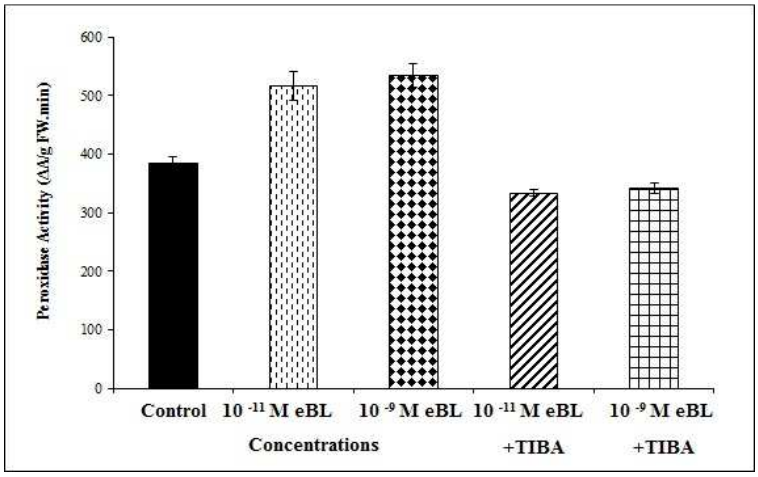

Figure 5: Comparison of the Peroxidase Activity in Cotyledons of the Harvested Seedlings Treated with 10^{-9} M Ebl, 10^{-11} M Ebl, 10^{-9} M Ebl+TIBA Ve 10^{-11} M Ebl+TIBA and the Average Green Space of the Control Plants' Cotyledons when they Reach to 50%

CONCLUSIONS

BRs are known to induce a broad spectrum of responses, including stimulation of longitudinal growth of young tissues via cell elongation and cell division (Zurek *et al.*, 1994, Hu *et al.*, 2000, Clouse, 2002) and vascular differentiation, which is a developmental process critical for plant growth (Verma *et al.*, 2012). This study examines effects of BRs on senescence, taking into account IAA as likelihood senescence signal (Sağlam-Çağ and Okatan, 2014) and synergistic interaction of BR and IAA (Kaplan-Dalyan and Sağlam-Çağ, 2013). Cotyledons of the seedlings treated with 10^{-9} M and 10^{-11} M eBL undergo senescence earlier than the controls do. This finding is in concordance with the findings of the studies reporting BRs induce senescence. Based on the knowledge that eBL concentration of 10^{-9} M induces senescence, and BRs cooperate with IAA synergetically (Yopp *et al.*, 1981; Katsumi, 1985; Mandava, 1988; Yalovsky *et al.*, 1990; Bajguz and Piotrowska-Niczyporuk, 2013), eBL was applied to the seedlings which were sprayed with TIBA known to inhibit IAA transfer to the leaves. By doing so, we prevented auxin from being transferred to cotyledons, allowing examination of the way eBL affects senescence solely. Combined TIBA and eBL surprisingly resulted in delayed senescence of cotyledons, leading to two inferences: Firstly, eBL can induce senescence only in the present of IAA; and secondly, a lack of IAA gives rise to delayed senescence by eBL. Studies by Mishra and Gaur (1980), and Singh *et al.* (1992) also declared that auxin accelerates senescence in leaf tissue (Even-chen *et al.*, 1978; Christine *et al.*, 2012). Even Christine *et al.* showed that two other ARF genes, *NPH4/ARF7* and *ARF19*, were also induced by senescence. Our findings are also confirmed by study of Sağlam-Çağ and Okatan, 2014 claiming that senescence signal may be in IAA architecture, or it is a substance which mimics IAA. Applied eBL concentrations of 10^{-9} M and 10^{-11} M caused decreases of 27% and 12%, respectively, findings being concordant with Sağlam-Çağ (2007) study in which 10^{-9} M eBL was applied to excised leaf segments of wheat. On the other hand, protein amount was not increased as much as chlorophyll content during senescence. Although protein destruction occurs in course of senescence a marked difference in protein amount was not seen between control and test cotyledons, since new proteins are synthesized at the same time. However, chlorophyll and protein amount was elevated in excised cotyledons of *Brassica oleracea* (red cabbage) treated with 10^{-9} M eBL when compared to chlorophyll and protein amount of leaf segments during senescence. Indeed, it was reported that exogenous application of 24-EpiBL arrested protein degradation and enhanced cell membrane stability (Yadava *et al.*, 2016). As senescence continues in cotyledons of soybean seedlings POD activity was increased by 39% and 34% with 10^{-9} M and 10^{-11} M eBL applications,

respectively in comparison with the controls. POD increments parallel to fast catabolic reactions in senescencing organs are somehow in accordance with some studies (He *et al.*, 1996; Kanazawa *et al.*, 2000; Sağlam-Çağ, 2007), while in contradiction with others (Palavan-Unsal *et al.*, 2004). The results indicate a negative correlation of POD activity with chlorophyll content in descending eBL concentrations, POD activity being higher when compared to decrease in chlorophyll content.

Senescence in cotyledons of the seedlings treated with eBL+TIBA was delayed for 8-9 days. This delay is quite confirmed by increased chlorophyll contents of 94% and 204% resulted from 10^{-9} M eBL and 10^{-11} M eBL applications, respectively. The concordance between test plants both treated with TIBA and untreated is quite intriguing. Similarly, protein amounts are parallel to chlorophyll contents. However, when compared to both the controls and other test groups, rate of increase is not as high as that of chlorophyll content (50% and 72%). As to POD, increase in activity is around 14% and 16%, while it is 39% and 34% in cotyledons of the seedlings treated with eBL without TIBA application, being of significance. The results revealing eBL has an inducing effect on POD activity are confirmed by He *et al.* (1996) and Sağlam-Çağ (2007). Both experimental series showed that rates of increase and decrease are nearly at the same level (16%-7%), and there is no pronounced difference compared to the controls. Substances which leak through cell membranes into surroundings during senescence are used as a good parameter (Halevy and Mayak, 1979; Yamane *et al.*, 2005). It is known that leakage rate is gradualy increased towards late stages of senescence (Noodén and Leopold, 1988). Moreover, pigments, sugars and electrolites are observed to leak into surroundings as senescence proceeds (Suttle and Kende, 1978). With the present study, leakage rate is the highest in cotyledons which are yellow (0% green).

One may elucidate from the results that eBL induces senescence by increasing peroxidase activity and destruction of chlorophyll and proteins, but delays it when applied in combination with TIBA. Along with clarifying information on senescence, this study also provides a conclusion that eBL induces senescence together with IAA, and does not act as senescence signal alone.

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