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EFFECTS OF AUXIN ON PHOTOSYNTHETIC PIGMENTS AND SOME ENZYME ACTIVITIES DURING DARK-INDUCED SENESCENCE OF TROPAEOLUM LEAVES

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Abstract

Effects of indole acetic acid (IAA), indole butyric acid (IBA) and naphthalene acetic acid (NAA) were investigated on some physiological parameters in detached leaves of *Tropaeolum majus* L., during dark-induced senescence. Auxin accelerated the loss of chlorophyll (Chl) and carotenoid content whereas it retarded the loss of protein amount at the end of senescing period in leaves significantly. Auxin (IAA and IBA) decreased meaningfully catalase (CAT; EC 1.11.1.6) activity at the second day of senescencing period but increased it at the end of senescence compared to the control group. On the other hand, peroxidase (POD; EC 1.11.1.7) activity was not changed with auxin application. H_2O_2 levels in leaves were unstable during senescing period in both treated and control groups however they were higher in treated leaves than the control group at 6th day of experiment. NAA was found to be the most effective auxin on protein and pigment destruction.

Introduction

Senescence is an oxidative process that involves a general deterioration of cellular metabolism in plants. Senescence symptoms include loss in chlorophyll, carotenoids, proteins and increase lipid peroxidation and membrane permeability resulting in injury, all of which lead to decrease in photosynthetic output (Nooden *et al*., 1997; Prochazkova *et al*., 2001). Besides, senescence is a genetically regulated process comprising many breakdown reactions some of which giving rise to the production of active oxygen species (Kukavica & Javanovic, 2004). In plant cell, reactive oxygen species (ROS) including the superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) are inevitably generated via a number of metabolic pathways (Kanazawa *et al*., 2000; Prochazkova *et al*., 2001; Ashraf, 2009; Noreen & Ashraf, 2009). Free radicals and lipid peroxidation are mostly considered to be major contributors to leaf senescence (Chang & Kao, 1998; Sairam *et al*., 2003, 2004).

Antioxidant enzymes and compounds scavenge on active oxygen species in plants (Farooq & Bano, 2006). However, increase in formation of active oxygen species through senescence change the balance in favor of antioxidant compounds that makes the plant more vulnerable for oxidative damage (Pastori & DelRio, 1997).

There are several factors that can accelerate or delay this breakdown of the photosynthetic apparatus. Plant hormones inducing senescence include ethylene, abcisic acid and jasmonates and those that retard senescence contain gibberellins, cytokinin and auxin (Lim *et al.*, 2003). Decrease in light intensity or darkness result in reduced expression of light-dependent genes and disappearance of photosynthetic proteins and chlorophyll (Wingler *et al.*, 1998).

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Effectiveness of an auxin in maintaining protein levels in detached leaves of *Prunus* were found to be greater in old leaves than in younger ones (Osborne & Hallaway, 1964). When kinetin and NAA were applied separately to excised leaf discs and petioles of detached leaves of broccoli and *Xanthium*, the loss of chlorophyll was strongly retarded by kinetin, but very slightly influenced by NAA. On the other hand, when the two substances were applied concurrently, the effect of kinetin was markedly reduced by NAA (Abrams & Pratt, 1966). IAA, 2,4-D or gibberellic acid did not cause major delay in chlorophyll loss in treated bean leaf discs after leaving the discs of primary (12 to 15 days-old) and younger leaves (30-40 days-old) of bean plants in test solutions or water for 8 days in darkness (Goldthwaite & Laetsch, 1967). Bean endocarp sections were treated with and without NAA and assayed at zero time and after aging for 15 hours. It is stated that auxin may suppress the amount or activity of RNase causing an increase in both RNA and protein and hence preventing senescence (Sacher, 1969). It was suggested that gibberellic acid (GA_3), kinetin and 3-IAA have retarded chlorophyll loss from leaf discs of romaine lettuce but accelerated chlorophyll loss in a high concentration of IAA (10^{-4} M) (Aharoni, 1989).

Many studies have suggested the correlation between auxin levels with senescence and abscission (Sexton & Roberts, 1982; Nooden & Leopold, 1998). One of them was reported that a gradient of auxin levels between the leaf blade and the stalk occurred in bean leaves. Auxin levels declined as leaf aged and senescence occurred when auxin levels between the leaf and stalk were approximately equal (Ellis *et al.,* 2005). Recently, auxin was reported to repress transcription of some genes whose expression is correlated with senescence and/or abscission (Tucker *et al.,* 2002).

According to the literary data, auxin may retard (Ellis *et al.,* 2005) or accelerate (Aharoni, 1989) senescence in some cases in plants. Also, the effect of auxins on the antioxidant enzymes participating senescence period has not been investigated yet. The purpose of this work was to search the effects of auxin on senescence parameters and the antioxidant enzyme activities in darkness-activated senescence period.

Material and Methods

1. Plant material and treatments: *Tropaeolum majus* L., was germinated and raised in growth chamber under 12 h light/25˚C and 12 h dark/18˚C. Laminal sections of leaves were ceased with petiole which is about 7 cm and placed into tubes containing 10^{-6} M IAA, IBA, NAA solutions or distilled water. Analyses were performed after the leaves were kept in darkness for 2, 4 and 6 days. In addition to that, starch content was determined after sun light treatment for 12 h for each dark period.

2. Chlorophyll content: For the extraction of chlorophyll and carotenoids, 0.25 g leaf was homogenized with 80% (v/v) acetone then crude extract was centrifuged at 3000 g for 5 min., at 4ºC. The absorbencies of supernatant were measured spectrophotometer at 450, 645 and 663 nm. The formulae of Arnon (1949) and Jaspars (1965) were used to calculate chlorophyll and carotenoids, respectively.

3. Starch content: The leaf tissues (0.25 g) were homogenized in 3 ml water and centrifuged at 3000 g for 5 min. Supernatants were boiled in hot water bath for 10 minutes and then centrifuged again at 3000 g for 5 min. 0.9 ml of supernatant was added into 0.1 ml of 7.8 mM iodine solution containing 2% potassium iodide (KI) (w/v) and the

mixture was incubated at dark for 5 min. Absorbance of the supernatants were recorded at 600 nm. A standard curve for starch was constructed to estimate the starch content for each sample (Dennis & Winfield, 1978).

4. H_2O_2 content: Leaf tissues (0.25 g) were homogenized in ice bath with 2.5 ml 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12000 g for15 min., and 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) with 1 ml 1 M KI. The absorbance of supernatants was recorded at 390 nm. The contents of H_2O_2 were calculated by standard curve (Velikova *et al.*, 2000).

5. Enzyme analysis and protein content: In order to enzyme extraction, leaf tissues (0.25 g) were homogenized in 50 mM potassium phosphate buffers (pH 7.0) containing 0.1% (w/v) polyvinyl pyrrolidone. The homogenate was centrifuged at 12000 g for 15 min and the supernatant was filtered through Whatman No. 4 filters, and then the supernatant was used as enzyme extract. Protein contents of samples were measured by method of Bradford (1976) utilizing bovine serum albumin as standard.

In order to determine CAT activity, the assay medium contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 30µl enzyme extract in a final volume of 3ml. The decrease in the absorbance of H_2O_2 was recorded at 240 nm for 2 min., using a spectrophotometer (Jasco V-530 UV/VIS, Japan). One unit of activity was defined as the amount of enzyme catalyzing the decomposition of 1 µmol H_2O_2 per min., calculated from the extinction coefficient (0.036 cm^2 μmol^{-1}) for H_2O_2 (Havir & Mchale, 1987).

POD activity was determined by measuring the rate of increase in absorbance of tetraguaiacol at 470 nm using a spectrophotometer (Jasco V-530 UV/VIS, Japan) for 2 min. The assay medium was contained 50 mM potassium phosphate buffer (pH 6.5), 7.5 mM H_2O_2, 10 mM guaiacol and 30µl enzyme extract in a final volume of 3 ml. One unit of enzyme represents the amount of enzyme catalyzing the oxidation of 1 µmol guaiacol min^{-1} using the extinction coefficient (26.6 mM^{-1} cm^{-1}) of tetraguaiacol (Angelini *et al*., 1990).

6. Statistical analysis: Figures in the text indicate mean values ± SE. Differences between control and treated leaves were analyzed by one-way ANOVA, taking $p<0.05$ as significant, according to Duncan's multiple range test.

Results

Biochemical parameters of senescence: The level of decrease in the chlorophyll and protein content are commonly used biochemical parameters to determine the senescence process in detached leaves. When the petioles of the excised leaves were placed in tubes containing water, 10^{-6} M IAA, IBA and NAA solutions in the dark, they gradually turned yellow because of the decrease in Chl content. On the second day, the contents of both Chl and carotenoids in the leaves treated with NAA were higher than those for control group, 6% and 24% respectively (Fig. 1). Whereas at the 4^{th} and 6^{th} days, the loss of Chl and carotenoids were accelerated significantly in the leaves treated with IAA, IBA and NAA. The maximum losses for these parameters were recorded for those treated with NAA.

The starch content of the leaves was assayed in both control and treated groups, degradation of starch accelerated with time for both of them (Fig. 1). This was conspicuous in leaves treated with IAA and IBA at 4^{th} and 6^{th} days, which was higher than the control group. The effect of IBA on the degradation of starch was also found to

be considerable. After each dark period, the leaves were lightened for 12 hours and the effects of auxin on the capacity of starch synthesis were determined (Fig. 1). On the second day, IBA treatment significantly increased the starch content. However, on the 4th and 6th days the levels of starch content for each treatment was considerably lower than those for control group. Auxin substantially increased the damages in starch synthesis mechanism throughout the dark period.

Auxins retarded the amount of protein loss in leaves during the period of senescence, which is most prominent in 6th day (Fig. 2).

Hydrogen peroxide content: The amount of H_2O_2 in both treated and control groups fluctuated throughout the dark period (Fig. 2). On the second day, the amount of H_2O_2 in IAA and NAA-treated leaves decreased considerably compared to control group. However, at the 4th day, IAA and NAA increased H_2O_2 level. At the 6th day; all auxin raised significantly the amount of H_2O_2. The maximum enhancement was observed in IAA treated leaves at the 6th day

Enzymatic changes during senescence: On the second day, CAT activity in IBA, IAA and NAA-treated leaves was lower than control group; however, the decrease for CAT activity in IBA and IAA-treated leaves was more significant (Fig. 2). But, IAA and IBA increased significantly at CAT activity at the 6th days. IAA and IBA treated leaves had about 67 and 68% of initial activity, respectively. Peroxidase activity gradually increased both in control and treated leaves with time during senescence (Fig. 2). Auxin had no effects significantly of peroxidase activity compared to control group.

Discussion

Chlorophyll and protein loss in leaves are conventionally used biochemical parameters to indicate senescence (Bora *et al*., 2007). Leaf senescence is marked by degradation in chlorophyll levels. In the present paper, a decline in chlorophyll and carotenoid content were evident in the leaves of both control and treated plants after their transfer to dark, but the extent of the decline in chlorophyll content in treated plants was significantly higher than in control plants. Our results are convenient to the previous study (Even-Chen *et al*., 1978) in which they showed that NAA treatment in 14 week-aged tobacco plants increased the loss of chlorophyll. Moreover high concentration levels of IAA in leaf discs of lettuce also accelerated the chlorophyll loss (Aharoni, 1989). However, IAA was suggested to prevent the loss of chlorophylls throughout the aging of chloroplasts in wheat both *in vivo* and *in vitro* conditions (Misra & Biswal, 1980). Plant tissues contain substantial amounts of carotenoids that serve as scavenger compounds to oxygen radicals (Lavelli *et al*., 2006).

Many protease genes show induced expression during senescence (Buchanan-Wollaston *et al.,* 2003). Nutrients convert to transportable form during senescence (Ali & Bano, 2008). Also, the numerous enzymes such as metallothionin and peroxidase increase their synthesis. These enzymes are required to protect cell function against released peroxides and metal ions (Buchanan-Wollaston, 1997; Nooden *et al*., 1997). In our study, protein content decreased in both control and treated plants senescing period. However, auxins retarded loss of protein content. IAA and IBA retarded loss of protein more than NAA during senescence.

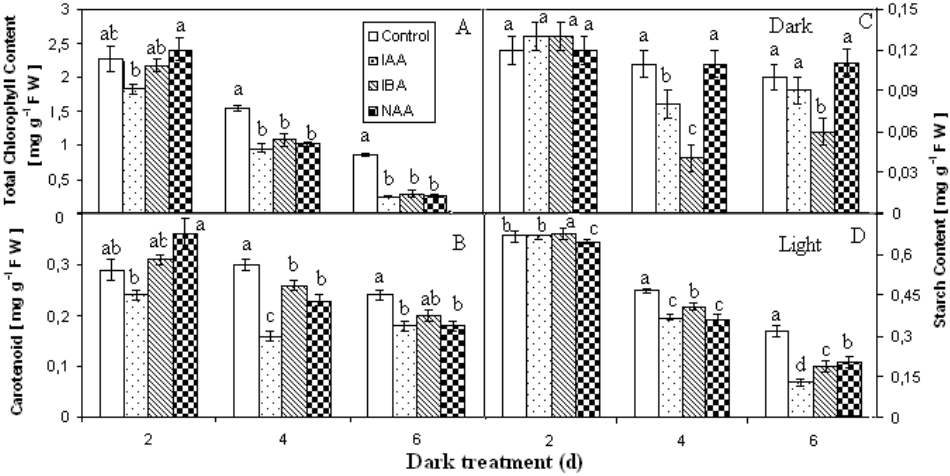

Fig. 1. Effects of IAA, IBA and NAA on contents of chlorophyll (A), carotenoid (B), starch (both dark, C and light, D) of *T. majus* leaves with time after detachment. Vertical bars indicate SE of mean (*n*=4).

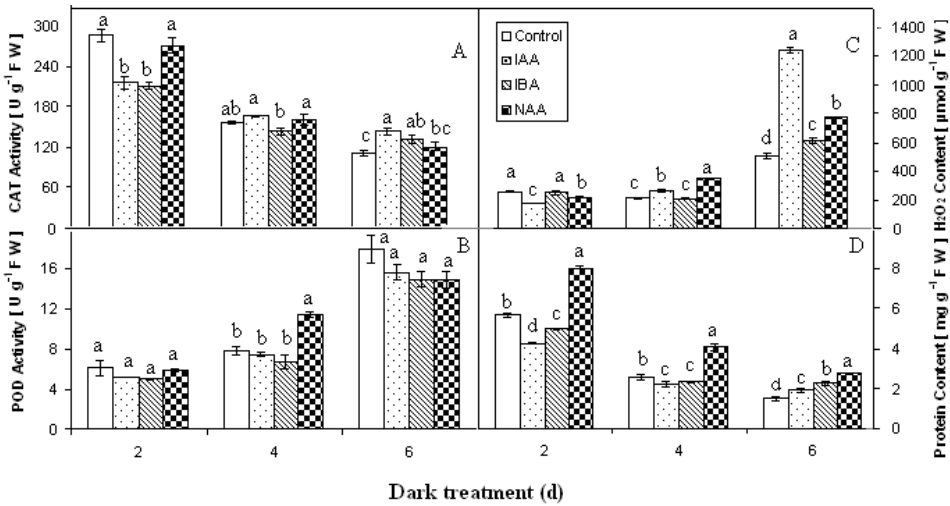

Fig. 2. Effects of IAA, IBA and NAA on CAT (A), POD (B) activities, H_2O_2 (C) and protein (D) contents of *T. majus* leaves with time after detachment. Vertical bars indicate SE of mean (*n*=4).

Although activities of protease increased during senescence, auxin may retard loss of protein because it induces synthesis of protease inhibitor. It was reported that auxin induced protease inhibitor genes expression in senescence period (Young *et al.*, 1994).

Of the various postulates concerned with senescence initiation in plants, the free radical hypothesis has recently attracted considerable attention (Bora *et al.*, 2007). Activated oxygen species such as superoxide ($O_2^{\cdot-}$) or H_2O_2 and their interaction products react with proteins, lipids and nucleic acids and their accumulation therefore may initiate leaf senescence (Bora *et al.*, 2007). Senescence is genetically regulated process

comprising many breakdown reactions, with some of them leading to the production of active oxygen species (Kukavica & Jovanovic, 2004; Rosenvasser *et al*., 2006). In our studies, H_2O_2 content increased with time and reached the highest level at the final stage of senescing. Various workers have also reported the enhancement in H_2O_2 content and lipid peroxidation during senescence (Chang & Kao, 1998; Finger *et al*., 1999; Hung &Kao, 2004). Also, the senescence-induced H_2O_2 leaking from peroxisomes might act as a second messenger in the cellular signal transduction pathway (Chang & Kao, 1998; Kukavica & Jovanovic, 2004; Zimmermann & Zentgraf, 2005). That induces a number of genes and proteins involved in stress defense enzymes e.g., superoxide dismutase, catalase, peroxidase, glutathione peroxidase and glutathione S-transferase (Levine *et al*., 1994; Zimmermann & Zentgraf, 2005). Morita *et al*., (1999) have shown that H_2O_2 is also involved in oxidative stress signaling which leads to the induction of cytosolic ascorbat peroxidase genes in rice. Therefore, H_2O_2 involvement is pointed out in abscisic acid-induced senescence of rice leaves (Hung & Kao, 2004).

Decline in antioxidant enzymes activity has been reported as the possible causes of leaf senescence in different plants by various workers (Kar & Mishra, 1976; Prochazkova *et al*., 2001). It was suggested that phytohormones can regulate the synthesis of basic antioxidant enzymes, and some of the isoforms of antioxidant enzymes are also implicated in phytohormone catabolism (Szechyńska-Hebda *et al*., 2007).

In this study, auxins generally did not affect CAT and POD activities except for CAT at sixth day during senescence period. However IAA and IBA retarded loss of protein in senescing period.

Conclusion

Senescens is a genetically regulated metabolic breakdown process. During this process synthesis of hydrolytic enzymes increase and therefore molecules like chlorophyll and proteins breakdown quickly. Auxins may retard loss of proteins since they encourage synthesis of protease inhibitors.

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