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Effects of Triacontanol on Senescence of Radish (*Raphanus sativus* L.) Cotyledons

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무우 자엽의 노쇠에 미치는 Triacontanol의 효과

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ABSTRACCT

Effects of triacontanol(TRIA) on several parameters of senescence including the changes of related enzyme activities were investigated in radish(*Raphanus sativus* L.) cotyledons developing in light. In senescing radish cotyledons, 1.0mg TRIA/l retarded the degradation of chlorophyll content. Moreover, it depressed the increases of malondialdehyde and H_2O_2 contents compared to the control. Catalase and superoxide dismutase activities were highly maintained but the increase of peroxidase activity was inhibited remarkably under the TRIA application.

These results suggested that TRIA participated in the regulation of senescence during the late part of cotyledon development where it delayed senescence through its action on free radical-associated enzymes and consequent metabolic turnover.

INTRODUCTION

The phenomenon of leaf senescence, in general, results from the deteriorative processes which normally occur during leaf ontogeny under the genetic control. Then, the progress of leaf senescence is accelerated or retarded through the influence of environmental factors(Dhindsa *et al.*, 1981).

The nature of the inducers starting such a senescence program in leaves are not yet understood(Kar and Feierabend, 1984). However, some possibilities have been presented up to date. Among the various possibilities that are discussed, such as source-sink competitions during redirection of nutrients within plants, changing hormonal controls, or change of the energy status of the cell, the free radical theory has recently attracted increasing attention(Brennan and Frenkel, 1977; Fridovich, 1976; Thomas and Stoddart, 1980; Leshem, 1981). During the last few years, some aspects of free radical metabolism, such as superoxide dismutase and lipid peroxidation(Dhindsa *et al.*, 1981), peroxide levels(Brennan and Frenkel, 1977), and the

behavior of some activated oxygen(Kar and Feicrabend, 1984) have been studied during senescence of leaves. In a view of the free radical theory, the large accumulation of free radicals are claimed to occur in senescing leaves. Also, the theory proposes that free radicals may oxidize cell membranes rapidly and result in serious damage of cell constituents in which they are present. Thus, free radicals may initiate the progress of leaf sennescence.

Since the plant growth regulating activity of triacontanol(TRIA: a 30-carbon primary fatty acid alcohol) was first observed in some crop plants in 1977(Rics *et al.*, 1977), there have been many attempts to elucidate the action mechanism of TRIA within growing plants(Hangarter *et al.*, 1978; Eriksen *et al.*, 1981; Haugstad et al., 1983; Houtz *et al.*, 1985 a and b). TRIA has recently shown the effect on retention of green leaf area in rice leaf senescence assay(Debata and Murty, 1981). Bhalla (1981) also found that TRIA was effective in causing the retardation of chlorophyll loss in senescing oat leaf.

In addition, TRIA reduced wound-induced ethylene synthesis in pea seedlings like kinetin ad benzyladenine(BA) which were known as senescence retardents (Saltveit and Dilley, 1979).

The above researches have shown the visible effect of TRIA applied to plants, however, the action mechanism of TRIA still remains to be answered. There is also a report that the senescence-retarding action of cytokinin is attributed to its ability to act as a free-radical scavenger(Leshem, 1981).

Therefore, the present study is aimed to investigate the possibility that the senescence-retarding effect of TRIA may appear through a modulation of free radical metabolism in radish cotyledons following TRIA application.

MATERIALS AND METHODS

Plant materials. Radish seed (*Raphanus sativus* L. cv Taiwang) surface was sterilized with 1% solution of sodium hypochlorite. The seeds were sown and allowed to germinated in glass-covered plastic containers which contained 3 layers of filter paper (Toyo No.2) moistened with distilled water or treatment solution. The radish seedlings in growth chamber were grown for 8 days without nutrient supply at 25+1℃ and continuously irradiated with daylight tube (General Electric, U.S.A.) giving an approximate intensity of 20W.m^{-2}. Cotyledons were harvested at daily intervals after sowing for biochemical and enzyme assay.

Preparation of treatment solutions. Treatment solutions consisted of aqueous 0.1%(v/v) Tween 20 containing TRIA (Sigma Chemical Co.) prepared from stock dissolved in acetone. The amount of stock added to distilled water was adjusted to achieve a final concentration of 0.1% Tween 20 and 1 mg/l TRIA.

Biochemical analyses. Chlorophyll content was determined by the procedure of Arnon(1949). Malondialdehyde (MDA, a product of lipid peroxidation) content was estimated as thiobarbituric acid (TBA)-reactive material from cotyledon extracts in 0.1% trichloroacetic acid (TCA) according to procedure of Dhindsa *et al.* (1981). Measurements were corrected for

unspecific turbidity by substracting the absorbance at 600nm from the value at 532nm. The concentration of MDA was calculated using its extinction coefficient of $155mM^{-1}cm^{-1}$ (Heath and Packer, 1968). Total inorganic peroxide was estimated from cotyledon extracts, as described by Bernt and Bergmeyer(1974). Inorganic peroxide content is expressed as H_2O_2 equivalent.

Enzyme extraction and assay. Ten pairs of cotyledons were ground on ice with 0.3g quartz sand in 4ml 70mM K-phosphate buffer (pH8.0). After the homogenate was centrifuged at 18,000 x g for 30min, the clear supernatant was used for enzyme assays. Catalase (EC 1.11.1.6) activity was assayed according to Chance and Maehly(1955). In a total volume of 3ml, the reaction mixture contained 0.05M K-phosphate buffer (pH 7.0), 10mM H_2O_2 and 0.05ml of enzyme extract. Enzyme activity was estimated from the decrease of absorbance at 240nm using an extinction coefficient of $0.44 \times 10^2 M^{-1}cm^{-1}$(Chance and Maehly, 1955). Superoxide dismutase (EC 1.15.1.1) activity was assayed by measuring its abillity to inhibit the photochemical reduction of nitroblue tetrazolium(NBT) using the method of Beauchamp and Fridovich(1971). The 3ml reaction mixture contained 50mM K-phosphate buffer (pH7.8), 13mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1mM EDTA, and 0-50 μl enzyme extract. The reaction was initiated and terminated by turning the light on and off. The reaction mixture lacking enzyme developed the maximum colour and this decreased with increasing volume of enzyme extract added. The absorbance at 560nm was plotted as a function of the volume of enzyme extract used in the reaction mixture. From the resultant graph, the volume of enzyme extract corresponding to 50% inhibition of the reaction was read and was considered as one enzyme unit (Giannopolitis and Ries, 1977). Peroxidase (EC 1.11.1.7) activity was assayed according to a modification of the procedure of Chance and Maehly(1955). The 3ml volume of reaction mixture contained 0.07M K-phosphate buffer (pH6.0), 5mM guaiacol, 10mM H_2O_2, and 0.1ml of enzyme extract. The reaction was started by the addition of H_2O_2 and estimated from the increase of absorbance at 430nm.

RESULTS AND DISCUSSION

Changes in the biochemical parameters of senescence. It is well known that the biochemical parameters frequently used to determine the senescence process in leaves are the decrease in the chlorophyll content and an increase in the malondialdehyde content(Kunert and Ederer, 1985; Pauls and Thompson, 1984) and total phenolics content(Kar and Mishra, 1976). Leaf chlorosis due to chlorophyll degradation is one of the most visible phenomena occurring during the leaf senescence. Thus, the effect of TRIA on the change of chlorophyll content was investigated during the development of radish seedlings (Fig 1). The chlorophyll contents of cotyledons in both the control-(DW treatment and Tween 20 only treatment) and the TRIA treatment increased during early cotyledon growth and reached to maximum values at day 5 after sowing. Thereafter, they showed a decline with onset of cotyledon senescence. However, the

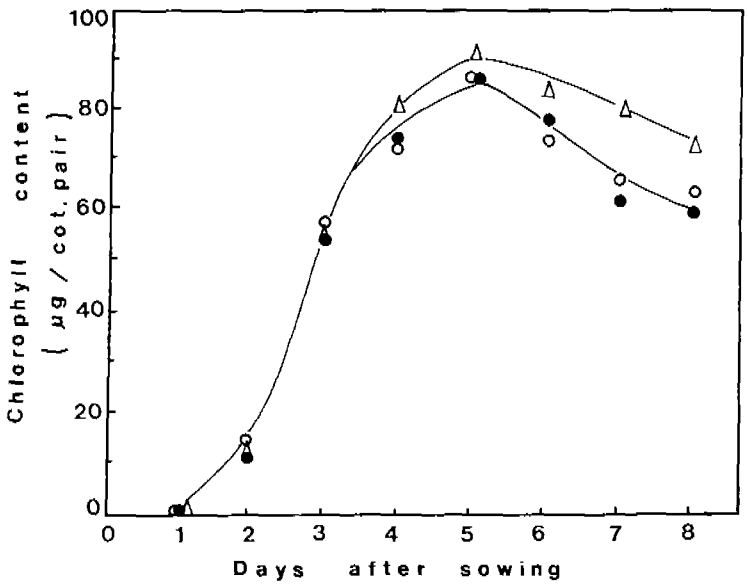

Fig. 1. The effect of triacontanol on chlorophyll content in the cootyledons of radish seedlings: -○-, D.W.; -●-, 0.1% tween 20; -△-, 1.0mg triacontanol+0.1% tween 20.

decrease of chlorophyll content in TRIA-treated cotyledon after day 5 was noticeably retarded as compared to the control. This result is consistent with the results of TRIA-induced chlorophyll retention in senescing leaves of oat and rice(Bhalla, 1981; Debata and Murty, 1981). These results, therefore, indicate the possibility of the senescence-retarding action of TRIA. On the chlorophyll breakdown in senescing leaves, even though the intermediate steps in its biodegradation are still obscure, the enzymatic degradative process and a directive photooxidation of pigment have been suggested(Kato and Shimizu, 1985). The photooxidation of pigment include the activated O_2 species such as superoxide or H_2O_2. It is also known that peroxidase, predominantly found in plant materials, bleaches chlorophyll in the presence of H_2O_2 and certain phenolics(Katoand Shimizu, 1985). In order to understand the effect of TRIA on chlorophyll retention in senescing cotyledon, we think, it seems to be essential to examine the possible role of TRIA in the free radical metabolism together with the associated enzyme system.

The trend of changes in malondialdehyde (MDA) content of cotyledon is shown in Fig.2. It has been known that the cellular membrane deterioration during leaf senescence may be a consequence of cumulative increase in the level of membrane lipid peroxidation (Kunert and Ederer, 1985;Dhindsa *et al.*, 1981). Pauls and Thompson(1984) found that MDA, a product of lipid peroxidation, was accumulated in older bean cotyledons. Thus, lipid peroxidation at present is considered to be an important process underlying cellular membrane degradation during leaf senescence. In our experiment using the radish cotyledons, the increasing patterns

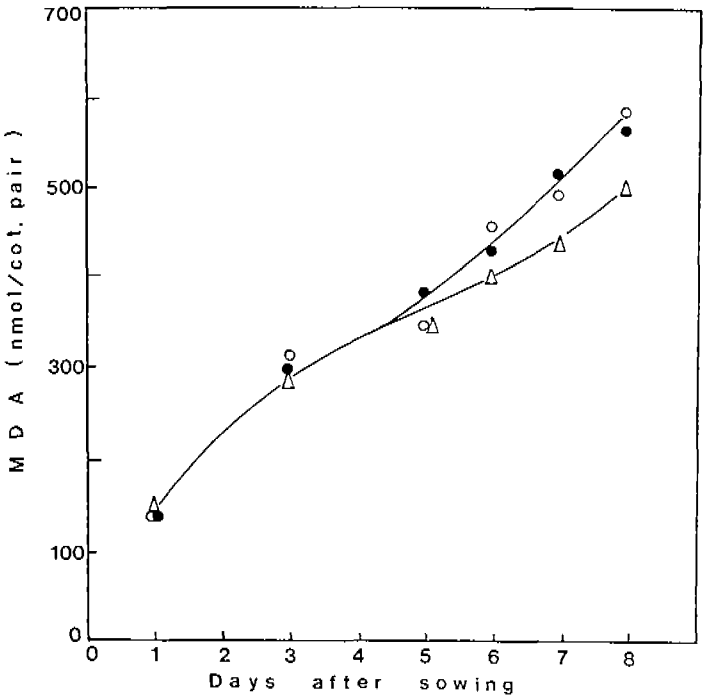

Fig. 2. The effect of triacontanol on changes in malondialdehyde (MDA, a product of lipid peroxidation)content in the cotyledons of raidsh seedlings: -○-, D.W.; -●-, 0.1%tween 20; -△-, 1.0mg/1 triacontanol+ 0.1% tween 20.

of MDA content showed no significant difference between the control and the TRIA otreatment during early cotyledon growth from day 1 to day 4. However, beginning with day 5 after sowing, the increase of MDA content in TRIA treated cotyledons was retarded more or less in comparison to the control cotyledons until day 8 after sowing.

As the primary leaves of bean senesce, increasing proportions of membrane lipids transform from the normal liquid-crystalline phase to the gel phase (McKersie and Thompson, 1978). In this case, lipid peroxidation contributes to the formation of gel-phase lipid which results in membrane permeability. In senescing tobacco leaves,

Dhindsa *et al.* (1981) also have correlated increased membrane permeability with lipid peroxidation. This increased membrane permeabillity seemes to involved further in damaging reactions for membrane and cellular constituents, such as proteins, DNA, and chlorophyll. Therefore, the inhibition effect of TRIA on MDA accumulation in radish cotyledons during the late growth period indicates that TRIA may have, in part, a regulating ability of cotyledon senescence through a modulation of lipid peroxidation in cellular membrane. Such result further suggests that TRIA may be involved in metabolic event of hydrogen peroxide that readily react with membrane lipids and may thus initiate senescence.

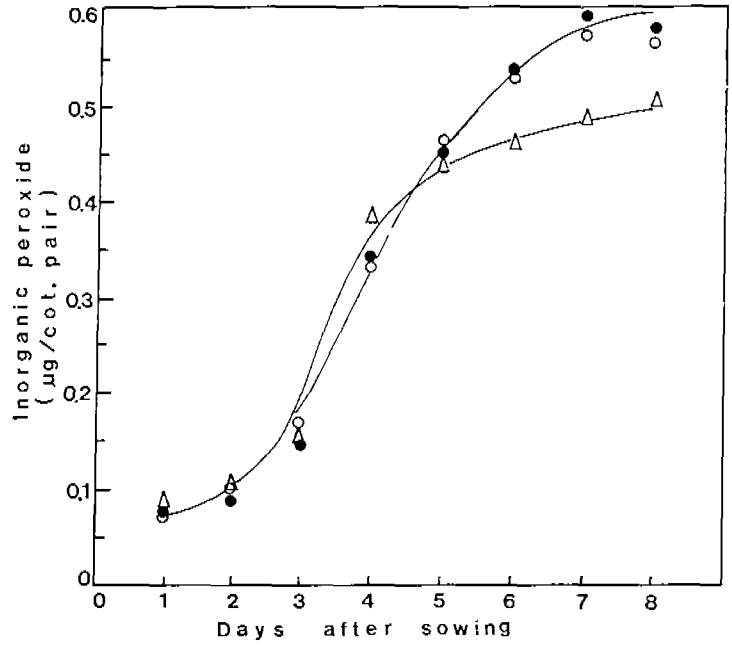

Fig. 3. Changes in inorganic peroxide content of radish cotyledons with time after sowing -○-, D.W.; -●-, 0.1% tween 20; -△-, 1.0mg/l triacontanol+0.1% tween 20.

Changes in the total inorganic peroxide(H_2O_2) levels. Free radicals participate, chiefly, in the form of activated O_2 species such as superoxide or H_2O_2, in several electron-transfer reactions of normal cell metabolism and are usually controlled by the appropriate protective mechanisms, such as superoxide dismutase(SOD), catalase, and peroxidase (Fridovich, 1976; Kar and Feierabend, 1984). It has been speculated that activated oxygen may leak away from faulty defense mechanism and, in addition, give rise to the generation of other, even more aggressive oxygen radicals, such as the hydroxyl radical, singlet oxygen, and lipid hydroperoxides, that readily react with nucleic acids, proteins and lipids and may thus initiate senescence (Leshem, 1981; Fridovich, 1976). In the present study, therefore, we have investigated some aspects of activated-oxygen metabolism, such as the behavior of SOD, catalase, peroxidase, lipid peroxidation and the total H_2O_2 levels. To begin with, we have examined the time course of H_2O_2 content in radish cotyledons in order to confirm the involvement of H_2O_2 in cotyledon senescence. Figure 3 shows the changes in endogenous H_2O_2 content in cotyledons with time after sowing. As presented in Figure 3, TRIA did not affect the level of H_2O_2 in comparison with the control in early growth period from day 1 to day 4, but the H_2O_2 accumulation in the late stage was conspicuously inhibited by TRIA treatment. Brennan and Frenkel(1977) showed that application of ethylene, a senescence-stimulating hormone, induced the increased peroxide levels in pear fruits and also that application of glycolate or xanthine, serving as substrates for the formation of H_2O_2, increased the peroxide content of the tissue and

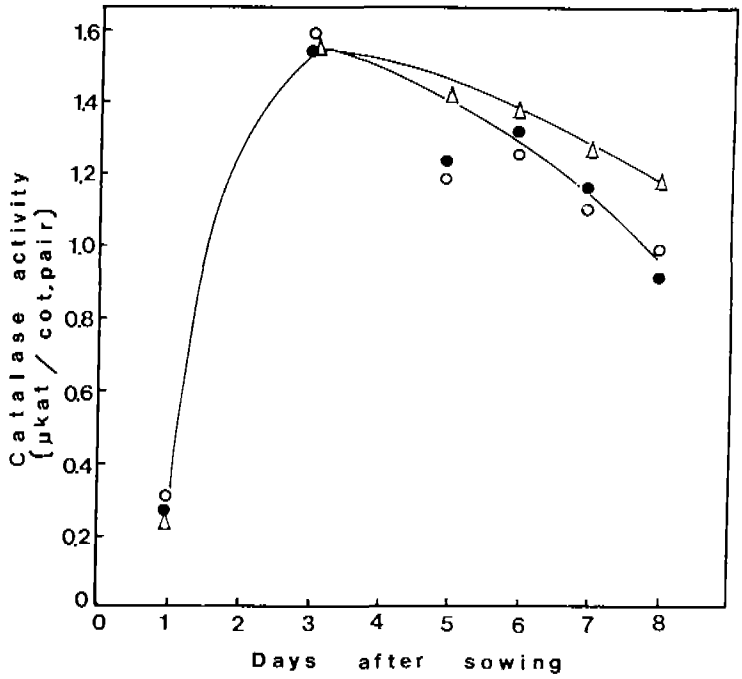

Fig. 4. The effect of triacontanol in catalase activity in the cotyledons during development of radish seedlings: -○-, D.W.; ●, 0.1% tween 20; -△-, 1.0mg/l triacontanol+0.1% tween 20.

accelerated the onset of ripening.

Hydrogen peroxide, therefore, must be involved in oxidative processes required in the initiation and promotion of senescence phenomena. From the above reports and our results, it was assumed that the retardation of radish cotyledon senescence by TRIA might be a consequence of an inhibition on cellular membrane oxidation due to scavenging of H_2O_2 possibly controlled by TRIA through a modulation of free radical metabolism. Our results, thus, further suggest the need to investigate the possibility that the senescence-retarding effects of TRIA may be occured through a regulation of the behaviors of catalase and SOD which destroy the H_2O_2 and superoxide radical, respectively.

Changes in the activities of catalase, SOD and peroxidase In order to discover the TRIA effect on catalase activity in radish cotyledons, its activity profile was investigated for 8 days of germination (Fig. 4). The activity of catalase increased rapidly during early cotyledon growth and reached a maximum value at day 3. Thereafter, it decreased gradually with the progress of senescence. In the TRIA-treated cotyledons, however, the decreasing trend of the activity in senescing stage was delayed remarkably over control cotyledons.

Dhindsa *et al.* (1981) examined the biochemical nature of the processes of membrane deterioration during senescence in order to better understand the causes of senescence. They showed from their experiments that tobacco leaf senescence might result from the membrane

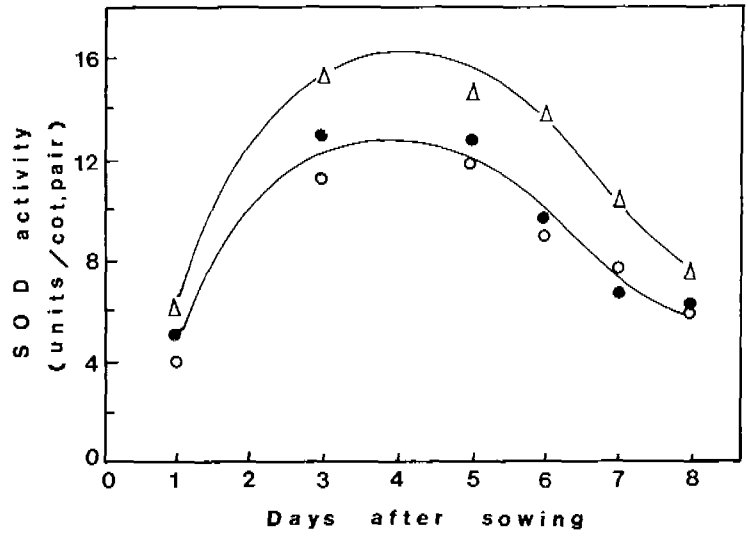

Fig. 5. The effect of triacontanol in SOD activity in the cotyledons during development of radish seedlings: -○-, D.W.; -●-, 0.1% tween 20; -△-, 1.0mg/l triacontanol+0.1% tween 20.

deterioration due to increasing level of membrane-lipid peroxidation controlled by the activities of SOD and catalase.

Brennan and Frenkel(1977) observed that the pear fruit senescence was promoted when H_2O_2 were conserved by inhibiting the activity of catalase with KCN. Also, Kar and Mishra(1976) found that kinetin treatment retarded the decrease of catalase actibity during rice leaf senescence and thus led to delay the process of senescence.

Therefore, it is likely that the senescence-retarding effects of TRIA may be exerted through a inhibition of H_2O_2 accumulation(Fig 3) subjected to catalase activity.

In the case of SOD activity profile, the changes of activities are presented in Figure 5. The overall fluctuation patterns in SOD activities were smiliar to each other in both the control and the TRIA treatment, however, the activity of TRIA-treated cotyledon was retained in high level compared to the control during the whole period of growth.

McRae and Thompson(1983) measured the formation of superoxide radicals by chlroplasts from senescing bean leaves and showed the propensity of chloroplasts to produce increased levels of superoxide radicals with advancing senescence.

Moreover, they observed that the peak in superoxide radical production during leaf senescence coincided with the initiation of lipid peroxidation and the formation of gel-phase lipid in chloroplast membranes leading to increased membrane permeability. Kar and Feierabend(1984) found that SOD activity declined with progress of senescence in wheat and rye leaves. Such a superoxide radical is converted by SOD to H_2O_2 which can then be removed by catalase(Dhindsa *et al.*, 1981). Accordingly, the activities of SOD and catalase can determine the abundance of superoxide radical and H_2O_2 in the tissue, and thereby control the level of

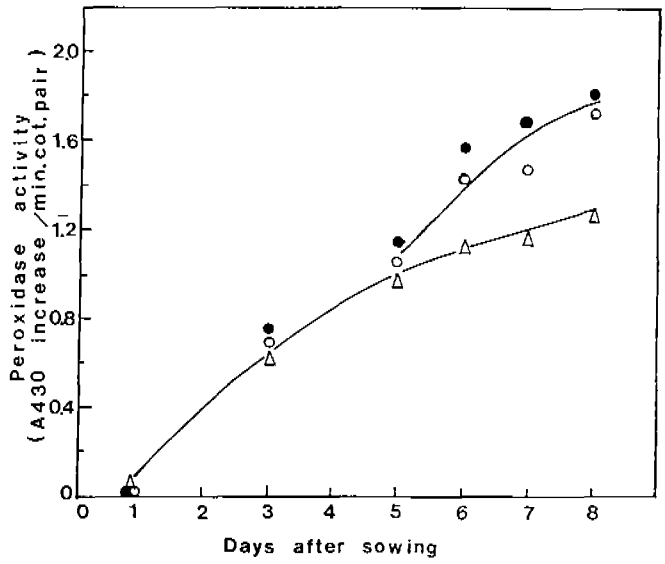

Fig. 6. The effect of triacontanol on peroxidase activity in the cotyledons during development of radish seedlings: -○-, D.W.; -●-, 0.1% tween 20; -△-, 1.0mg/l triacontanol+0.1% tween 20.

lipid peroxidation. In conclusion, the senescence-retarding action of TRIA in radish cotyledons is probably attributed to its ability to act as a regulator of some aspects of free radical metabolism.

Peroxidase is rich in all higher plants (Dunleavy and Ramaraje, Urs, 1978) and cytochemical localizations of it have shown that this enzyme is present in vacuoles as well as in the endomembrane system and in cell walls(Matile, 1980; Martinola et al., 1982). In the presence of H_2O_2, peroxidase can oxidize some of phenolics, organic matters, and chlorophylls(Huff, 1982; Martinola *et al.*, 1982; Kato and Shimizu, 1985). Moreover, the increase in peroxidase activity, can be taken as one of the reliable indicators of senescence due to its ability to oxidize IAA(Birecka *et al.*, 1979) and the above-mentioned implications of peroxidase. Therefore, the change of peroxidase acitivity from cotyledons was investigated during the development of radish seedlings after apliction of TRIA. As presented in Figure 6, peroxidase showed a increasing activity toward the end of cotyledon senescence regardless of TRIA-treatment. However, the increasing activity in TRIA treated cotyledons was noticeably retarded in contrast to the control during the senescing stage of cotyledons. Patra and Mishra (1979) reported that peroxidase activity was high in the senescent rice leaves and there existed a correlation between chlorophyll content and peroxidase activity. Henry and Gordon(1980) found that pea tissues treated with TRIA(0.1mg/l)plus 10 μm GA_3 showed a 20% decrease in peroxidase activity compared to the control.

In a view of the peroxidase that can oxidize IAA and, in turn, promote the senescence processes, an alternate action mechanism of TRIA in senescence retardation can be suggested.

Indeed, Galston(1949), in a study of pea pith tissue, suggests that IAA oxidase is a peroxidase with the molecule having perhaps two different active sites; 1) acting as an IAA oxidase to destroy IAA; and, 2) possibly acting as a regular oxidative peroxidase enzyme(cited in Henry and Gordon, 1980).

In conclusion, our present data suggested that TRIA participated in the regulation of senescence during the late phase of cotyledon development where it delayed senescence through its action of free radical-associated enzymes and consequent metabolic turnover.

적 요

광조건하에서 무우 유식물의 발달중, 자엽의 노쇠에 관한 생화학적 요인 및 그와 관련된 효소의 활성도에 미치는 triacontanol(TRIA)의 효과를 조사하였다. 노쇠중인 자엽내에서, 1.0mgTRIA/l로 처리된 자엽은 처리되지 않은 자엽에 비하여 엽록소의 분해가 억제되었으며 더우기 TRIA처리는 MDA, 및 H_2O_2등의 함량의 증가를 지연시켰다. 또한, catalase와 superoxide dismutase의 활성도가 높게 유지 되었으나 발달중 peroxidase의 활성도 증가는 현저하게 억제되었다.

이상의 결과로부터, TRIA는 후기 자엽발달동안에 있어서 자유라디칼에 관련된 효소활성도의 조절 및 그로인한 자유라디칼 대사의 조절을 통해 무우 자엽의 노쇠억제에 관여하고 있음을 알 수 있었다.

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