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Effects of Aqueous Extracts of *Pinus rigida* on Protein and Isozyme Patterns during Radish Germination

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리기다소나무의 수용추출액이 무 종자의 발아과정에서 단백질과 동위효소 패턴에 미치는 영향

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ABSTRACT

Aqueous extracts of *Pinus rigida* changed the electrophoretic patterns of total proteins and of hydrolytic enzymes such as peroxidase, esterase and amylase during the germination of radish (*Raphanus sativus* var. *hortensis* for. *acanthiformis*). When the extract treatment was finished, at the late stage of radish germination, aqueous extracts of *P. rigida* had suppressed the expression of 24 KD and 60 KD proteins. The extract induced new isozyme bands, indicating concomitant activity of peroxidases, especially in the cathodic region. Although it reduced the number of isozyme bands of esterases, esterase activities were stimulated in the cathodic region. The activity of amylase was enhanced by the extract.

Key words: *Pinus rigida* extract, Peroxidase, Esterase, Amylase, Isozyme, *Raphanus sativus* var. *hortensis* for. *acanthiformis*.

INTRODUCTION

It is known that allelopathy is an important ecological phenomenon that inhibits seed germination and seedling growth in plant-plant interaction. The allelochemicals of plants repress photosynthesis and cell division (Bhowmik and Doll 1984), and change photorespiration, permeability, protein synthesis and enzyme activity (Lodhi and Killingbeck 1981, Del Moral 1972, Dieterman *et al.* 1964). As seed germination proceeds, stored protein content decreases because amino acids are hydrolyzed (Evans and Bhatt 1977). There are many isozymes of peroxidase, amylase, esterase and catalase that affect the growth and differentiation of the plant cell (Corcoran *et al.* 1972).

Peroxidase is sensitive to non-optimal growth conditions, such as wounding, noxious materials, dryness and coldweather, is easily damaged and exerts an influence upon seedling growth, differentiation and organic formation of the cell (Seeni and Gnanam 1981). Esterase mostly appears during nonspecific substrate-reactions, and is concerned with hormone reactions, growing period, and features of genetic revelation by plant part (Abbot *et al.* 1984). Amylase has been classified as α-amylase or β-amylase according to its mode of action and is an important enzyme beca-

use it hydrolyzes starch in the early part of seed germination.

Accordingly, the study of enzymes concerned with seedling growth of plants pursue only the pathways of physiological metabolism in a physiological and ecological unit. Thus, no study has been done on the increase of cells in seed germination or enzymatic changes as they mutually represent plants in states of natural growth.

This paper describes aqueous extract of *Pinus rigida* that had effects on protein and isozymes of peroxidase, esterase and amylase in the processes of seed germination of radish (*Raphanus sativus* var. *hortensis* for. *acanthiformis*).

MATERIALS AND METHODS

Aqueous extracts and radish germination

Aqueous extracts were made from the fresh leaves of *Pinus rigida*. One liter of distilled water was added to 200 g of fresh leaves at 80℃ and then distillated for 48 hr, and each aqueous extract was filtered through a 150 mm filter paper. The filtrates were centrifuged at 1,000 g for 30 min (Centrikon T.-1045, Kontron Co) and the supernatants were used as the materials of this experiment.

The radish germination test was carried out in glass Petri dishes (d, 12 cm) on two pieces of filter paper wet with various concentrations (3%, 12%, 25%, 50%, 75% and 100%) of the aqueous extracts. Distilled water was used for the control. Each dish containing 50 seeds was placed in a 28℃ incubator (Hotpak). Three replications were used.

Electrophoresis

When germinating seeds of radish were treated with various concentrations of extract, the threshold concentrations were 60% and 25%, respectively. So each of seeds was treated at 60% and 25% to execute the germination experiment, and 20 individuals were harvested every day for 7 days, kept in a deepfreezer and used for the electrophoretic experiment. Protein extraction was performed as follows: 20 seeds were added to 0.9 ml of distilled water, homogenized, and then centrifuged for 15 min at 15, 000 rpm. The supernatant was mixed with sample buffer (1:1) and treated with boiling water for 5 min, then used for electrophoresis. Electrophoresis (SDS-PAGE) was carried out as described by Laemnli (1970). The gel was 12% and electrophoresis was accomplished at a constant current of 15 mA for approximately 5~7 hr. The gel was stained with a solution of 0.12% Comassie Brilliant Blue R-250 for two hours or more and then destained (acetic acid : methanol : water=1:5:4, v/v).

The intact electrophoretic sample was used as an isozymic assay sample, and IEF-PAGE was assayed by a modified procedure of Osterman (1984) and Stegmann *et al.* (1985). When the gel was standardized at 30 ml total volume, polyacrylamide 5.5 ml (0.3 g/ml) was added by 1.5 ml of phamalytes (pH 3~10, pH 4~6.5) to control pH range. IEF-PAGE was placed onto an electrophoresis apparatus (7 cm× 22 cm×0.75 cm) and an electrode strip of 0.1 M ethanolamine and 0.04 M aspartic acid was applied as the cathode and the anode, respectively, also this strip was stood in the center of a line and added to 12 l of sample, and energized to 100 V for 1 hr and then to 200~500 V for approximately 3 hr.

Isozyme activity and staining

The staining of the isozymes of peroxidase, esterase and amylase were performed as follows. Peroxidase isozyme was stained with 2 ml of dimethyl formamide made from 100 ml of 0.1 M acetate buffer (pH 4.5) and 0.01 g of 3-amino-9-ethylcarbazole and then dyed in a solution of 1 ml of 1 M $CaCl_2$ and 100 μl of H_2O_2 (30%) for 40 min. Esterase isozyme was stained with solution made from 250 ml of phosphate buffer (0.2 M, pH 7.0), 40 mg of α-naphthy acetate and 100 mg of fast blue RR salt at 37℃ for 30 min. Amylase isozyme was precipitated in a solution of 30% soluble starch,

washed with distilled water and then, negative staining was performed for 10 min in mixed solution made from 20 mM I_2, 28 mM KI, acetic acid and distilled water.

Peroxidase activity was measured using guaiacol as a substance. 0.1 ml of each sample was added to the reaction solution which included 0.005 ml of 12.3 mM guaiacol, 0.05 ml of 0.042% H_2O_2 and 3.0 ml of 10 mM potassium phosphate buffer (pH 7.8) and reacted at 30 ℃ and absorptance measured at 436 nm, using a spectrophotometer (UV-120-02, Shimadzu, Japan) (Worthington enzyme manual 1972).

RESULTS AND DISCUSSION

The pattern and activity change of protein

During radish germination, we treated them with the 60% extract of *P. rigida* and studied the change of protein every 4 hr which led to the results shown in Fig. 1. In the early part of germination, they appeared the same as control until 34 hr, afterwards strong new activity was formed in the 24 KD and 60 KD bands, and when compared with the bands of early germination, they later decreased (Fig. 1A). In comparison with the control group, the treated group was almost the same but the 24 KD and 60 KD bands did not appear until 60 hr (Fig. 2B).

When the differences of the synthesis of protein are investigated according to the period of germination after treatment of extracts by the SDS-PAGE, there are no differences between the control groups and the treated groups at the beginning time of germination (18 hr), but there is a difference at the late period of germination (60~105 hr). Namely, 24 KD and 60 KD bands come out only in the control group. This result indicates that the synthesis of protein is inhibited at the late period of germination. This corresponds with the report (Kil and Yim 1983) that allelochemicals have a stronger inhibitory effect at the late period of germination than at the beginning of germination and it confirms that the synthesis of protein is inhibited at the late period of germination.

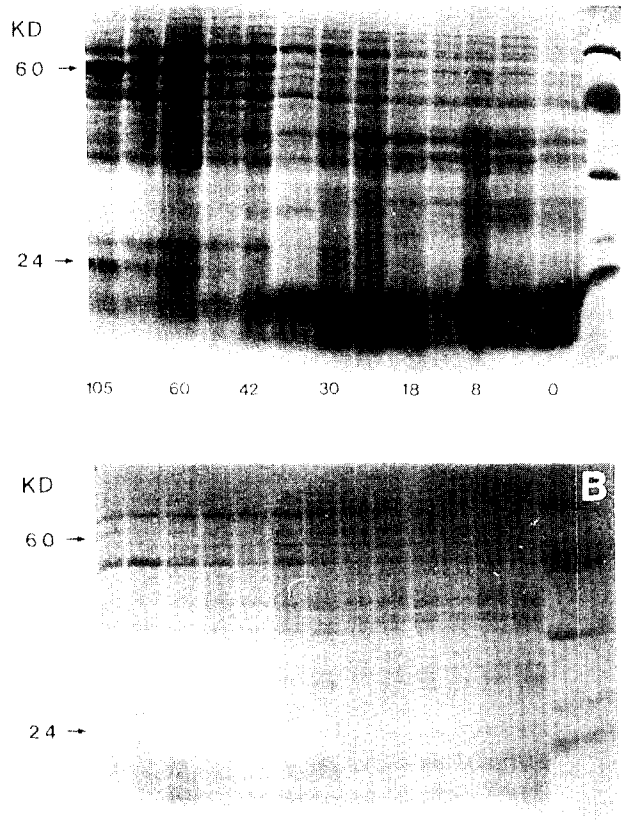

Fig. 1. Comparison of protein bands in germinating radish seeds between control (A) and treatment (B) with aqueous extracts of *P. rigida* by SDS-PAGE.

Krylov (1970) found that the quantity of protein in apple trees decreases because toxic materials from potatoes inhibit the growth of apple trees when they were cultivated together. Danks *et al.* (1978) and Kolesnichen and Aleikina (1976) reported that cinnamic acid and ferulic acid inhibit the synthesis of protein in plants. van Sumere *et al.* (1971) reported that the growth of lettuce and barley is retarded because of a decrease of the synthesis of protein as it inhibits transfer of amino acids when ferulic acid and p-coumaric acid are treated on them. The appearance that the synthesis of protein is inhibited by the extract of *P. rigida* in this experiment corresponds with those results.

Isozyme assay

When the extract is treated in the process of germination of radish, the a and b bands of peroxidase come out on the fourth day and the c, d and e bands come out on the second day in accordance with the increase of enzyme activity (Fig. 2). If treated with a phenolic compound such as caffeic acid, the treated seed had a higher activity than control, and in the control one band appeared at 13 hr while in the treated the same band did so at 24 hr (Fig. 3). As we determined the peroxidase activity, compared with the control band (A, B, C and D), the treated band (1, 2, 3 and 4) indicated a higher activity which increased and then decreased from the beginning of germination to 36 hr, again showing high after 48 hr (Fig. 4). Yoo and Kim (1988) described that the activity of peroxidase in root heavily increases but there is almost no change in the activity of peroxidase in cotyledon or hypocotyl in the process of time. In this experiment, the a and b bands were those of peroxidase related to the growth of leaf and the c, d and e bands were those of peroxidase related to the differentiation of root. Therefore, the increase of the thickness of the c, d and e bands indicate that the activity of peroxidase of the treated group suddenly increased because of stunting in the root by allelochemicals. Kim *et al.* (1987) reported that the growth rate of cells, isozymes and activity rate have the tendency of inverse proportionality because the activity of peroxidase decreases related with the increase of the growth rate of the cell and the activity increases according to the decrease of the rate. In the case of radish, the activity of peroxidase is low in the control group showing a high growth rate while the activity is high in the treated group showing a low growth rate. These results are the same as the experiment of Kim *et al.* (1987). Also, the treated group has a higher activity rate than the control group when the activity is measured by spectrophotometer. Gaspar *et al.* (1985)

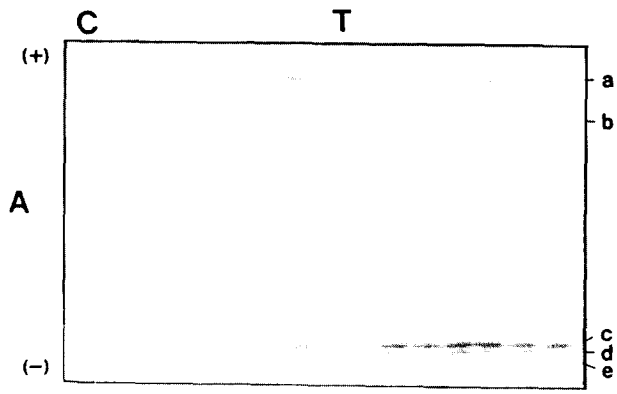

Fig. 2. Comparison of peroxidase isozymes between control (C) and treatment of *P. rigida* extract (T) during the seed germination of *R. sativus* var. *hortensis* for. *acanthiformis* analyzed by IEF in the range of pH 3~10.

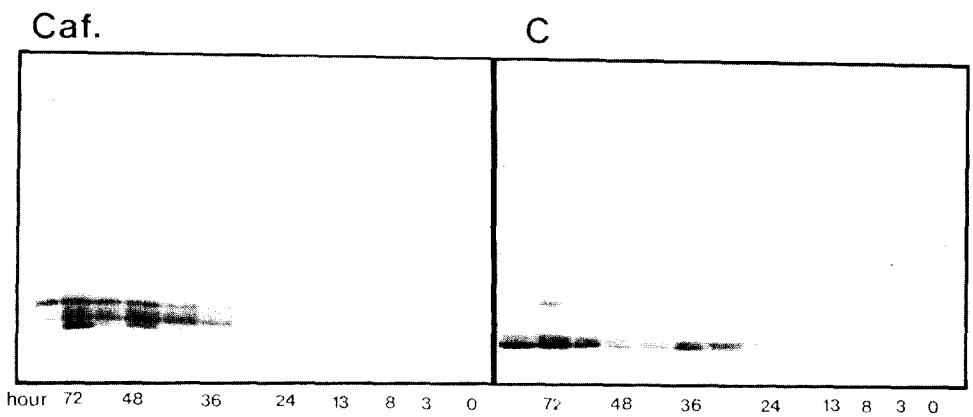

Fig. 3. Comparison of peroxidase isozymes between control (C) and 10^{-3}M caffeic acid treatment (Caf.) during the seed germination of *R. sativus* var. *hortensis* for. *acanthiformis* by IEF in the range of pH 3~10.

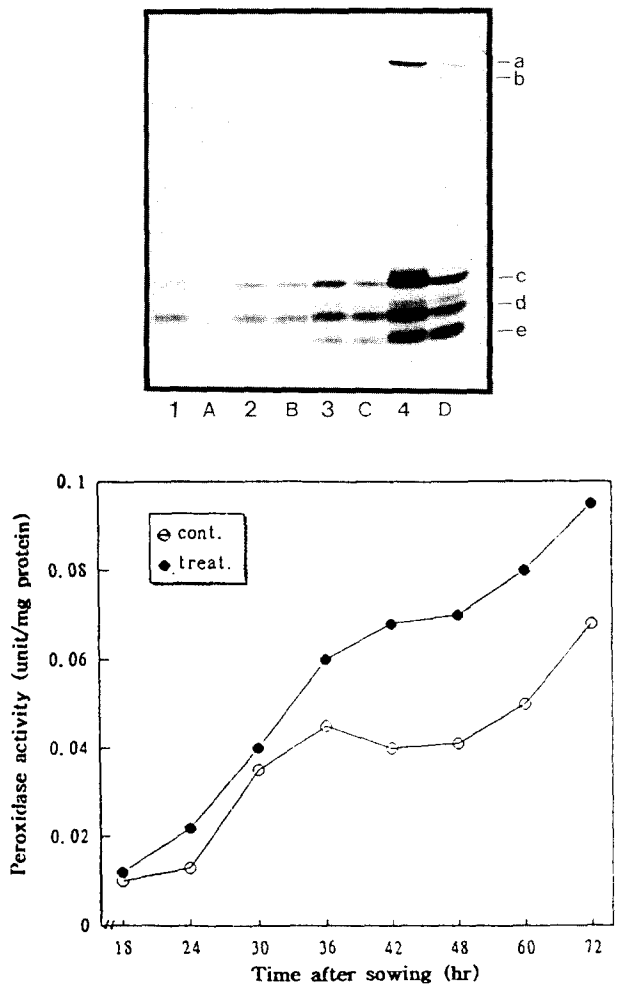

Fig. 4. Comparison of peroxidase activity between control (A:24, B:36, C:48, D:72 hr) and treatment of *P. rigida* extract (1:24, 2:36, 3:48, 4:72 hr) during the seed germination of *R. sativus* var. *hortensis* for. *acanthiformis* analyzed by IEF in the range of pH 3~10. cont.: control, treat.: *P. rigida* extract.

and Dzyubenko and Petrenko (1971) reported that the extract of *Lupinus albus* and maize inhibited the growth of *Chenopodium album* var. *centrorubrum* and *Amaranthus mangostanus*, and increased the activity of peroxidase. Loebenstein and Linsey (1961) reported that the activity of peroxidase increased when plant growth was inhibited because of infection from a pathogenic organ. But Rice (1984) reported that allelochemicals inhibit the activity of various enzymes including proteinase, catalase and peroxidase. The result of this experiment is the same as those of the experiments of Gaspar *et al.* (1985) and Loebenstein and Linsey (1961) regarding stress. In the case of treatment by caffeic acid only, the activity of peroxidase increased more highly than the activity of the control group and the band came out at 13 hr after germination in the control group while came out at 24 hr after germination in the treated group. This result indicates that the control group had a high rate of organ differentiation and low activity, and the treated group had a low rate of organ differentiation and high activity.

The esterase band of radish was that one band appeared in the cathodic on the first or second day in the control but the corresponding band in the treated group showed a high activity on the first day and unclear bands (b, e and f) which continually existed for 7 days (Fig. 5). In the control group, esterase bands, e and f that were coming out on the 1st and 2nd days vanished after that time but in the treated group the bands were maintained continuously. This result indicates that the activity of the cathodic band increases due to external stimuli to the root and is similar to the report of Yoo and Kim (1988).

The amylase of radish was that in the control the activity of b and c bands was high on 2 days, then decreased, and in the treated it elevated weakly on the third day (Fig. 6). In the case of amylase of the

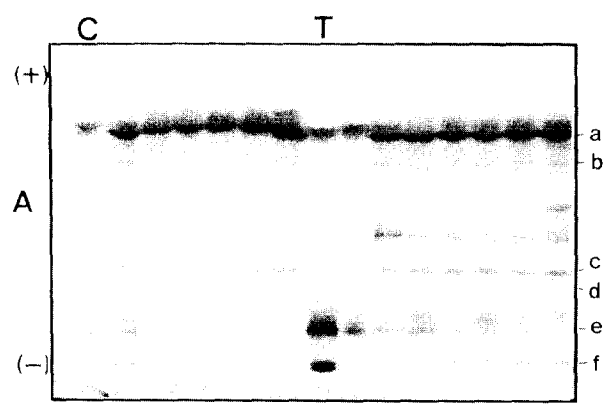

Fig. 5. Comparison of esterase isozymes between control (C) and treatment of *P. rigida* extract (T) during the seed germination of *R. sativus* var. *hortensis* for. *acanthiformis* by IEF in the range of pH 4~6.5.

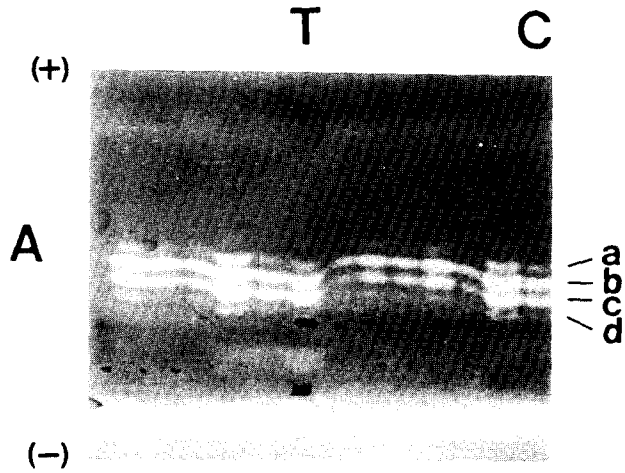

Fig. 6. Comparison of amylase isozymes between control (C) and treatment of *P. rigida* extract (T) during the seed germination of *R. sativus* var. *hortensis* for. *acanthiformis* by IEF in the range of pH 3~10.

control group, the a, b, c, d and e bands came out on 1st or 2nd days but the a and d bands began to vanish on the 3rd day and activities decreased. In the case of the treated group, a, b, c, d and e of the amylase bands existed for 7 days after seed germination and enzyme activity was high. Specifically, the activities of the isozymes of peroxidase, esterase and amylase were heightened more by allelochemicals, namely the extract of *P. rigida*. Especially, the activity of the cathodic region increased, and this result is the same as that of Espelie *et al.* (1986) and Mader *et al.* (1977) that the root region is affected the most strongly by extract which simulates stress.

적 요

리기다소나무의 수용 추출액은 무 종자의 발아에서 peroxidase, esterase, amylase의 활성과 단백질의 밴드 패턴에 영향을 주었다. 추출액이 처리된 무 종자의 발아후기에 24 KD와 60 KD의 단백질 밴드가 억제되었다. 무의 peroxidase는 발아가 진행되면서 대조구에 비하여 처리구의 밴드 수가 많아지고 특히 cathodic 부분에서는 활성이 증가하였다. Esterase는 추출액 처리구의 밴드 수는 줄었지만 cathodic 부분의 활성이 강하게 나타났다. Amylase는 대조구에 비하여 전반적으로 처리구의 활성이 증가하였다.

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