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Effects of *Pinus rigida* Allelochemicals on Isozyme Activities during Seed Germination of *Cassia mimosoides* var. *nomame*

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Pinus rigida Allelochemicals가 차풀종자의 발아과정에서 동위효소의 활성에 미치는 영향

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ABSTRACT

Eleven phenolic compounds including caffeic acid were identified through analyzing the aqueous extracts of *Pinus rigida* by HPLC. Among them, protocatechuic acid was the maximum amount of 6.84 ppm.

Seed germination of *Cassia mimosoides* var. *nomame* was significantly stimulated by the extract of *P. rigida* leaves in the proportion to concentration. However, root growth was elevated at a threshold concentration below 25%, but it was inhibited at high concentrations. In 50% extract of *P. rigida*, upward root tip of *C. mimosoides* var. *nomame* showed negageotropism which the root end showed necrosis.

New isozyme bands were induced indicating concentration activity of peroxidase from the extract of *C. mimosoides* var. *nomame*, especially in the cathodic region. Although it reduced the number of isozyme bands of esterase, esterase activities were stimulated in the anodic region of *C. mimosoides* var. *nomame*. The activity of amylase was not remarkably different between control and treatment.

Key words : *Pinus rigida* extract, Phenolic compound, Seed germination, Negageotropism, Peroxidase, Esterase, Amylase, *Cassia mimosoides* var. *nomame*.

INTRODUCTION

It is known that allelopathy is an important ecological phenomenon as inhibition of seed germination and seedling growth in plant-plant interaction. The allelochemicals of plants repress photosynthesis and cell division(Bhowmik and Doll 1984), change photorespiration, permeability, protein synthesis and enzyme activity(Lodhi and Killingbeck 1981, Del Mo-

ral 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 analogs of peroxidase, amylase, esterase and catalase that affect the growth and differentiation of the plant cell (Corcoran *et al.* 1972). In plant, phenolic compounds exist in the form of glycosides and these toxic substance are well combined with sugar and protein, therefore get a protoplasmic function in the cell (Whittaker and Feeny 1971). Among the phenolic compound, caffeic acid and ferulic acid prohibit the activation of phosphorylase in potato (Rice 1984) and tannin inhibit activity of proteinase, peroxidase and catalase (Kakiuchie *et al.* 1985).

Peroxidase is sensitive to non-optimal growth conditions, such as wounding, noxious materials, dryness and cold-weather, and it 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 non-specific substrate-reactions, and is concerned with hormone reactions, growing period, and features of genetic revelation in plant part (Abbott *et al.* 1984). Amylase has been classified as α-amylase or β-amylase according to its mode of action and concerned as an important enzyme because it hydrolyzes starch in the early part of seed germination.

Accordingly, the studies 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 state of natural growth.

This paper describes the phenolic compounds of *Pinus rigida* that has effects on peroxidase, esterase and amylase isozymes in the processes of seed germination of *Cassia mimosoides* var. *nomame*.

MATERIALS AND METHODS

Pinus rigida extract and seed germination

Aqueous extracts were provided 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 hrs, and each aqueous extract was filtered through a 150 mm filter paper. The filtrates were centrifuged at 1,000 g for 30 minutes (Centrikon T.-1045, Kontron Co.) and the supernatant were used as the materials of this experiment. The germination test of *C. mimosoides* var. *nomame* was carried out in the Petri dishes (d, 12cm) on which two sheets of filter paper were wet with varied concentrations (3, 12, 25, 50, 75 and 100%) of the aqueous extracts. Distilled water was used for the control. Each dish containing 25 seeds was placed in a 28℃ incubator (Hotpack) and repeated 3 times.

Identification of phenolic compounds by HPLC

HPLC (Waters, USA) was used to identify the allelochemicals from *P. rigida* extract. Purification of the sample was carried out with the Kil method (1992). We used commercial compounds obtained from Sigma (Sigma Chemical Co. USA) as a standard. The HPLC conditions were as follows: detector UV absorbance, 250, 254, 284 nm columm, μ Bonda-Pak C_{18} Radial Pak(0.8×10m), mobile phase, acetonitrate and sodium acetate buffer (A pump : acetonitrile, B pump : 0.02 M sodium acetate buffer pH 4.3 with acetic acid), flow rate, 1.3 ml/min and injection volume, 20 μl.

Isoelectric focusing(IEF)

The electrophoretic sample was used intact *C. mimosoides* var. *nomame* during the seed germination. 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, 1.5 ml of phamalytes(pH 3~10, pH 4~6.5) was added to polyacrylamide 5.5 ml(0.3 g/ml) to control pH range. IEF-PAGE was placed onto an electrophoresis apparatus (7 cm×22 cm×0.75 mm) and an electrode strip of 0.

Table 1. Recipe for gel preparation using polyacrylamide gel isoelectric focusing(PAGIEF) system

Stock solution	Composition		Gel solution(ml)
Acrylamide solution (T:30%, C:5%)	Acrylamide Bisacrylamide H_2O	29.1g 0.9g 100ml	5.5
Carrier ampholyte	–	–	1.5
TEMED solution	TEMED H_2O	0.2ml 19.8ml	0.9
Ammonium persulphate solution	Ammonium persulphate H_2O	0.2g 10ml	0.4
H_2O	–	–	21.7
Total volume			30ml

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 12 μl of sample, and energized to 100 V for 1hr and 200~500 V for 3 hrs(Table 1). Peroxidase isozyme was stained with 2 ml dimethyl formamide made of 0.1 M acetate buffer(pH 4.5) 100 ml and 3-amino-9-ethylcarazole 0.01 g and then dyed with a solution of 1 M $CaCl_2$ 1 ml and H_2O_2(30%) 100 μl for 40 min. Esterase isozyme was stained with solution made of 250 ml phosphate buffer(0.2 M, pH 7.0), 40 mg α-naphthy acetate, and 100 mg fast blue RR salt at 37℃ for 30 min. Amylase isozyme was precipitated in a solution of 30 % soluble starch, and washed with distilled water, then, negative staining was performed for 10 minutes in mixed solution made of 20 mM I_2, 28 mM KI, acetic acid and distilled water.

RESULTS AND DISCUSSION

The components of *P. rigida* extract were analyzed using HPLC(Fig. 1). The concentration of protocatechuic acid was the maximum amount of 6.84 ppm among 11 chemical compounds. That of ρ-hydroxybenzoic acid, vanillic acid, benzoic acid, ρ-coumaric acid and ferulic acid was 0.72, 0.61, 2.06, 1.10 and 0.17 ppm, respectively. Among phenolic compound, vanillic acid, protocatechuic acid, ρ-coumaric acid and ferulic acid have the closest relation in plant growth and division, and play

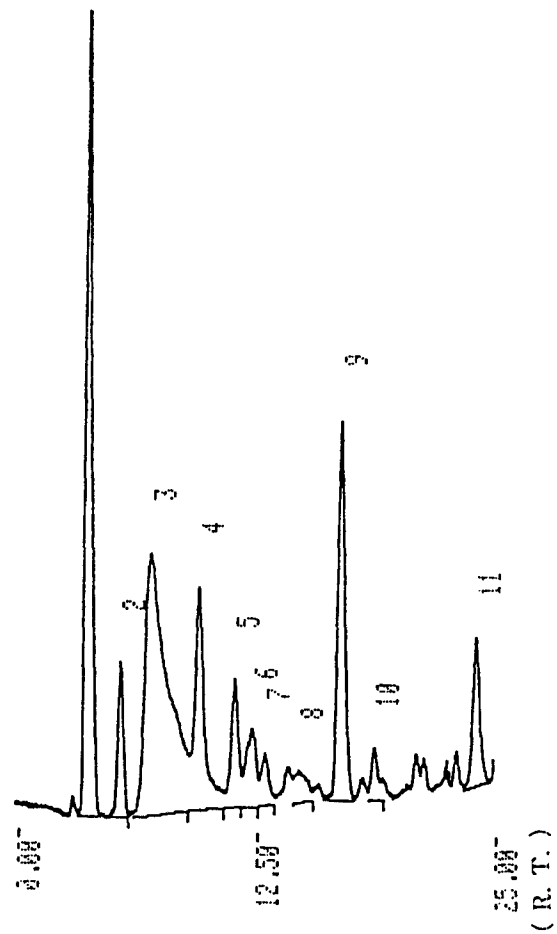

Fig. 1. Identification of chemical compounds from *Pinus rigida* by high performance liquid chromatography.
1. Unknown, 2. ρ-hydroxybenzoic acid, 3. Protocatechuic acid, 4. Unknown, 5. Vanillic acid, 6. Unknown, 7. Benzoic acid, 8. Unknown, 9. ρ-coumaric acid, 10. Ferulic acid, 11. Unknown ; R.T., retention time(min).

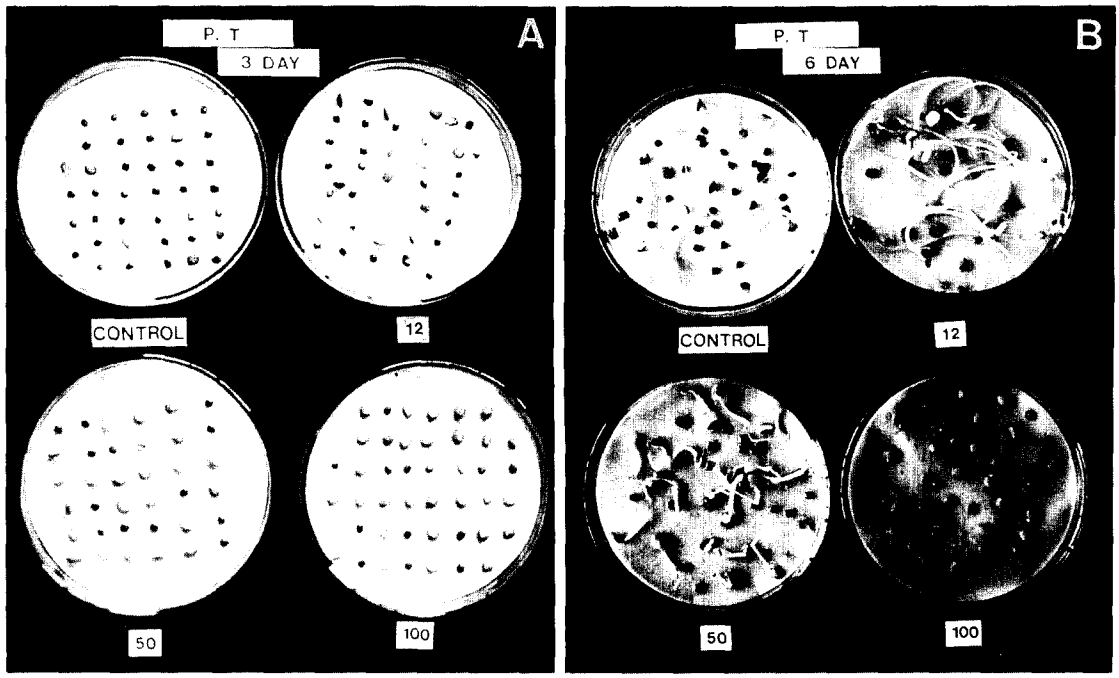

Fig. 2. Effect of different concentrations of *P. rigida* extract on the seed germination and seedling growth of *C. mimosoides* var. *nomame* at 3 days(A), 6 days(B) after sowing. P.T : treatment of *P. rigida* extract.

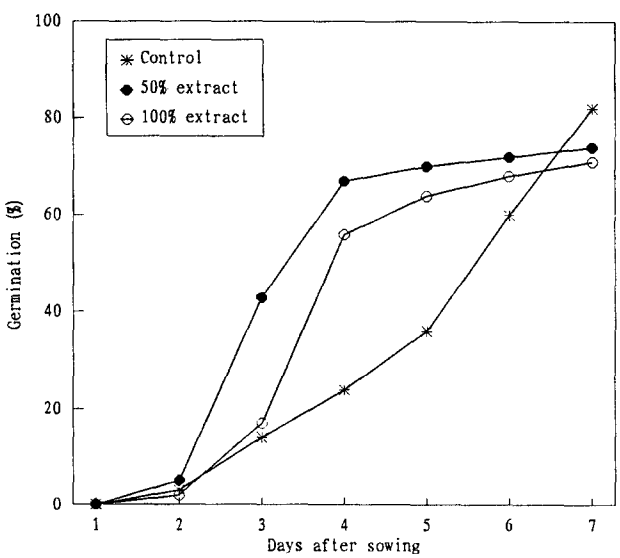

Fig. 3. Changes of germination rate of *C. mimosoides* var. *nomame* with different concentrations of *P. rigida* extract.

a strong role as an inhibitor(Kim 1993, Kim and Lee 1996). Seed germination of *C. mimosoides* var. *nomame* was stimulated when treated with 50 and 100% concentation of *P. rigida* extract (Fig. 2, 3) and seedling growth was stimulated by treating with 3, 6 and 12% extracts, but it was inhibited at 50 and 100% concentration(Fig. 4A). Accordingly, the growth stimulating phenomenon is in accordance with Lodhi(1976) concerning yield enlargement. In 50% extract of *P. rigida*, root tip of *C. mimosoides* var. *nomame* necrosed and its showed negageotropism which the root end was upward(Fig. 4B).

We also treated with 25% extract on the seed germination of *C. mimosoides* var. *nomame* and studied the peroxidase pattern. We found that e and f bands would not appear until the fourth day in early germination of control, but in the treated group they appeared continuously as clear bands from the beginning of germination to the end(Fig. 5). The control group of *C. mimosoides* var. *nomame* had a faint f band of peroxidase. The treated group had a, b, c and d bands in the anodic region and e and f bands in the cathodic region during the late period of ger-

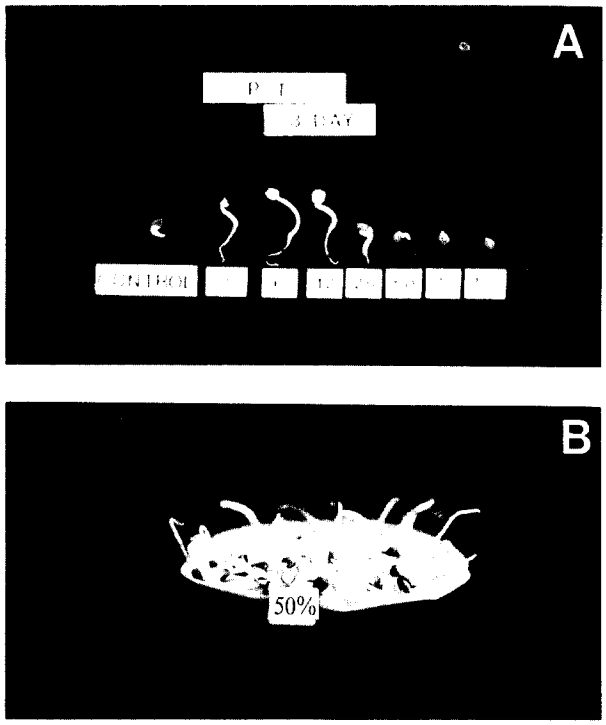

Fig. 4. Comparison of seedling growth at 3 days after sowing of *C. mimosoides* var. *nomame* with different concentration(A) and negageotropism of radicles on 50% extract of *P. rigida*(B).

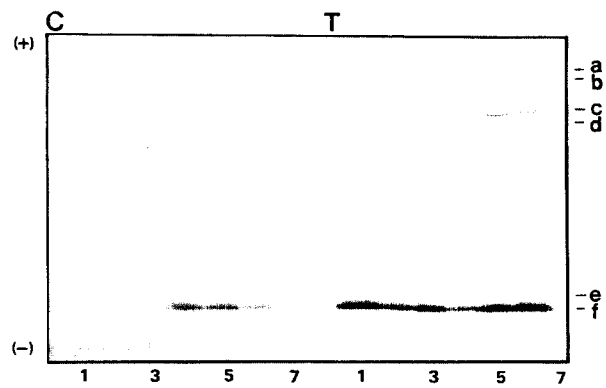

Fig. 5. Comparison of peroxidase isozymes between control(C) and treatment of *P. rigida* extract(T) during seed germination of *C. mimosoides* var. *nomame* by IEF in the range of pH 3~10.

mination, and the f band had very strong activity during all processes of germination. The esterase band of *C. mimosoides* var. *nomame* in the control appeared on the first day and then disappeared,

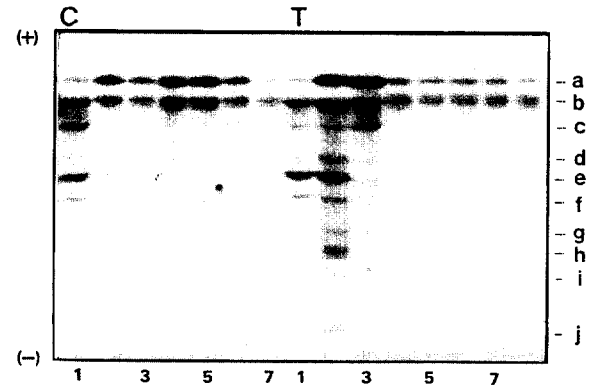

Fig. 6. Comparison of esterase isozymes between control(C) and treatment of *P. rigida* extract(T) during seed germination of *C. mimosoides* var. *nomame* by IEF in the range of pH 4~6.5.

while in the treated, the same band showed all through the first, through third days then disappeared. Also, in the control esterase were showed not only a, b, c and d bands but also e, f, g, h, i and j bands(Fig. 6). The control group had the band of esterase in the anodic region on the 1st day and had no band after that time because the band of *C. mimosoides* var. *nomame* was changed in the anodic region, whereas the treated group had esterase band for 3 days. It is able to be interpreted as due to the specificity among species. Also, this result is in discord with tannin prohibit activity of peroxidase and catalase (Kakiuchie *et al.* 1985) and caffeic acid and ferulic acid retard phosphorylase in potato (Rice 1984). Finally, the amylase of *C. mimosoides* var. *nomame* was not markedly different between control and treated, as shown in Fig. 7. The pattern of isozyme bands reveals that the treated group had higher activity than the control group except for amylase. This result indicates that in the case of *C. mimosoides* var. *nomame*, cell division is vigorous at low concentrations but is inhibited accordingly as the extract increase and seedling growth is ultimately influenced.

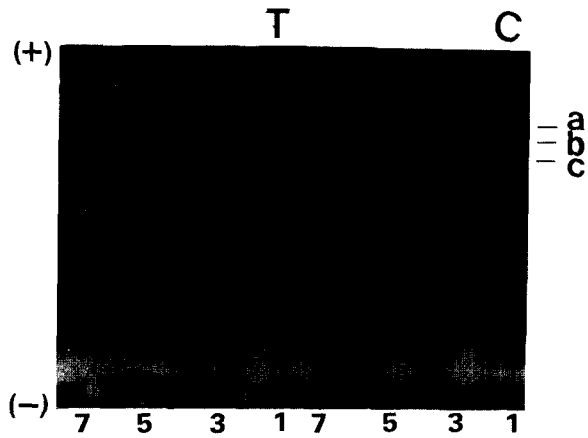

Fig. 7. Comparison of amylase isozymes between control(C) and treatment of *P. rigida* extract(T) during seed germination of *C. mimosoides* var. *nomame*(B) by IEF in the range of pH 3~10.

적 요

*Pinus rigida*의 추출액을 HPLC로 성분분석한 결과, 11개의 phenolic compound가 분석되었으며 그들 중 protocatechuic acid가 6.84 ppm으로 가장 높게 나타났다.

차풀은 수용추출액의 모든 농도에서 발아 촉진현상을 나타냈으나 유근생장은 3, 6 및 12%의 농도에서 촉진되었고 그 이상의 농도에서는 억제되었다. *Pinus rigida*의 50% 추출액에서는 차풀의 뿌리 끝이 고사되면서 위로 향하는 근단의 음성굴지성이 관찰되었다.

추출액의 농도를 25%로 처리하였을 때 peroxidase 밴드는 발아 초기부터 후기까지 대조구보다 처리구의 밴드가 강하게 나타났다. Esterase 밴드는 대조구보다 처리구의 밴드활성이 강하였으며 amylase는 대조구와 처리구간의 밴드에 있어서 큰 차이를 보이지 않았다.

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