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Effects of *Pinus rigida* Extract on Isozyme Patterns of *Glycine max* Callus

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대두 캘러스의 동위효소 패턴에 미치는 리기다소나무 추출액의 영향

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ABSTRACT

The seeds and callus induced from the root of *Glycine max* were used as test materials. When the seed was treated with the different concentrations of *Pinus rigida* extract, there was a more striking inhibition of seedling growth than of seed germination. The callus of the control group was in good condition, but when treated with 5% extract there was generalized browning and cell division was decreased in the upper part of the callus. There was no difference in the fresh and dry weights in 2% extract treatment but there was dramatic repression at concentrations higher than 5%. The band activity of peroxidase isozyme in treated callus was elevated, while that of esterase was inhibited.

Key words : *Pinus rigida* extract, Seedling growth, Seed germination peroxidase, Esterase, *Glycine max* callus

INTRODUCTION

Allelochemicals are causal substances for allelopathy in plant. They are secondary metabolic materials which are composed of phenolic compounds, volatile substances, terpenoids, flavonoids and alkaloids. Among them, phenolic compounds are present as the largest component in the cell (Einhellig and Rasmussen 1973, Lodhi 1976, Whittaker and Feeny 1971). 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, Rice 1984). Also, allelochemicals including phenolic compounds are known as an inhibitory substances in physiological mechanism like germination, radicle growth, cell division, photosynthesis, respir-

ation, membrane permeability, protein synthesis, enzyme activity and many other aspects (Horsley 1976, Rice *et al.* 1981, Bhowmik and Doll 1984). It has also been shown that similar substances produced by higher plants can be used as "natural herbicides" (Putnam and Defrank 1983).

Seed germination and seedling growth can be repressed by factors besides allelochemicals. Cells, especially callus, are also sensitive to stress. *Glycine max* callus treated by *Abutilon theophrasti* extract, repressed fresh and dry weights (Colton and Einhelling 1980). When *Lactuca sativa* callus was incubated in media containing 5~100% wormwood extract, it was poor and induction did not occur at concentrations higher than 50%(Kil 1992). Besides these extracts, the activity of peroxidase in callus increased due to kinetin treatment which reduced cell number(Kim *et al.* 1980). Accordingly, the study of enzymes concerned with seedling growth of plants pursues only the pathways of physiological metabolism in a physiological and ecological unit.

Thus, no study has been made on the increase of cells in seed germination or enzymatic changes as they mutually represent plants in the state of natural growth. As a part of our study which looked into the plant mechanisms change by allelochemicals, this paper describes that the leaf extract of *Pinus rigida* had effects on activities of peroxidase and esterase isozymes in the process of seed germination and the callus growth of *G. max*.

MATERIALS AND METHODS

Pinus rigida extract and seed germination

Aqueous extracts were made from the fresh leaves of *P. rigida*. One liter of distilled water was added to 200 g of fresh leaves at 80℃, then distilled for 48 hrs, and each aqueous extract was filtered through a 150 mm filter paper, then centrifuged at 1,000 g for 30 minutes (Centrikon T.-1,045, Kontron Co). The supernatant was used for sample source.

The germination test of *G. max* was carried out in the 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 25 seeds was placed in a 28℃ incubator (Hotpat) for 7 days and it repeated 3 times.

Treatment of *Pinus rigida* leaf extract on the medium of *G. max* callus

The seed of *G. max* was sprouted in the dark for 3 days and the root was cut at the length of 0.2 cm and maintained 3~4 weeks on B5 media (B5 salt solution+ 1 mg/l 2,4-D + 0.1 mg/l kinetin + 0.8% agar) at 27℃ and subcultured every 3 weeks. The extract of *P. rigida* was filtered through a 0.2 μm sieve, then the filtrate was diluted into 2, 5, 10, 20, 30, 50 and 100% B5 media. After plating in flasks, 1.5 g callus was added to compared with the control group (Krikorian and Katz, 1968; Hogan and Manners, 1990). Each experiment was repeated six times. After 17-day-incubation, the fresh weight of callus was supplemented with mg units for the different concentrations of extract and dry weights were oven-dried at 50℃ for 10 hrs, and all the statistics were tabulated. After treatment of 20% extract, callus was sampled at 5, 8 and 12 days.

Isoelectric focusing (IEF)

Isoelectric focusing analysis was carried out to study the band patterns of peroxidase and esterase. The electrophoretic sample used *G. max* callus as an isozymic assay, and IEF-PAGE of peroxidase and esterase was assayed by a modified procedure of Kim *et al.* (1997).

RESULTS AND DISCUSSION

Growth inhibition of *G. max* callus

When the seed of *G. max* was treated with the different concentrations of *P. rigida* extract, there was a

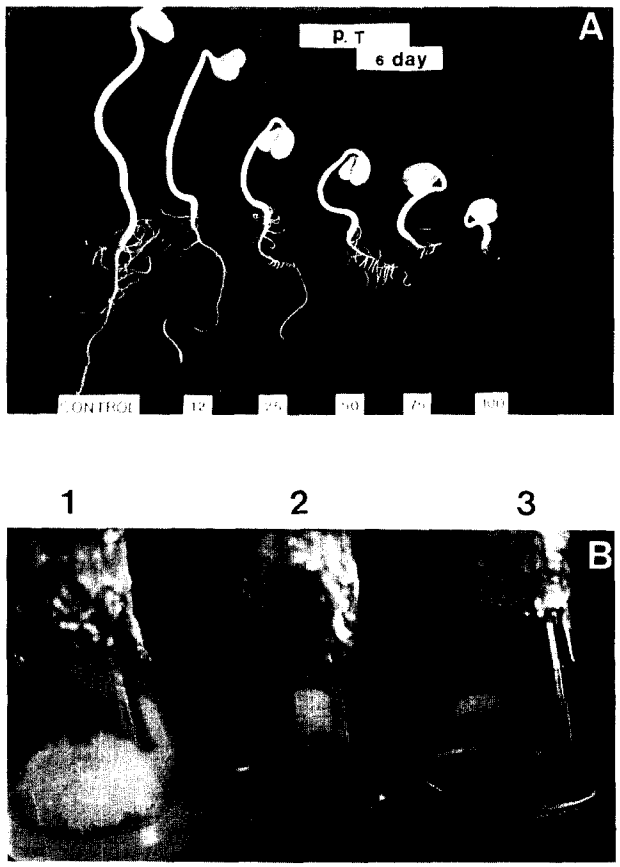

Fig. 1. Effect of *P. rigida* extract on the seedling growth at 6 days after sowing (A) and callus growth of *Glycine max* (B). 1. control 2. 5% extract treatment 3. 20% extract treatment P.T : treatment of *P. rigida* extract

more striking inhibition of seedling growth than of seed germination in direct proportion to extract concentration (Fig. 1-A). After 17 days, the callus of the control group was in good condition, but at 5% extract, there was generalized browning and little cell division in the upper part of the callus. The callus extracted with 20% extract was totally necrosised (Fig. 1-B). Therefore, phenolic compounds considerably influence the growth of callus as well as seed germination. According to Kil (1992), when callus induction was in media containing 5~100% extract of wormwood, the condition of the callus of *Lactuca sativa* was poor and did not even occur at concentrations higher than 50%. Investigation for

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	29.1g	5.5
	Bisacrylamide	0.9g	
	H_2O	100ml	
Carrier ampholyte	–	–	1.5
TEMED solution	TEMED	0.2ml	0.9
	H_2O	19.8ml	
Ammonium persulphate solution	Amm. pers.	0.2g	0.4
	H_2O	10ml	
H_2O	–	–	21.7
Total volume			30ml

growth inhibition in the cell of *Antennaria microphylla* after phenolic compound treatment was largly reported (Hogan and Manner, 1990)

There was no difference in the fresh and dry weights in 2% extract treatment, but dramatic repression was showed at concentrations higher than 5%. Table 2 suggests that F value of fresh weight and dry weight was 25.70 and 14.84 respectively. These inhibitory effects were statistically significant at the 1% level because this values were heigher than $F_{0.01}(5,24) = 4.22$. According to the investigation of relations between dry/fresh weights and the each concentrations of the extracts, correlation coefficient was 0.94 in dry weight and 0.93 in fresh weight, and the weights abruptly decrease in the 2% and 5% extract treatments (Fig. 2). When we investigate the growth rates of the callus at 2, 5, 10, 20, 30, 50 and 100% extracts and the callus of the control group, there was no difference of growth rates between the control group and callus treated with 2% extract. But fresh weight and dry weight decreased at concentrations higher than 5% and, like the experiment of Kil (1992), the growth rate of callus radically decreased at concentrations between 2% and 5%. Therefore, it is expected that when the concentrations of extract are more finely fractionized, the correlation coefficient will be higher. This is in agreement with Colton and Einhelling (1980), who showed the effect of the extract of *Abutilon*

Table 2. Total fresh and dry weights of *Glycine max* callus at different concentrations of *Pinus rigida* extract

Treatments (%)	Mean values		% of inhibition	
	F.W(mg)	D.W(mg)	F.W(mg)	D.W(mg)
Control	10.03±4.50a	0.30±0.0379a	0	0
2	9.36±3.28a	0.28±0.0405a	6.68	6.7
5	5.85±0.48b	0.23±0.0195b	41.67	23.3
10	5.30±2.13b	0.21±0.0397b	46.17	30.0
20	3.19±0.05c	0.20±0.0003b	68.20	33.3
30	1.56±0.09c	0.14±0.0001c	84.45	53.3
50	–	–	–	–
100	–	–	–	–

F.W : Fresh weight D.W : Dry weight
Mean values with the same letter are not significantly different
(Duncan's new multiple test, P>0.01)

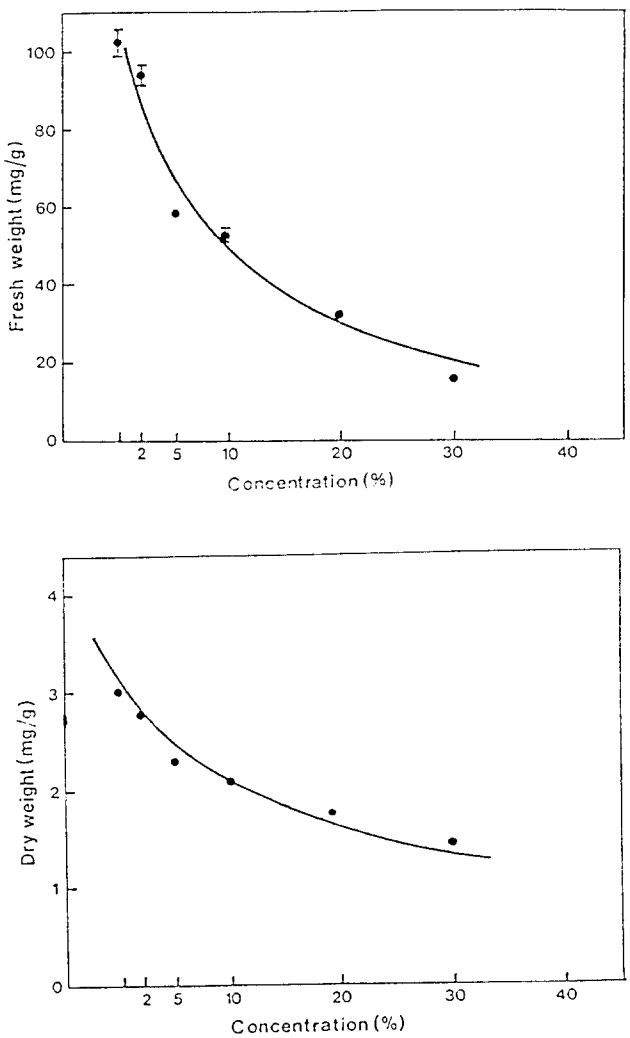

Fig. 2. Dose response of concentration of *P. rigida* extract on fresh weight (A) and dry weight (B) of *G. max* callus.

theophrasti to the growth of *G. max* callus.

Activity change of peroxidase and esterase

At 5, 8 and 12 days, the *G. max* callus in the 20% extract was sampled for comparison of isozyme activity. The band patterns of the peroxidase in the control group showed that the anodic region were a and b bands, and the cathodic region were c, d, e, f and g bands, and as the treatment time passed, the activity of the a band became considerably denser. There were the same results in the treated group, but as time passed, the activities of the a and g bands intensified while gradually decreasing in the b band. Comparing the peroxidase band of the control group with that of the treated group, we know that there showed a higher activity in the treated group than the control group at the b band of the anodic but it decreased later. And the c, d, e and f bands of the cathodic weakend in the treated group whereas the g band strengthened afterward (Fig. 3). In the case of *G. max* callus, there was no difference between the bands of the control group and the bands of callus treated by the 20% extract but the enzyme activity of the treated group was high. This result is the same as Kim's report (Kim, 1993) that the activity of peroxidase of callus increased when cell numbers were not as high as anticipated because of kinetin treatment.

But this is in discord with the band pattern of esterase showed that the anodic were a and b bands

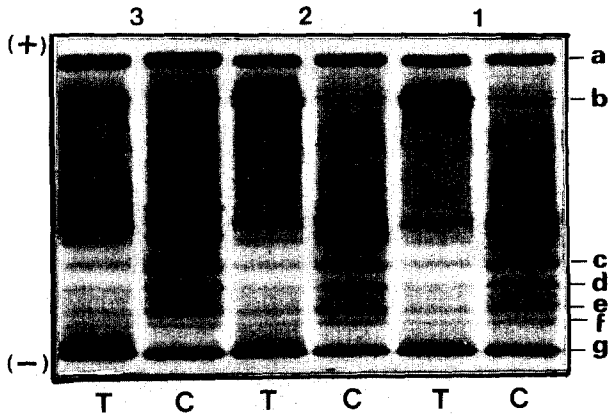

Fig. 3. Comparison of isozymes of peroxidase between control (C) and treated callus (T) at 5 (1), 8 (2) and 12 days (3) on B5 medium with 20% extract of *P. rigida* by IEF in the range of pH 3~10.

and the cathodic were c, d, e and f bands. Comparing the control group with the treated group, the a band of the anodic was much the same, but the c, d, e and f bands of the cathodic decreased somewhat in the treated group, also the f band did not appear until 5 to 8 days in the treated group, but became visible at 12 days in the control group (Fig. 4). The activity of esterase of the control group was higher than the treated group.

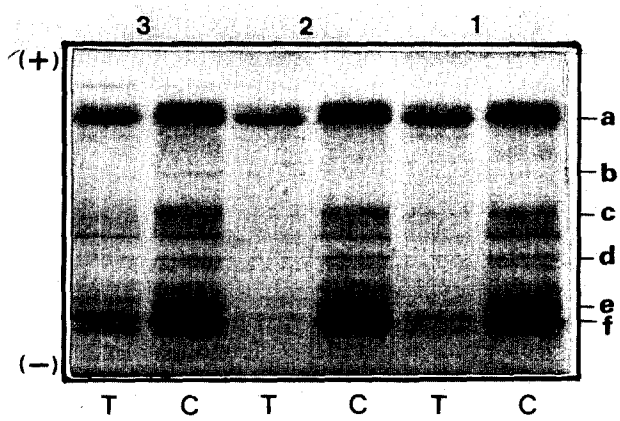

Fig. 4. Comparison of isozymes of esterase between control (C) and treated callus (T) at 5 (1), 8 (2) and 12 days (3) on B5 medium with 20% extract of *P. rigida* by IEF in the range of pH 3~10.

적 요

Pinus rigida 추출액을 농도별로 대두종자에 처리하였을 경우 농도가 증가함에 따라 발아보다는 유근생장에 현저한 억제현상을 나타냈다. 추출액이 처리된 대두 callus는 5%의 추출액에서도 세포분열이 억제되어 갈색으로 변화했으며 그 이상의 농도에서는 거의 callus 증식이 없었다. 추출액을 농도별로 처리한 후의 생중량, 건중량은 대조구와 2% 농도에서는 큰 차이가 없으나 5% 농도 이상부터 급격히 억제되었다. 대두의 peroxidase 밴드는 처리구가 대조구보다 강하였으며 특히 anodic의 a, b 밴드의 활성이 높았다. Esterase 밴드는 처리구보다 대조구의 밴드가 강하였으며 특히 cathodic의 f 밴드의 활성이 높았다.

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