

# Effects of an aqueous red pine (*Pinus densiflora*) needle extract on growth and physiological characteristics of soybean (*Glycine max*)

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## Abstract

The effect of allelochemicals on growth, root nodule nitrogen fixation activity, and ion patterns of soybeans were investigated. We prepared 50 g/L (T50), 100 g/L (T100), and 200 g/L (T200) extract concentrations by soaking fresh red pine needles in a nutrient solution. Adding needles to the nutrient solution increased the content of total phenolic acids, osmolality, and total ions. The total phenolic content in the T50, T100, and T200 extracts were  $206 \pm 12.61$ ,  $335 \pm 24.16$ , and  $603 \pm 12.30$  mg gallic acid equivalents, respectively. The  $K^+$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $PO_4^{3-}$  content increased by adding needles to the nutrient solutions, whereas  $SO_4^{2-}$  content decreased. The growth inhibition of soybeans was proportional to the needle extract concentrations, and the T100 and T200 concentrations resulted in remarkable growth inhibition. On day 20 after treatment, dry weight and nitrogen fixation activity of the root nodules were reduced by the T100 and T200 treatments, whereas the T50 treatment was not difference from the control. After day 10, total ion content in all treatment groups was not different in comparison with the control. However, total ionic content in all treatment groups decreased significantly compared with that in the control after day 20. The lowest total ion value was found for the T200 concentration. The T200 treatment also resulted in significantly reduced  $SO_4^{2-}$  content. The amounts of  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $Mn^{2+}$  were higher than those of the control for the T50 treatment on day 10 and for T100 on day 20 after treatment. A significant increase in osmolality was observed in the T200 treatment on day 10 and in the T100 treatment on day 20. These results suggest that under severe allelochemical stress conditions, a remarkable reduction in nodule formation, nitrogen fixation activity, and ion uptake eventually resulted in a decrease in leaf production. Furthermore, increased  $K^+$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ , and osmolality in soybeans exposed to lower concentrations of allelochemicals than the critical stress level helped overcome the stress.

**Key words:** allelopathy, mineral uptake, nitrogen fixation activity, osmolality, red pine, soybean

## INTRODUCTION

Red pine (*Pinus densiflora* Siebold & Zucc.) has been reported to possess allelopathic potential (Lee and Monsi 1963, Kil 1989). Aqueous extracts of several *Pinus* sp. needles inhibit seed germination, and seedling growth in many plant species as well as nitrification and nitrifying bacteria (Lodhi and Killingbeck 1982, Fernandez et al.

2006). In nature, foliar leachates and root exudates may contain different classes of organic compounds such as phenolics, terpenoids, polyacetylenes, and alkaloids. Among them, phenolic compounds are the most common under field conditions (Rice 1984) and their effects have been studied extensively (Baziramakenga et al.

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1994, Blum and Gerig 2005). The extent of the allelopathic effect depends on the concentration of compounds in the mixture (Alsaadawi et al. 1986, Kil 1989, Inderjit et al. 2002), and allelopathic activity is thought to be due to the joint action of several allelochemicals rather than due to one allelochemical (Einhellig 1996). Plant leachates, root exudates, and debris leachates are composed of various phenolic acids, other organic molecules, and inorganic ions, and organic and inorganic ions particularly affect the action of phenolic acids released by plants (Blum et al. 1999). Phenolic compounds decrease plant growth and reduce nodulation and the amount of leghemoglobin (Rice et al. 1981, Batish et al. 2007). The sensitivity of *Rhizobium* to allelochemicals leads to poor nodulation and a subsequent reduction in nitrogen available to legumes (Blum and Rice 1969). Although the effects of red pine needle extracts on seed germination and plant growth have been studied, the total phenolic acid content in red pine needles and its effects on dry weight (DW), nitrogen fixation activity, ion uptake, and osmolality of legumes treated with red pine needle extract are unknown.

The objectives of this study were to identify differences in total phenolics (TP), osmolality, and total ions based on the quantity of red pine needles, and to understand allelopathic effects on plant growth by investigating nitrogen fixation activity of the root nodules, total nitrogen (TN) content, osmolality, total ions, and inorganic ion content in soybeans by red pine needle extract concentration and treatment period.

## MATERIALS AND METHODS

### Red pine needle extract

Fresh pine needles were collected from Korean red pine trees (*P. densiflora* Sieb. et Zucc.) on Palgon Mountain, Korea and stored at -20°C until use. Fifty g (T50), 100 g (T100), and 200 g (T200) of fresh red pine needles were extracted by immersion in 1 L of modified Hoagland's solution (0.5 mM  $\text{CaSO}_4 \cdot 1/2\text{H}_2\text{O}$ , 0.625 mM  $\text{K}_2\text{SO}_4$ , 0.5 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25 mM  $\text{KH}_2\text{PO}_4$ , 1 mM  $\text{KNO}_3$ , 0.095 mM Fe-EDTA, and trace elements) at room temperature for 24 h. Modified Hoagland's solution was the control. The extracts were then vacuum filtered through GF/C filter paper (pore size, 1.2  $\mu\text{m}$ ; Whatman International Ltd., Banbury, England). The pH of all extracts was adjusted to 5.0 with 0.1 M sodium hydroxide.

### TP Measurement

TP concentrations in the needle extracts were measured using Folin-Ciocalteu reagent, as described by Singleton and Rossi (1965) with gallic acid as a standard. The TP content was expressed as milligram of gallic acid equivalents per needle extract.

### Plant material, growth conditions, and harvest

Uniform seeds of soybean obtained from the National Institute of Crop Science were soaked for 2 h in distilled water, and then one seed was sown per plastic pot (10 cm  $\times$  15 cm) filled with perlite in a growth chamber (30/20°C - day/night, relative humidity of 60/70% - day/night, 420  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , photoperiod of 12 h). Three pots per treatment concentration were used. Modified Hoagland's solution was applied to the seedlings the second day after sowing. When the unifoliated leaves were fully expanded, soybean nodule extract (5 g/L distilled water) was supplied at 100 mL per pot for 2 days to inoculate the soil with *Rhizobium* sp. When the first trifoliated leaves fully emerged and opened, 100 mL of red pine needle extracts per pot was applied to the soybean seedlings every day. Soybean leaves were harvested after treatment on days 10 and 20. After determining fresh weight, the samples were dried at 80°C for 72 h, and DW was determined. The dried plant material was ground to a homogenous powder and extracted in distilled water at 95°C for 1 h. Osmolality and total ionic content in the soybean water extracts were determined quantitatively.

### Nitrogen fixation activity and TN content

The fresh roots of three plants from each treatment were collected and placed into 100 mL Erlenmeyer flasks with rubber stoppers; 0.1 atm of  $\text{C}_2\text{H}_2$  (10 mL) was added and incubated at 30°C to determine  $\text{N}_2$ -fixation activity. Gas samples (0.5 mL) were removed from the Erlenmeyer flasks with a 1 mL syringe at intervals of 1 h and analyzed for peak ethylene production using a gas chromatograph (GC-8 APF; Shimadzu, Kyoto, Japan) equipped with a  $\text{H}_2$ -frame ionization detector and a Porapak R column (182 cm  $\times$  0.32 cm). Nitrogen fixation activity was calculated at a conversion ratio of 1.5:1 (acetylene reduced: $\text{N}_2$  fixed) (Fishbeck et al. 1973). TN was determined by the micro-Kjeldahl method.

### Determination of total ions, osmolality, and inorganic ions in needle extracts and soybean leave

Total ionic content (calculated as NaCl equivalents)

was determined using a conductivity instrument (Mettler Check Mate 90; Mettler, Toledo, OH, USA), and the osmotic potential of the solutions was measured using an osmometer (Micro-Osmometer; Precision System Inc., Natick, MA, USA).  $K^+$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $Mn^{2+}$  were determined using inductively coupled plasma mass spectrometry (JY 38 plus; Jobin Yvon, Longjumeau, France).  $SO_4^{2-}$  and  $PO_4^{3-}$  were determined using ion chromatography under the following conditions: column, Star-ion A-300,  $1,000 \times 4.6$  mm; eluent, 1.7 mM sodium hydrogen carbonate/11.8 mM sodium carbonate; flow rate, 1.5 mL/min.

### Photography and data analysis

The surfaces and sections of nodules were observed with a stereoscope, and the results are shown as means  $\pm$  standard errors of three independent replicates. Statistical analyses were conducted with one-way and two-way (concentration and period) analyses of variance. Duncan's multiple range test was used to identify significant differences ( $P < 0.05$ ) among groups using SPSS ver. 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Chemical characteristics of the needle extracts

Total phenolics, osmolality, total ions,  $K^+$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $PO_4^{3-}$  of red pine needle extracts were higher than those of the control, and these values increased with an increase in the amount of needles added. However, the opposite trend was observed for  $SO_4^{2-}$  content (Table 1).

### Effect on growth and physiological characteristics

After day 10, treatment with the needle extract resulted in a significant reduction in DW of leaves, but no significant differences were found within the treatment groups. A significant decrease in leaf production based on the needle extract concentrations was observed after day 20 of treatment and the DWs of the leaves in the T50, T100, and T200 concentration groups decreased by 12.8%, 38.3% and 53.8% compared with the control, respectively (Table 2). Treatment with the needle extracts resulted in a significant reduction in TN after day 20. TN content decreased significantly with the two highest extract con-

**Table 1.** Changes in osmolality, total ionic content, total phenolic content, and inorganic ion content in red pine needle extracts adjusted to pH 5.0

	Osmolality (mOsm/kg H <sub>2</sub> O)	Total ions (mM)	Total phenolics (mg GAE)	Inorganic ions (mM)					
				K <sup>+</sup>	Mg <sup>2+</sup>	Ca <sup>2+</sup>	Mn <sup>2+</sup>	SO <sub>4</sub> <sup>2-</sup>	PO <sub>4</sub> <sup>3-</sup>
Con	2.33 $\pm$ 0.58	4.43 $\pm$ 0.40	n.d.	2.50	0.50	0.50	0.10	1.83	0.25
T50	8.42 $\pm$ 4.82	6.08 $\pm$ 0.24	206 $\pm$ 12.61	3.87	0.80	0.63	0.04	1.61	0.28
T100	17.66 $\pm$ 4.08	8.10 $\pm$ 0.25	335 $\pm$ 24.16	4.25	0.88	0.64	0.09	1.50	0.31
T200	33.07 $\pm$ 4.82	11.66 $\pm$ 0.72	603 $\pm$ 12.30	6.00	0.90	0.65	0.17	1.39	0.41

Mean values of three replicates with standard deviation.

GAE, gallic acid equivalents; Con, control; n.d., not detected; T50, 50 g fresh red pine needles/L modified Hoagland's solution; T100, 100 g fresh red pine needles/L modified Hoagland's solution; T200, 200 g fresh red pine needles/L modified Hoagland's solution.

**Table 2.** Comparison of dry weights (DW) (g) and total nitrogen (TN) content (g) in soybean leaves on days (d) 10 and 20 after treatment with red pine needle extracts

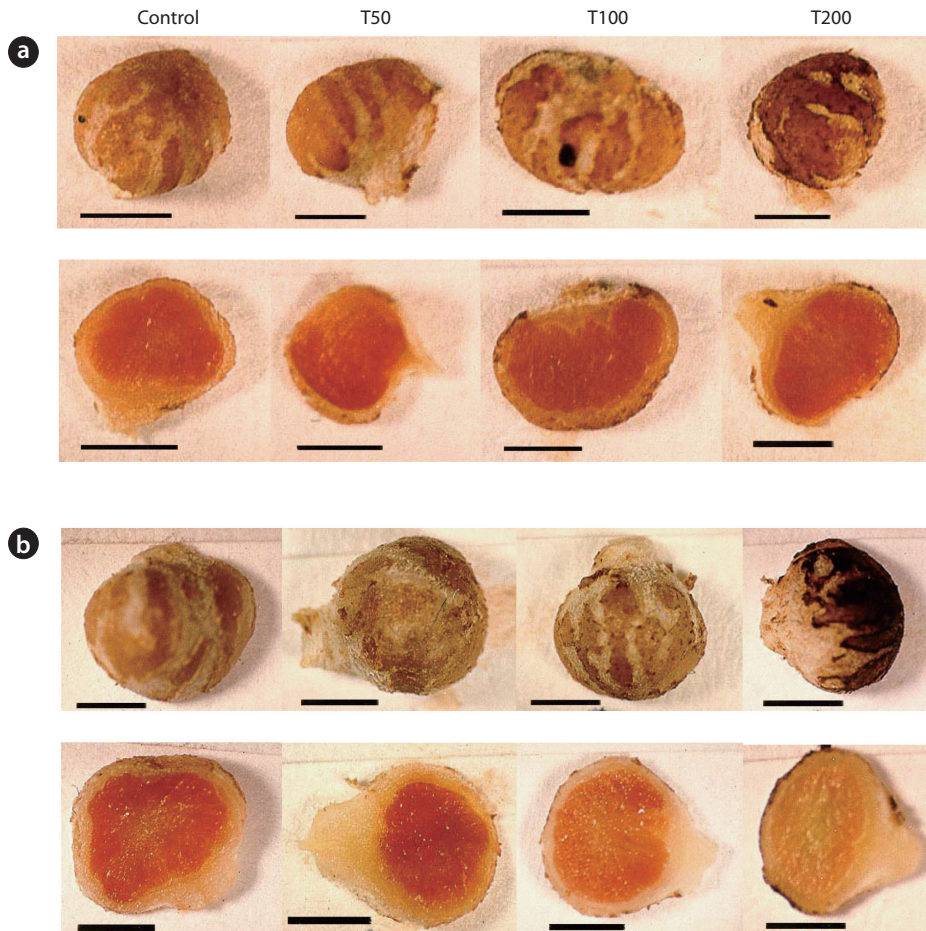
		Control	T50	T100	T200
DW	10 d	0.49 $\pm$ 0.02 <sup>a</sup>	0.36 $\pm$ 0.06 <sup>b</sup>	0.33 $\pm$ 0.04 <sup>b</sup>	0.30 $\pm$ 0.10 <sup>b</sup>
	20 d	0.89 $\pm$ 0.06 <sup>a</sup>	0.77 $\pm$ 0.06 <sup>b</sup>	0.55 $\pm$ 0.02 <sup>c</sup>	0.41 $\pm$ 0.03 <sup>d</sup>
TN	10 d	0.015 $\pm$ 0.001 <sup>a</sup>	0.009 $\pm$ 0.001 <sup>b</sup>	0.007 $\pm$ 0.001 <sup>b</sup>	0.007 $\pm$ 0.002 <sup>b</sup>
	20 d	0.029 $\pm$ 0.002 <sup>a</sup>	0.023 $\pm$ 0.002 <sup>b</sup>	0.016 $\pm$ 0.000 <sup>c</sup>	0.012 $\pm$ 0.001 <sup>d</sup>

Mean values of three replicates with standard deviation (tested with a one-way analysis of variance [ANOVA] and Duncan's multiple range test;  $P < 0.05$ ).

Two-way ANOVA of DW and TN by concentration and period are as follows. DW: concentration<sup>\*\*\*</sup>, period<sup>\*\*\*</sup>, concentration  $\times$  period<sup>\*\*\*</sup>. TN: concentration<sup>\*\*\*</sup>, period<sup>\*\*\*</sup>, concentration  $\times$  period<sup>\*\*\*</sup>.

<sup>\*\*\*</sup> $P < 0.001$ .

T50, 50 g fresh red pine needles/L modified Hoagland's solution; T100, 100 g fresh red pine needles/L modified Hoagland's solution; T200, 200 g fresh red pine needles/L modified Hoagland's solution.



**Fig. 1.** Surface and cross section of soybean nodules treated with red pine needle extract on day 10 after treatment (a) and on day 20 after treatment (b). T50, 50 g fresh red pine needles/L modified Hoagland's solution; T100, 100 g fresh red pine needles/L modified Hoagland's solution; T200, 200 g fresh red pine needles /L modified Hoagland's solution. Scale bars = 3.5 mm.

centrations by 50.8% and 57.4% compared with that in the control, after days 10 (Table 2). The T100 and T200 extract concentrations inhibited nodule DW and total nitrogen fixation activity (TNA) (Table 3). However, nodule DW and TNA were not significantly different compared with those in the control for the lowest extract concentration, except for TNA on day 10 of treatment. The surface of the nodules exposed to the extract was discolored brown to black, and the inner color of the nodules faded gradually in comparison with that in the control, except for the nodules treated with the T50 extract (Fig. 1).

### Osmolality and total ionic content

After day 10 of treatment, soybean osmolality increased following addition of the needle extracts and was highest for the T200 concentration. However, a significant increase in osmolality was observed for the T100

treatment after day 20 of treatment (Table 4). Total ionic content was not significantly different between the control and all treatments after 10 days. However, total ionic content in all treatment groups decreased significantly compared with the control after day 20 of treatment. The lowest total ionic content value occurred in plants exposed to the T200 concentration (Table 4).

### Inorganic ions

Soybeans showed different trends for inorganic ion content based on the extract treatment concentration and treatment period (Table 5). The contents of  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $Mn^{2+}$  were higher than those of the control in plants treated with the T50 concentration on day 10 and for those treated with the T100 concentration on day 20 after treatment. The highest  $K^+$  value occurred in the control after day 10 of treatment and in the control and

**Table 3.** Total number of nodules, dry weight of nodules (g), and total nitrogen fixation activity (TNA,  $\mu\text{M C}_2\text{H}_4 \text{ plant}^{-1} \text{ h}^{-1}$ ) of soybean root nodules on days (d) 10 and 20 after treatment with red pine needle extracts

		Control	T50	T100	T200
Total nodule number	10 d	14.7 ± 1.2 <sup>a</sup>	15.0 ± 3.6 <sup>a</sup>	10.7 ± 0.6 <sup>a</sup>	11.0 ± 4.4 <sup>a</sup>
	20 d	24.3 ± 9.3 <sup>ab</sup>	35.3 ± 11.4 <sup>a</sup>	14.7 ± 4.6 <sup>b</sup>	14.3 ± 4.7 <sup>b</sup>
Dry weight	10 d	0.063 ± 0.002 <sup>a</sup>	0.057 ± 0.004 <sup>ab</sup>	0.042 ± 0.004 <sup>c</sup>	0.045 ± 0.012 <sup>bc</sup>
	20 d	0.125 ± 0.010 <sup>a</sup>	0.130 ± 0.005 <sup>a</sup>	0.097 ± 0.004 <sup>b</sup>	0.079 ± 0.011 <sup>c</sup>
TNA	10 d	4.79 ± 0.58 <sup>a</sup>	3.37 ± 0.51 <sup>b</sup>	2.46 ± 0.20 <sup>b</sup>	1.46 ± 0.57 <sup>c</sup>
	20 d	14.72 ± 1.64 <sup>ab</sup>	17.30 ± 2.08 <sup>a</sup>	11.97 ± 1.13 <sup>b</sup>	7.21 ± 0.80 <sup>c</sup>

Mean values of three replicates with a standard deviation (tested with a one-way analysis of variance [ANOVA] and Duncan's multiple range test;  $P < 0.05$ ). Two-way ANOVA of total nodule number, dry weight of nodule, and TNA by concentration and period are as follows. Total nodule number: concentration<sup>n.s.</sup>, period<sup>†</sup>, concentration × period<sup>n.s.</sup>. Dry weight of nodule: concentration<sup>\*\*\*</sup>, period<sup>\*\*\*</sup>, concentration × period<sup>\*\*</sup>. TNA: concentration<sup>\*\*\*</sup>, period<sup>\*\*\*</sup>, concentration × period<sup>\*\*\*</sup>.

<sup>†</sup> $P < 0.05$ ; <sup>\*\*</sup> $P < 0.01$ ; <sup>\*\*\*</sup> $P < 0.001$ ; n.s., not significant.

T50, 50 g fresh red pine needles/L modified Hoagland's solution; T100, 100 g fresh red pine needles/L modified Hoagland's solution; T200, 200 g fresh red pine needles/L modified Hoagland's solution.

**Table 4.** Osmolality ( $\mu\text{osm/g}$  plant water) and total ionic content ( $\mu\text{eq/g}$  plant water) in soybean leaves on days (d) 10 and 20 after treatment with red pine needle extracts

		Control	T50	T100	T200
Osmolality	10 d	527 ± 19.1 <sup>b</sup>	580 ± 39.6 <sup>ab</sup>	609 ± 53.6 <sup>ab</sup>	676 ± 40.3 <sup>a</sup>
	20 d	634 ± 21.1 <sup>b</sup>	649 ± 37.4 <sup>b</sup>	761 ± 72.1 <sup>a</sup>	558 ± 39.8 <sup>b</sup>
Total ions	10 d	319 ± 12.6 <sup>a</sup>	301 ± 23.0 <sup>a</sup>	297 ± 30.4 <sup>a</sup>	267 ± 36.3 <sup>a</sup>
	20 d	344 ± 9.3 <sup>a</sup>	295 ± 17.3 <sup>b</sup>	308 ± 21.7 <sup>b</sup>	232 ± 17.6 <sup>c</sup>

Mean values of three replicates with standard deviation (tested with a one-way analysis of variance [ANOVA], and a Duncan's multiple range test;  $P < 0.05$ ). Two-way ANOVA of osmolality and EC by concentration and period are as follows. Osmolality: concentration<sup>†</sup>, period<sup>†</sup>, concentration × period<sup>\*\*</sup>. Total ionic contents: concentration<sup>\*\*\*</sup>, period<sup>n.s.</sup>, concentration × period<sup>n.s.</sup>.

<sup>†</sup> $P < 0.05$ ; <sup>\*\*</sup> $P < 0.01$ ; <sup>\*\*\*</sup> $P < 0.001$ ; n.s., not significant.

T50, 50 g fresh red pine needles/L modified Hoagland's solution; T100, 100 g fresh red pine needles/L modified Hoagland's solution; T200, 200 g fresh red pine needles/L modified Hoagland's solution.

**Table 5.** The contents of inorganic ions in soybean leaves ( $\mu\text{mol/g}$  plant water) on days (d) 10 and 20 after treatment with red pine needle extracts

		Control	T50	T100	T200
K <sup>+</sup>	10 d	229 ± 8.29 <sup>a</sup>	200 ± 13.65 <sup>ab</sup>	189 ± 16.60 <sup>b</sup>	185 ± 24.67 <sup>b</sup>
	20 d	216 ± 7.17 <sup>a</sup>	181 ± 10.45 <sup>b</sup>	228 ± 21.56 <sup>a</sup>	165 ± 11.72 <sup>b</sup>
Mg <sup>2+</sup>	10 d	36.90 ± 1.34 <sup>b</sup>	42.57 ± 2.91 <sup>a</sup>	35.55 ± 3.13 <sup>b</sup>	28.53 ± 3.81 <sup>c</sup>
	20 d	31.38 ± 1.04 <sup>b</sup>	33.95 ± 1.96 <sup>b</sup>	38.77 ± 3.67 <sup>a</sup>	20.28 ± 1.44 <sup>c</sup>
Ca <sup>2+</sup>	10 d	26.39 ± 0.96 <sup>c</sup>	37.60 ± 2.57 <sup>a</sup>	31.36 ± 2.76 <sup>b</sup>	17.76 ± 2.37 <sup>d</sup>
	20 d	24.41 ± 0.81 <sup>b</sup>	30.19 ± 1.74 <sup>a</sup>	32.83 ± 3.11 <sup>a</sup>	12.39 ± 0.88 <sup>c</sup>
Mn <sup>2+</sup>	10 d	16.33 ± 0.59 <sup>b</sup>	18.83 ± 1.29 <sup>a</sup>	15.73 ± 1.38 <sup>b</sup>	12.62 ± 1.68 <sup>c</sup>
	20 d	13.88 ± 0.46 <sup>b</sup>	15.02 ± 0.87 <sup>b</sup>	17.15 ± 1.63 <sup>a</sup>	8.97 ± 0.64 <sup>c</sup>
SO <sub>4</sub> <sup>2-</sup>	10 d	48.57 ± 1.76 <sup>a</sup>	35.95 ± 2.46 <sup>b</sup>	32.80 ± 2.89 <sup>b</sup>	21.47 ± 2.87 <sup>c</sup>
	20 d	75.05 ± 2.49 <sup>a</sup>	28.16 ± 1.62 <sup>b</sup>	26.50 ± 2.51 <sup>b</sup>	15.24 ± 1.09 <sup>c</sup>
PO <sub>4</sub> <sup>3-</sup>	10 d	23.60 ± 0.86 <sup>a</sup>	23.77 ± 1.62 <sup>a</sup>	21.89 ± 1.93 <sup>a</sup>	23.74 ± 3.17 <sup>a</sup>
	20 d	28.20 ± 0.94 <sup>ab</sup>	26.26 ± 1.51 <sup>b</sup>	27.28 ± 2.58 <sup>b</sup>	31.83 ± 2.27 <sup>a</sup>

Mean values of three replicates with standard deviation (tested with a one-way analysis of variance [ANOVA] and Duncan's multiple range test;  $P < 0.05$ ). Two-way ANOVA of the contents of K<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup>, SO<sub>4</sub><sup>2-</sup>, and PO<sub>4</sub><sup>3-</sup> by concentration and period are as follows. K: concentration<sup>\*\*\*</sup>, period<sup>n.s.</sup>, concentration × period<sup>†</sup>. Mg: concentration<sup>\*\*\*</sup>, period<sup>\*\*\*</sup>, concentration × period<sup>\*\*</sup>. Ca: concentration<sup>\*\*\*</sup>, period<sup>\*\*</sup>, concentration × period<sup>†</sup>. Mn: concentration<sup>\*\*\*</sup>, period<sup>\*\*\*</sup>, concentration × period<sup>\*\*</sup>. SO<sub>4</sub><sup>2-</sup>: concentration<sup>\*\*\*</sup>, period<sup>n.s.</sup>, concentration × period<sup>\*\*\*</sup>. PO<sub>4</sub><sup>3-</sup>: concentration<sup>n.s.</sup>, period<sup>\*\*\*</sup>, concentration × period<sup>n.s.</sup>.

<sup>†</sup> $P < 0.05$ ; <sup>\*\*</sup> $P < 0.01$ ; <sup>\*\*\*</sup> $P < 0.001$ ; n.s., not significant.

T50, 50 g fresh red pine needles/L modified Hoagland's solution; T100, 100 g fresh red pine needles/L modified Hoagland's solution; T200, 200 g fresh red pine needles/L modified Hoagland's solution.

T100 concentration plants after day 20. Among the inorganic ions,  $\text{SO}_4^{2-}$  content decreased significantly with an increase in extract concentration.  $\text{SO}_4^{2-}$  content was reduced by 55.8% and 79.7% compared with that in the control for plants in the T200 treatment group after days 10 and 20, respectively. After day 10,  $\text{PO}_4^{3-}$  content in all treatment groups was not significantly different compared with that in the control. However,  $\text{PO}_4^{3-}$  content in plants exposed to the T200 concentration was higher than that of the T50 and T100 concentration after day 20 of treatment.

## DISCUSSION

Applying red pine needle extracts resulted in physiological changes to nitrogen fixation activity of the root nodules, TN content, osmolality, and total ionic contents in soybean leaves. Growth inhibition was considered to be due to allelochemicals in the needle extracts (Kil 1989, Kato-Noguchi et al. 2009) or related to the concentration of phenolic compounds in the mixture as well as the treatment duration (Alsaadawi et al. 1986, Inderjit et al. 2002).

Adding red pine needles to the nutrient solution resulted in an increase in osmolality and total ions as well as total phenolic content (Table 2). All aqueous plant extracts will exert negative osmotic and allelochemical effects on test plants (Bell 1974). In the present study,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{PO}_4^{3-}$  and total phenolic content increased with increasing needle quantity added to the nutrient solution, which was correlated with the increase in total ionic content, but  $\text{SO}_4^{2-}$  content decreased. Phenolics have significant effects on nutrient cycling by forming nutrient complexes (Appel 1993). This suggests that phenolics released from the pine needles may have affected  $\text{SO}_4^{2-}$  availability to the soybean and reduced  $\text{SO}_4^{2-}$  content in the leaves. Despite the increase in total ionic content,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  content in the needle extract and the contents of inorganic ions in the leaves of the treated plants showed different patterns according to the treatment concentration and duration. Phenolic compounds can cause depolarization of plant membranes and peroxidation of membrane lipids, which leads to the disruption of water balance and perturbations in ion uptake at the roots (Lyu and Blum 1990, Barkosky and Einhellig 1993, Baziramakenga et al. 1995). Therefore, changes in total leaf ions and inorganic ions could be explained as a consequence of alterations in membrane function by allelochemicals in the pine needle extracts.

Nitrogen nutrition in soybeans is ensured by both nitrogen fixation and nitrogen mineral assimilation, as in other legumes (Appleby 1984). Rice et al. (1981) reported that phenolic compounds reduce both nodule numbers and leghemoglobin content of nodules in two bean (*Phaseolus vulgaris*) varieties. Takahama and Oniki (1997) reported that oxidation of phenolic compounds results in yellow-brown colored nodules, which are observed when plant cells are damaged. Nitrogen fixation activity is closely related to respiration, cytosolic protein content, and leghemoglobin levels within the nodule (Walsh 1995). Leghemoglobin content of the nodules was not measured in this study, but Fig. 1b shows the changes of leghemoglobin content indirectly according to extract concentration, which may have been related to a significant inhibition of TNA at higher concentrations. O'hara et al. (1988) reported that mineral nutrient deficiency is a major constraint limiting legume nitrogen fixation and yield. Nitrogen fixation activity and TN content are related to soil nutrient status and plant ion uptake. At higher extract concentrations, the reduction in TN content of soybean leaves was correlated with inhibited nodule formation, nitrogen fixation activity, and a reduction of inorganic ions in the leaves. Eventually, low nitrogen content is associated with a reduction in leaf DW (Bergmark et al. 1992). As reported by Morgan (1984), osmotically active solutes, including organic and inorganic ions, play an important role in osmotic adjustment. Accumulating organic solutes demand more energy than accumulating inorganic ions, and limit plant growth by reducing organic compounds available for growth (Munns 2002). In this study, the inverse relationship between osmolality and total ionic contents was thought to be associated with an increase in organic osmotica (Table 5). Despite a reduction in total ionic content,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Mn}^{2+}$  were higher in soybean plants treated with the T100 extract concentration on day 20 after treatment than those in the control. These results suggest that  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , and  $\text{Mn}^{2+}$  may contribute to osmotic adjustment in plants under allelopathic stress. However, if soybeans are exposed to a critical stress level, such as the T200 concentration for an extended period, osmotic adjustment through ion uptake will not play an important role.

In conclusion, the DWs and TN of soybean were reduced by a red pine needle extract, indicating that allelochemicals within the red pine needle extract may have a negative effect on soybean growth. The extent of growth inhibition was related to the concentration of phenolic compounds in the extract and treatment duration. Total nodule number and nodule DW were not affected by the

low phenolic compound concentration. TN activity and the low concentration of phenolic compounds resulted in a significant decrease in nitrogen fixation compared with the control after day 10 of treatment, but was not affected when soybeans were exposed for a long duration, indicating that the low concentration of phenolic compounds had a positive effect on nitrogen fixation activity. Under severe allelochemical stress conditions, a significant reduction in nodule formation, nitrogen fixation activity, and ion uptake resulted in a reduction in plant growth. The change in ion fluxes and the accumulation of osmotic solutes in soybeans exposed to lower pine needle extract concentrations than a critical level may help to overcome allelopathic stress and maintain plant growth against allelochemical exposure.

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