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# Pathogenesis mechanism of *Pestalotiopsis funerea* toxin (Pf-toxin) on the plasmalemma of needle cells of different pine species

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The Pf-toxin (C<sub>5</sub>H<sub>11</sub>O<sub>5</sub>N) has been genetically associated with the pathogenesis mechanism in plasmalemma cells of pine needles in previous reports. In this study, a toxin was obtained from *Pestalotiopsis funerea* (called Pf-toxin) by concentrating and column chromatography. Responses of the needles of eight pine species against the toxin were investigated. The O<sub>2</sub><sup>-</sup> production rate, malondialdehyde (MDA) content, fatty acid composition, relative conductivity, and lesion length of the needles were determined. The severest damage and lipid peroxidation were exhibited by the needle plasmalemma of *Pinus massoniana*, *Pinus yunnanensis*, and *Pinus tabuliformis*. *Pinus elliottii* and *Pinus taeda* followed. *Pinus armandi*, *Pinus radiata* and *Pinus thunbergii* came last. The resistance capability of resistant species against the Pf-toxin precedes that of susceptible species.

**Key words:** *Pestalotiopsis funerea*, *Pestalotia* needle blight, *Pinus*, resistance.

## INTRODUCTION

*Pestalotia* needle blight caused by *Pestalotiopsis funerea* (Desm.) Stey is a common and serious disease in young pine trees. This has been the most important conifer disease in Chinese forests since 1980 (Qiu et al., 1980; Wu and Wei, 1987). To date, many pine species have been infected by this disease. Such species include *Pinus massoniana* Lamb., *Pinus yunnanensis* Franch., *Pinus armandii* Franch., *Pinus tabulaeformis* Carr., *Pinus thunbergii* Parl., *Pinus elliottii* Engelm, *Pinus caribaea* Morelet, *Pinus taeda* Linn., and *Pinus latteri* Mason. *P. massoniana* and *Pinus tabuliformis* are the most seriously susceptible to the diseases. Their foliage turns brown and their twigs die. Successive years of severe infection result in decreased growth, and ultimately, death. Previous studies have focused mainly on the pathogen and symptoms (Zhao and He, 1993; Huang and He, 2000; Sutarman et al., 2004), the hosts and

regularity (Liang et al., 2002; Jeewon et al., 2004), as well as disease control (Qiao et al., 2006; Jiang et al., 2007; Pan et al., 2010). However, the toxicity of the compounds produced by *P. funerea* (Pf-toxin) has only been reported by us. We have studied the cultivating conditions (Zheng and Zhu, 2006), isolation and purification (Zhu et al., 2003), as well as the structure (Zhu et al., 2005) of the Pf-toxin. Therefore, the pathogenesis mechanisms of the Pf-toxin on pines are still unknown.

Pathogens damage hosts mainly producing toxins, enzymes, and/or altering the metabolism of phytohormones (Heitefuss and Williams, 1991). Previous studies have demonstrated that the toxin destroys the structure and function of the plasmalemma, cell nucleus, mitochondria, chloroplasts, as well as ribosomes (Damann et al., 1974; Holden, 1984; Ye et al., 2000; Manning and Ciuffetti, 2005; Potrich et al., 2009). Changes in the plasmalemma permeability are an ordinary reaction of plant tissues upon toxin exposure. These changes are usually characterized by electrolyte leakage (EL) as well as depolarization and hyperpolarization of

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the membrane electric potential energy (Shah, 2005). Thus far, these effects have been reported to be exhibited by toxins such as those involved in fusariosis on pineapple (Hidalgo et al., 1998), Ptr ToxA on wheat (Rasmussen et al., 2004), as well as AK-I, AK, and AM-I on pear (Park et al., 1987; Shimizu et al., 2006). In addition, Zhang et al. (2006) and Jiang et al. (2007) have suggested that microbial toxins cause increased potential differences and the eventual disruption of the host cell. However, Cahill (1996) has indicated that, in *Eucalyptus marginata* seedling infected by Pc-toxin, EL may be a resistance reaction and not a result of infection. Nevertheless, the Pf-toxin has been confirmed as one of the major factors in the pathogenesis of *Pestalotia* needle blight on pine trees (Luo and Zhu, 2002; Zhu et al., 2003, 2005). The introduction of the mature toxin into the pine needles has resulted in a typical response similar with the disease symptoms induced by the pathogen. Such symptoms include chlorosis, necrotic bands on live needles, and ultimately, death of the needles. However, the mechanisms by which the Pf-toxin acts on the plasmalemma and of lipid peroxidation have not been reported until now. Moreover, a lot of reports had claimed that a series of reactions of the plasmalemma might reflect the resistance of plants against the toxin, and then indirectly revealed their resistance level against pathogens (Lu et al., 2004; Zhen and Li, 2004; Yang et al., 2011). Although so far, the data of the relationship between Pf-toxin and pines' resistance is still lacking.

The Pf-toxin is usually removed from *P. funerea* by column chromatography. On this basis, the present study aimed to determine the effects of this toxin on needle cells of different pine species. The parameters evaluated were the production rate of the superoxide anion radical ( $O_2^-$ ), malondialdehyde (MDA), which is an indicator for lipid peroxidation, membrane fatty acid composition, relative conductivity, and lesion length in the needles of different pine species. The pathogenesis mechanism of the Pf-toxin on the plasmalemma was proposed, and the resistance of different pine species was also determined by above tests.

## MATERIALS AND METHODS

### Isolation and purification of the Pf-toxin from culture filtrates

*P. funerea* (Desm.) Stey (provided by the Laboratory of Forest Protection, Sichuan Agricultural University) was statically cultured in liquid potato dextrose agar at 25°C for 27 days. The culture was filtered through double gauze, and the filtrate was centrifuged at 10 000  $\times g$  for 30 min. The supernatant was filtered through a 0.45 mm millipore filter and used as a crude toxin extract (Dubery and Smit, 1994). The crude toxin was loaded onto silica gel for column chromatography (100 to 200 mesh) with the selective phase (*n*-butanol: methanol:  $H_2O$  = 4:1:2). The flow rate was kept constant at 2 ml·min<sup>-1</sup>. The compound was confirmed as  $C_5H_{11}O_5N$  (Mw = 165) using mass spectrometry, nuclear magnetic resonance, and infrared spectroscopy (Zhu et al., 2005). The purified toxin was diluted by sterile distilled water to a concentration of 100  $\mu g \cdot ml^{-1}$  and was

stored at 4°C.

### Plant materials and toxin treatments

Five-year-old pines were planted at the arboretum of Sichuan Agricultural University. These included the susceptible species *P. massoniana*, *P. tabuliformis*, and *P. yunnanensis*, as well as the resistant ones *P. armandi*, *P. elliotii*, *P. taeda*, *Pinus radiata*, and *P. thunbergii*. One-year-old needles were used for seven toxin treatments (0, 6, 12, 24, 48, 72, and 96 h) with the impregnation method (Ho et al., 1996); the clean needles from the shoots were cultured in solution containing 1 ml of 100  $\mu g \cdot ml^{-1}$  purified toxin in centrifuge tubes at 25°C. A control treated with sterile distilled water was used. 10 g needles were used per one treatment and each treatment was repeated five times (total 400 g needles per species). All treated needles were measured lesion lengths firstly, and then used to assay the other items.

### Determination of lesion lengths in the pine needles

After 0, 6, 12, 24, 48, and 96 h of toxin treatment, lesion lengths (mm) in the pine needles were measured.

### Measurement of the superoxide anion radical ( $O_2^-$ ) production rate

The  $O_2^-$  production rate was determined by the hydroxylamine oxidation method (Elstner and Heupel, 1976; Wang and Luo, 1990) with some modifications. About 0.5 g of needle samples was homogenized with 3 ml of 65 mmol·l<sup>-1</sup> potassium phosphate buffer (pH 7.8). The solution was then centrifuged at 10 000  $\times g$  for 15 min. Subsequently, 0.5 ml of the supernatant was mixed with 0.5 ml of 65 mmol·l<sup>-1</sup> potassium phosphate buffer (pH 7.8) and 1 ml of 10 mmol·l<sup>-1</sup> hydroxylamine chloride. The homogenized mixture was warmed for 20 min at 25°C. About 1 ml of 58 mmol·l<sup>-1</sup> *p*-aminobenzene sulfonic acid and 1 ml of 7 mmol·l<sup>-1</sup>  $\alpha$ -naphthylamine were added. The mixture was warmed for 20 min at 25°C. About 4 ml of *n*-butyl alcohol was added, and the final supernatant was used for measuring the absorbance at 530 nm. A standard curve was constructed using the nitrogen dioxide radical ( $NO_2^-$ ) to calculate the production rate of  $O_2^-$ . This rate was expressed in  $\mu mol \cdot min^{-1} \cdot g^{-1} FW$ .

### Analysis of the fatty acid composition

Membrane fatty acids were extracted following the procedure of Su et al. (1980) with slight modifications. About 2 g of needle samples were heated for 5 min at 100°C to inactivate enzymes. Homogenization with chloroform-methanol (1:2, v/v) followed. The homogenized mixture was centrifuged at 10 000  $\times g$  for 10 min, and the supernatant was mixed with 2 ml of chloroform for washing. Subsequently, 2 ml of 0.76% NaCl were added. After standing and layering, the subnatant liquid was mixed with 1 ml of methanol, and was washed three times with petroleum ether (boiling temperature,  $T_b$ , range = 90 to 120°C). The thrice-washed subnatant liquids were mixed back together, and were re-washed twice with petroleum ether before removing the superstratum. The extract was vacuum dried with drops of 0.4 N KOH and 1 ml of petroleum ether ( $T_b$  range = 30 to 60°C)/benzene (1:1, v/v). After allowing the extract to stand for 15 min, distilled water was added. The mixture was allowed to stand for another 5 min. The supernatant was used in the fatty acid analyses.

The analyses were performed on a gas chromatograph (HP 6890, Hewlett Packard, Avondale, PA, USA) equipped with a mass

selective detector (Agilent 5973, Hewlett Packard). A capillary column (60 m × 0.25 mm; BPX 70, SGE, Victoria, Australia) was used. Helium was utilized as the carrier gas (1.2 ml·min<sup>-1</sup>), and the injection volume was 1 µl. The injection was done in the splitless mode for 2 min. The oven temperature was increased from 65 to 230°C at 5°C·min<sup>-1</sup>, and was maintained for 10 min at 230°C. The temperature during both injection and detection was 230°C. The results were expressed as relative percentages of each fatty acid, which were calculated as the ratio of the surface area of the considered peak to the total area of all peaks. All analyses were made in triplicate. All chemicals used were analytical grade.

### Assessment of lipid peroxidation

The level of lipid peroxidation was measured by the amount of MDA, a product of unsaturated fatty acid peroxidation. The method of Heath and Packer (1968) was used with slight modifications. About 0.5 g of needle samples were homogenized in 8 ml of 10% trichloroacetic acid and the homogenate was centrifuged at 4000 ×g for 20 min. About 2 ml of 0.6% thiobarbituric acid were added to 2 ml of the supernatant. The sample was then incubated at 100°C for 20 min. The reaction was stopped by placing the reaction tubes in an ice bath. The samples were then centrifuged at 10 000 ×g for 30 min. The supernatant was removed, and the absorptions at 532, 600, and 450 nm were obtained. The concentration was calculated according to the following formula:

$$C_{\text{MDA}} (\mu\text{mol}\cdot\text{l}^{-1}) = 6.45 (OD_{532} - OD_{600}) - 0.56 OD_{450}.$$

The MDA content was computed according to:

$$[\text{MDA}] (\mu\text{mol}\cdot\text{g}^{-1}\text{FW}) = C_{\text{MDA}} \times \text{extract volume (ml)} / \text{fresh weight (g)}.$$

### Assessment of relative electrical conductivity

Relative electrical conductivity (EL) was measured as described by Ye et al. (2000) with slight modifications. The conductivity was determined using an automatic conductivity meter (DDS-307). The initial conductivity was described as  $E_1$  with the needle sample treated by the toxin. On the other hand,  $E_2$  represented needle samples treated by sterile distilled water. The formula of the relative conductivity is:

$$\text{Relative conductivity (\%)} = (E_1 - E_2) / E_2 \times 100.$$

### Statistical analyses

All data were subjected to one-way analysis of variance (ANOVA) to determine the significance of individual differences between different Pf-toxin treatments at  $P < 0.05$  level. Significant means were compared using the least significant difference (LSD) test. All statistical analyses were conducted using the commercial SPSS statistical package (Version 13.0 for Windows, SPSS Inc., Chicago, USA).

## RESULTS

### Superoxide anion radical production rate in the pine needles

$\text{O}_2^-$  is the mono-negatron reduction product of  $\text{O}_2$ , and is the active oxygen species initially produced by an organism. The rate of  $\text{O}_2^-$  production gradually increased

before 12 h, and varied afterwards among the eight pine species studied (Figure 1). For *P. massoniana*, *P. tabuliformis*, and *P. yunnanensis* (first group), their  $\text{O}_2^-$  production rates did not significantly differ from one another before 12 h. From 12 to 48 h, the rates declined and then increased thereafter. The  $\text{O}_2^-$  production rate of *P. massoniana* was significantly higher than the others. For *P. elliotii* and *P. taeda* (second group), the rates gradually increased before 48 h, and then decreased thereafter. The succeeding rates were always higher than the initial rates. The rates of *P. elliotii* and *P. taeda* were close. For *P. radiata*, *P. thunbergii*, and *P. armandi* (third group), the rates inconspicuously increased for the entire experiment. The rate of *P. radiata* was the highest in this group. Overall, the  $\text{O}_2^-$  production rate had the trend: first group > second group > third group.

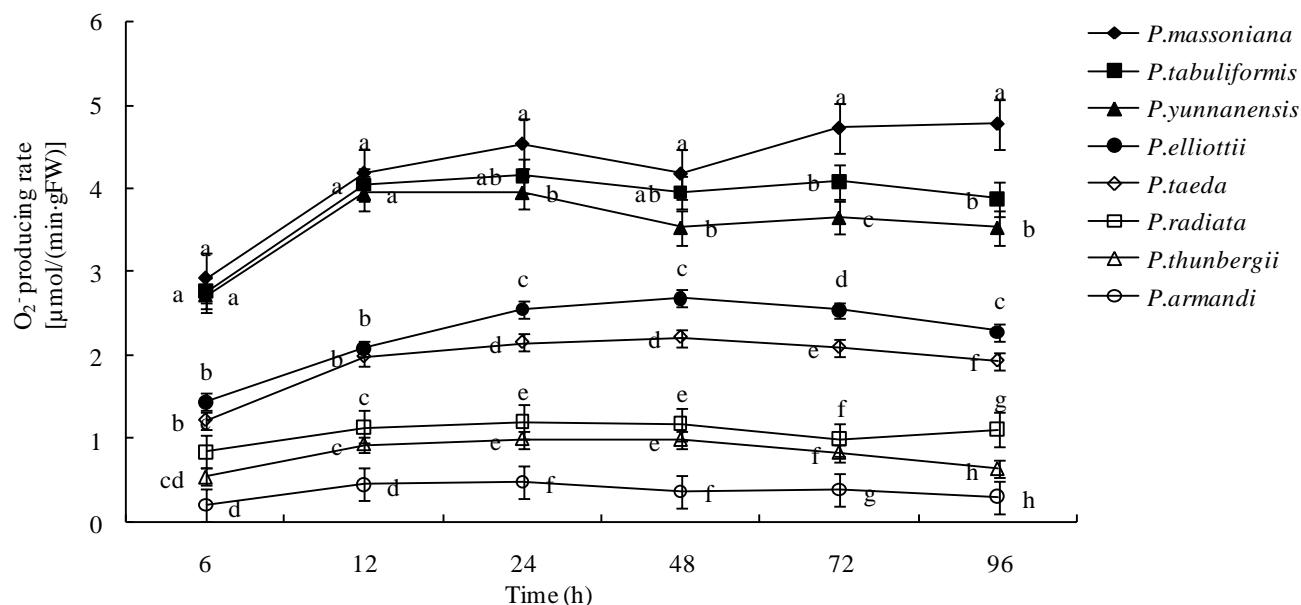
### Fatty acid components of pine needles

The fatty acid components of the plasmalemma of eight pines species were cetylic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids (Table 1). In saturated fatty acids (SFAs), the content of C16:0 was higher than that of C18:0. In addition, C18:3 content was the highest in unsaturated fatty acids (USFAs). The content of C16:0 in the needles of *P. massoniana* and *P. tabuliformis* was the highest. In the other species, the content of C18:3 was the highest. The content changes in these components differed from one another according to the toxin treatment time. The SFA contents (C16:0 and C18:0) declined from 0 to 12 h, and then increased afterwards. The USFA contents (C18:1, C18:2 and C18:3) had the opposite trend (Figure 2).

Among the eight pine species, the SFA content was the highest in *P. massoniana*, and was significantly different from the other species. In contrast, the SFA content was the lowest and relatively constant in *P. armandi*. On the other hand, the USFA content was significantly highest in *P. armandi* and remained constant. The USFA content rapidly declined after 12 h in *P. massoniana*, *P. tabuliformis*, *P. yunnanensis*, and *P. elliotii*. Moreover, the results of index of unsaturated fatty acids (IUFA) (Figure 3) indicated a trend similar with USFA content. The IUFA of all eight pine species decreased after Pf-toxin treatment. However, the IUFA of *P. armandi* showed a relatively smooth change, whereas that of *P. massoniana* and *P. tabuliformis* rapidly decreased.

### Malondialdehyde (MDA) content in pine needles

Lipid peroxidation measured as an increase in MDA content is known to be a good indicator of oxidative damage to membrane lipids. In this study, the MDA content increased with the Pf-toxin treatment compared with the control. The differences were significant between the control and toxin-treated groups for all eight pine



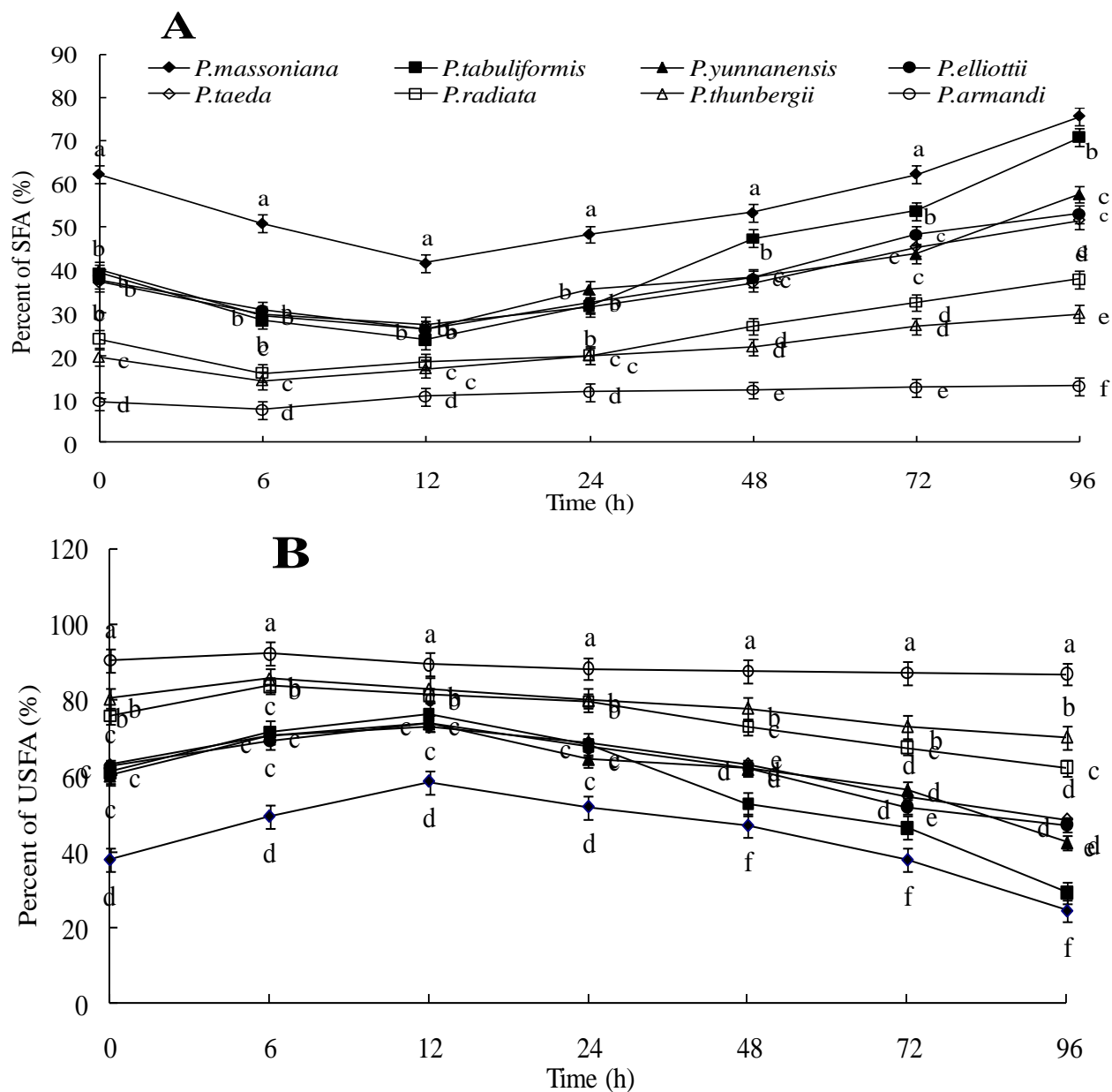
**Figure 1.**  $O_2^-$  production rate in the plasmalemma of pine needles treated with the Pf-toxin.  $O_2^-$  production rate was indirectly replaced with the absorbance amount at 530 nm ( $A_{530}$ ), and  $A_{530}$  was converted to the concentration of  $[NO_2^-]$  according to the standard curve of nitrous acid colour reaction (Elstner and Heupel, 1976).  $O_2^-$  production rate ( $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}\text{FW}$ ) = ( $[NO_2^-]$  concentration  $\times 2 \times$  total volume of solution) / (warmed time  $\times$  fresh weight of plant tissue). Data in the same column followed by different lowercase letters indicate significant differences by the LSD test ( $P < 0.05$ ,  $n = 5$ ). LSD, Least significant difference.

**Table 1.** Fatty acid components of pine needles treated with the Pf-toxin.

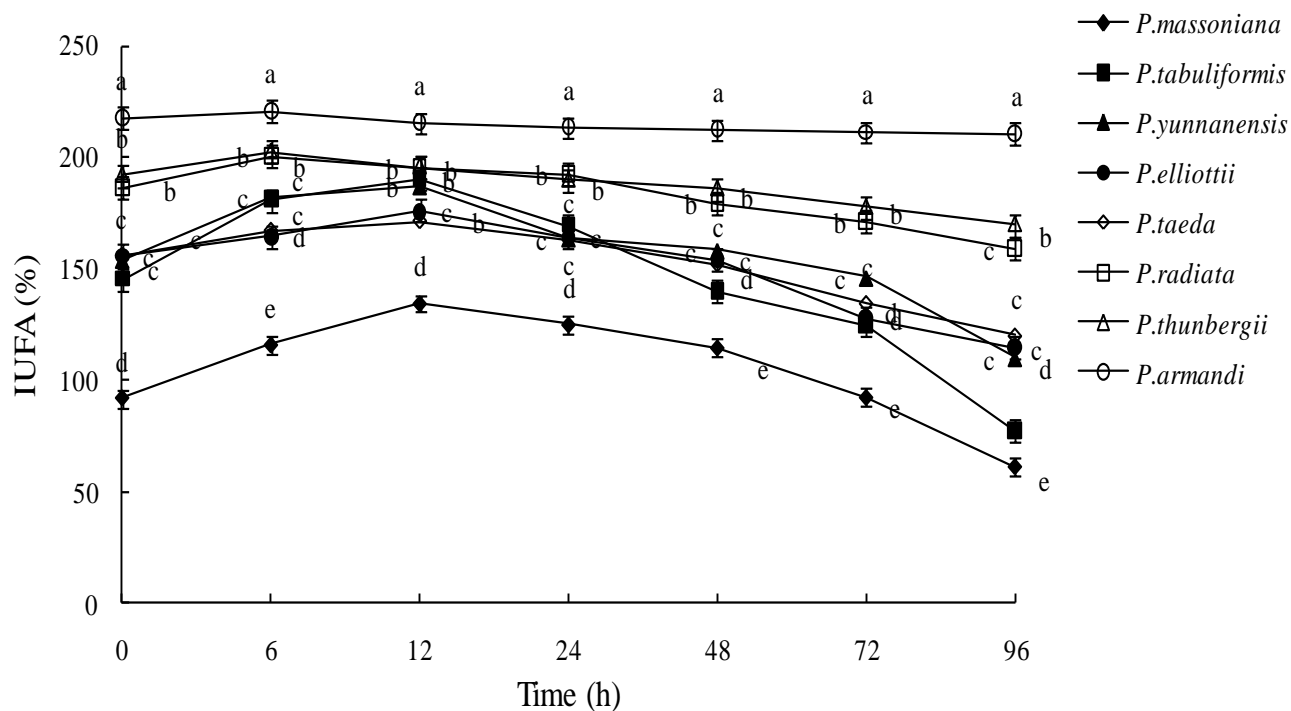
Species	Time (h)	Component (%)					Species	Time (h)	Component (%)				
		C16:0	C18:0	C18:1	C18:2	C18:3			C16:0	C18:0	C18:1	C18:2	C18:3
<i>P. massoniana</i>	0	48.15	14.01	2.25	17.22	18.37	<i>P. tabuliformis</i>	0	33.54	5.69	8.99	19.26	32.52
	6	38.75	12.02	5.00	22.00	22.23		6	24.46	3.91	11.27	11.61	48.75
	12	32.56	9.03	6.45	28.05	23.91		12	21.25	2.57	12.14	14.06	49.98
	24	36.26	12.00	4.08	22.28	25.38		24	24.66	7.17	7.72	19.82	40.63
	48	38.71	14.51	2.34	21.33	23.11		48	37.17	10.17	1.26	15.84	35.56
	72	47.14	15.08	2.06	17.25	18.47		72	42.51	11.21	1.05	12.34	32.89
	96	60.03	15.56	0.50	11.55	12.36		96	55.96	14.84	0.26	10.00	18.94
<i>P. yunnanensis</i>	0	29.89	10.01	2.54	21.38	36.18	<i>P. elliottii</i>	0	27.67	9.95	3.01	25.40	33.97
	6	23.00	6.40	1.59	26.51	42.50		6	22.02	8.74	6.35	30.50	32.39
	12	20.80	5.33	1.89	30.85	41.13		12	19.48	6.67	7.34	31.05	35.46
	24	25.60	9.91	1.26	27.45	35.78		24	22.55	9.73	5.09	28.77	33.86
	48	26.04	12.13	0.67	26.12	35.04		48	27.60	10.43	3.12	25.53	33.32
	72	29.11	14.55	0.35	22.56	33.43		72	33.87	14.32	2.56	22.77	26.48
	96	39.19	18.36	0.33	17.08	25.04		96	36.65	16.30	1.99	22.81	22.25
<i>P. taeda</i>	0	27.54	9.61	3.49	25.86	33.50	<i>P. radiata</i>	0	20.12	3.97	7.41	26.69	41.81
	6	21.59	8.13	6.87	30.32	33.09		6	14.06	2.05	10.55	30.25	43.09
	12	20.16	7.09	7.97	31.25	33.53		12	15.69	2.99	10.01	28.77	42.54
	24	22.20	9.05	6.89	29.78	32.08		24	16.41	3.78	9.50	28.00	42.31
	48	26.14	10.79	5.36	26.67	31.04		48	19.03	7.97	7.20	25.15	40.65
	72	32.97	12.35	3.38	23.09	28.21		72	23.30	9.19	4.41	22.88	40.22
	96	35.99	15.50	2.04	21.69	24.78		96	25.54	12.31	2.79	21.95	37.41

Table 1 Contd.

	0	16.36	3.38	9.99	29.07	41.20		0	9.51	–	12.34	29.17	48.98
	6	12.52	1.87	11.65	31.19	42.77		6	7.62	–	13.27	30.06	49.05
	12	14.14	2.98	11.47	30.15	41.26		12	10.17	0.41	12.46	28.12	48.84
<i>P. thunbergii</i>	24	15.33	4.74	11.03	28.16	40.74	<i>P. armandi</i>	24	11.22	0.45	12.09	27.67	48.57
	48	16.20	5.93	10.41	27.04	40.42		48	11.75	0.46	11.94	27.31	48.54
	72	19.00	8.02	8.14	25.35	39.49		72	12.33	0.46	11.78	26.95	48.48
	96	21.21	8.59	8.07	24.94	37.19		96	12.66	0.46	11.7	26.74	48.44



**Figure 2.** Percent saturated fatty acids (SFA) (a), and unsaturated fatty acids (USFA) (b), in the plasmalemma of pine needles treated with the Pf-toxin. The content of SFA was the sum of C16:0 and C18:0 content; the content of USFA was the sum of C18:1, C18:2 and C18:3 content. Percent SFA (%) = SFA content / total content of fatty acid; percent USFA (%) = USFA content / total content of fatty acid. Data in the same column followed by different lowercase letters indicate significant differences by the LSD test ( $P < 0.05$ ,  $n = 5$ ). LSD, Least significant difference.



**Figure 3.** Index of unsaturated fatty acids (IUFA) in the plasmalemma of pine needles treated with the Pf-toxin. IUFA (%) =  $1 \times C18:1(\%) + 2 \times C18:2(\%) + 3 \times C18:3(\%)$ . Data in the same column followed by different lowercase letters indicate significant differences by the LSD test ( $P < 0.05$ ,  $n = 5$ ). LSD, Least significant difference.

species (Table 2). In the Pf-toxin treatment group, the MDA content increased from 6 to 12 h, and decreased from 12 to 24 h among all the pine species. The MDA content in *P. massoniana* was the highest, and was significantly different from the other pines. The MDA content in *P. thunbergii* was the lowest during the entire experiment. Figure 4 shows that the changes in MDA content were similar for all pines. The MDA content increased from 6 to 12 h, and peaked at 12 h. The MDA content then decreased rapidly until 24 h, except in *P. massoniana* (48 h). After 24 h, the changes remained constant. The increase rate was significantly highest in *P. tabuliformis*, followed by *P. massoniana* and *P. yunnanensis* with non-significant differences. *P. elliottii*, *P. taeda*, *P. radiata*, *P. thunbergii*, and *P. armandi* came last with non-significant differences.

#### Relative electrical conductivity (EL) in pine needles

The effects of the Pf-toxin on the structure and function of the plasmalemma is usually expressed as the EL, which is measured as the relative conductivity (Figure 5). The relative conductivity indices of each pine species increased until the peak was reached during Pf-toxin treatment, and then became steady. However, the degrees of relative conductivity changed differently for each pine species. In *P. massoniana*, *P. tabuliformis*, and

*P. yunnanensis*, the changes in the relative conductivity were similar as the index rapidly increased from 6 to 24 h; afterwards, the high levels were maintained. On the other hand, the degrees of relative conductivity in *P. elliottii* and *P. taeda* were significantly lower than those of the aforementioned three pine species, although in a proportional manner. Moreover, the increased amplitudes of *P. radiata*, *P. thunbergii*, and *P. armandi* were less inconspicuous than the aforementioned five pine species. These indices increased up to 48 h of toxin treatment.

#### Effects of Pf-toxin on lesion length of pine needles

Disease spots are the visible symptoms of pine needles infected by Pf-toxin. The lesion length is one of the criteria for determining the degree of infection. In this study, changes in the lesion lengths are shown in Figure 6. The lesion length increased with the time of toxin treatment. From 0 to 24 h, the lesion lengths sharply increased. However, from 24 to 96 h, these indices steadily increased. All the lesion lengths of the eight pine species had significant differences after 6 h of treatment time. There were three tendencies in lesion length changes. First is the high level length increase (*P. massoniana*, *P. tabuliformis*, and *P. yunnanensis*), second is the middle level increase (*P. elliottii* and *P. taeda*), and third is the low level increase (*P. radiata*, *P. thunbergii* and

**Table 2.** Malondialdehyde (MDA) content ( $\mu\text{mol g}^{-1}\text{FW}$ ) in the plasmalemma of pine needles.

Species	Treatment	Time (h)					
		6	12	24	48	72	96
<i>P. massoniana</i>	Pf-toxin	3.12 ± 0.01 <sup>Ab</sup>	3.56 ± 0.03 <sup>Ba</sup>	2.36 ± 0.03 <sup>Bd</sup>	1.60 ± 0.01 <sup>Ef</sup>	2.24 ± 0.02 <sup>Ae</sup>	2.80 ± 0.02 <sup>Ac</sup>
	Control	3.28 ± 0.01 <sup>Ba</sup>	2.40 ± 0.02 <sup>Dc</sup>	2.16 ± 0.01 <sup>Cd</sup>	1.44 ± 0.03 <sup>Gf</sup>	2.00 ± 0.04 <sup>Ce</sup>	2.56 ± 0.01 <sup>Bb</sup>
<i>P. tabuliformis</i>	Pf-toxin	2.72 ± 0.01 <sup>Cb</sup>	3.60 ± 0.02 <sup>Aa</sup>	2.48 ± 0.03 <sup>Ac</sup>	2.24 ± 0.03 <sup>Ad</sup>	1.92 ± 0.02 <sup>Ee</sup>	1.80 ± 0.04 <sup>Ff</sup>
	Control	2.64 ± 0.01 <sup>Da</sup>	1.76 ± 0.01 <sup>Jc</sup>	1.60 ± 0.01 <sup>Hd</sup>	2.00 ± 0.03 <sup>Cb</sup>	1.76 ± 0.01 <sup>Fc</sup>	1.60 ± 0.01 <sup>Hd</sup>
<i>P. yunnanensis</i>	Pf-toxin	2.56 ± 0.03 <sup>Eb</sup>	3.44 ± 0.02 <sup>Ca</sup>	2.16 ± 0.01 <sup>Cd</sup>	2.24 ± 0.03 <sup>Ac</sup>	1.60 ± 0.02 <sup>Hf</sup>	1.76 ± 0.01 <sup>Ge</sup>
	Control	2.48 ± 0.02 <sup>Fa</sup>	2.28 ± 0.02 <sup>Fb</sup>	2.00 ± 0.02 <sup>Ec</sup>	2.00 ± 0.03 <sup>Cc</sup>	1.48 ± 0.02 <sup>Ie</sup>	1.60 ± 0.01 <sup>Hd</sup>
<i>P. elliotii</i>	Pf-toxin	2.16 ± 0.03 <sup>Gb</sup>	2.36 ± 0.02 <sup>Ea</sup>	2.00 ± 0.02 <sup>Ed</sup>	2.08 ± 0.02 <sup>Bc</sup>	2.04 ± 0.03 <sup>Bcd</sup>	2.00 ± 0.01 <sup>Dd</sup>
	Control	2.08 ± 0.03 <sup>Ha</sup>	2.00 ± 0.03 <sup>Gb</sup>	1.84 ± 0.02 <sup>Fd</sup>	1.92 ± 0.01 <sup>Dc</sup>	1.96 ± 0.03 <sup>Dc</sup>	1.96 ± 0.02 <sup>Ec</sup>
<i>P. taeda</i>	Pf-toxin	2.00 ± 0.03 <sup>Id</sup>	2.28 ± 0.03 <sup>Fa</sup>	2.12 ± 0.04 <sup>Dbc</sup>	2.00 ± 0.04 <sup>Cd</sup>	2.00 ± 0.01 <sup>Cd</sup>	2.08 ± 0.03 <sup>Cc</sup>
	Control	1.88 ± 0.01 <sup>Jc</sup>	1.88 ± 0.03 <sup>Ic</sup>	2.00 ± 0.02 <sup>Ea</sup>	1.92 ± 0.02 <sup>Dbc</sup>	1.96 ± 0.03 <sup>Dab</sup>	2.00 ± 0.03 <sup>Da</sup>
<i>P. radiata</i>	Pf-toxin	1.76 ± 0.01 <sup>Kc</sup>	2.00 ± 0.02 <sup>Ga</sup>	1.84 ± 0.04 <sup>Fb</sup>	1.60 ± 0.02 <sup>Ed</sup>	1.76 ± 0.01 <sup>Fc</sup>	1.60 ± 0.03 <sup>Hd</sup>
	Control	1.68 ± 0.01 <sup>Lb</sup>	1.68 ± 0.02 <sup>Jb</sup>	1.76 ± 0.02 <sup>Ga</sup>	1.60 ± 0.05 <sup>Ec</sup>	1.68 ± 0.02 <sup>Gb</sup>	1.60 ± 0.03 <sup>Hc</sup>
<i>P. armandi</i>	Pf-toxin	1.68 ± 0.02 <sup>Lb</sup>	1.92 ± 0.02 <sup>Ha</sup>	1.60 ± 0.01 <sup>Hc</sup>	1.52 ± 0.02 <sup>Fd</sup>	1.44 ± 0.03 <sup>Jf</sup>	1.48 ± 0.02 <sup>Ie</sup>
	Control	1.60 ± 0.03 <sup>Mb</sup>	1.68 ± 0.01 <sup>Ka</sup>	1.52 ± 0.02 <sup>Ic</sup>	1.44 ± 0.01 <sup>Gd</sup>	1.44 ± 0.02 <sup>Jd</sup>	1.44 ± 0.01 <sup>Jd</sup>
<i>P. thunbergii</i>	Pf-toxin	1.60 ± 0.02 <sup>Mb</sup>	1.76 ± 0.01 <sup>Ja</sup>	1.52 ± 0.03 <sup>Ic</sup>	1.52 ± 0.02 <sup>Fc</sup>	1.40 ± 0.01 <sup>Kd</sup>	1.28 ± 0.03 <sup>Ke</sup>
	Control	1.60 ± 0.01 <sup>Ma</sup>	1.52 ± 0.03 <sup>Lb</sup>	1.52 ± 0.02 <sup>Ib</sup>	1.52 ± 0.01 <sup>Fb</sup>	1.36 ± 0.03 <sup>Lc</sup>	1.24 ± 0.01 <sup>Ld</sup>

Data in the same row followed by different lowercase letters indicate significant differences between different exposure times by the LSD test ( $P < 0.05$ ,  $n = 5$ ). Data in the same column followed by different capital letters indicate significant differences within pine species by the LSD test ( $P < 0.05$ ,  $n = 5$ ). LSD, Least significant difference.

*P. armandi*).

### Correlation analysis between physiological indices and lesion lengths

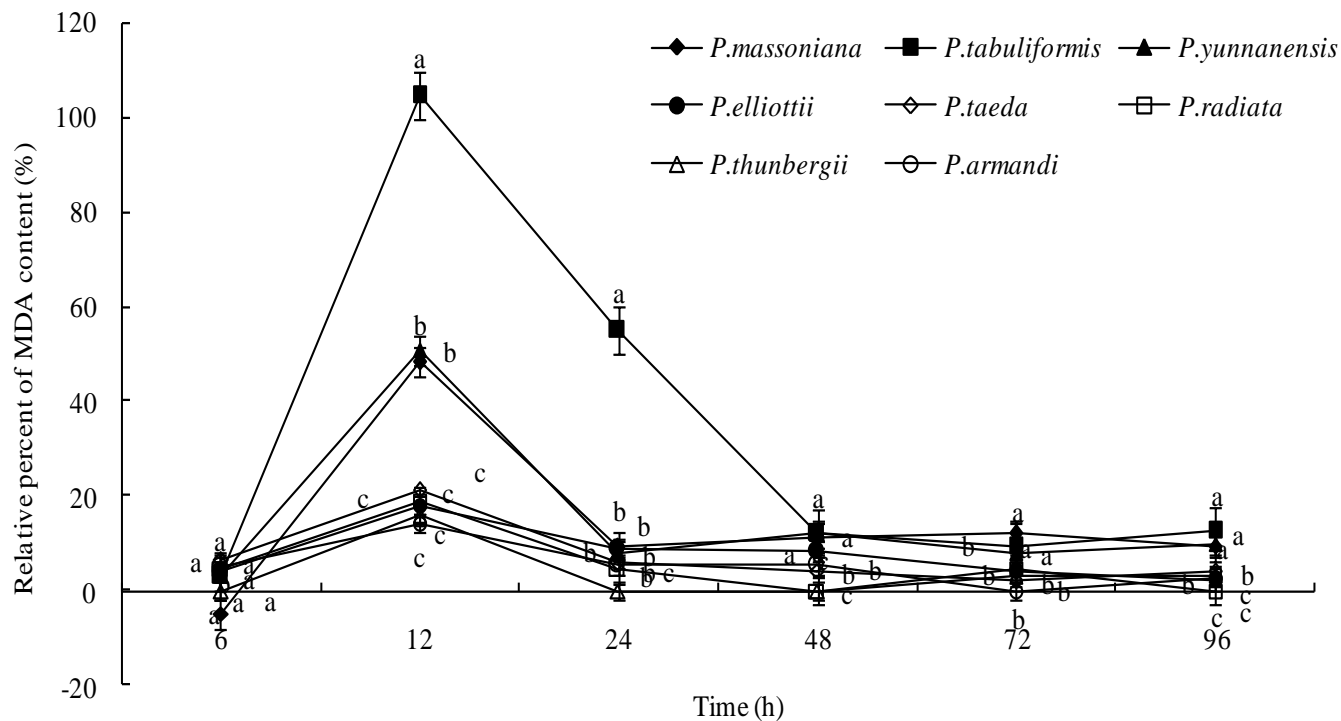
Correlation coefficients were determined, and the results are shown in Table 3. All indices reached or exceeded significant levels. The correlation coefficients of *P. thunbergii* and *P. armandi* were also lower than the others, except for IUFA. The opposite was true for *P. massoniana* and *P. tabuliformis*.

### DISCUSSION

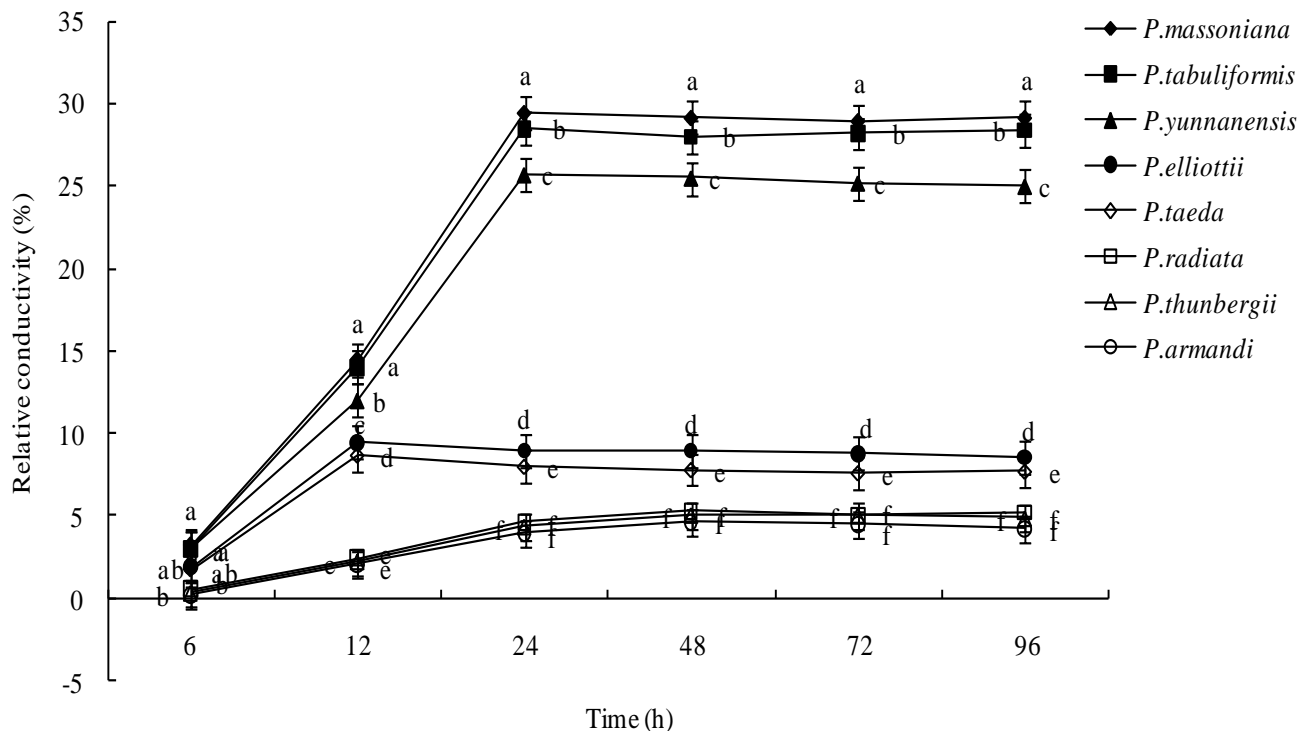
Increased permeability and decreased stability are the indications that the plasmalemma of a plant cell has been exposed to a phytotoxin. These indicators accurately reveal that the plasmalemma is the initial toxin action site (Hartung, 1987; Yang et al., 2000). The results of the present study have suggested that the Pf-toxin may alter the permeability of pine needle plasmalemma and cause

disease spots. These effects became more apparent with longer treatment times. Moreover, the capability of pine species in resisting toxic effects depended on the resistance of the plasmalemma against the Pf-toxin. Zhen and Li (2004) had confirmed that *Verticillium dahliae* toxin made different damage degrees to cell wall and plasmalemma in different cotton species; Yang et al. (2011) had reported that *Phytophthora infestans* toxin had effected different reactions of three potato species; on the basis of these theories, this study has indicated that the plasmalemma of resistant pine species incurred less damaged pine needles than that of susceptible species. Therefore, we believe that the plasmalemma is the action site of the Pf-toxin.

The first reaction during a phytotoxin-induced oxidative burst is believed to be the one-electron reduction of molecular oxygen to form the superoxide anion ( $\text{O}_2^-$ ) (Mehdy, 1994; Gechev et al., 2006). Keppler and Novacky (1987) reported that lipid peroxidation and pathogen-induced changes in membrane components were virtually the results of an  $\text{O}_2^-$  startup. Similarly, in this study, the rate of  $\text{O}_2^-$  production increased with increased toxin treatments in eight species of pines in varying degrees. Moreover,

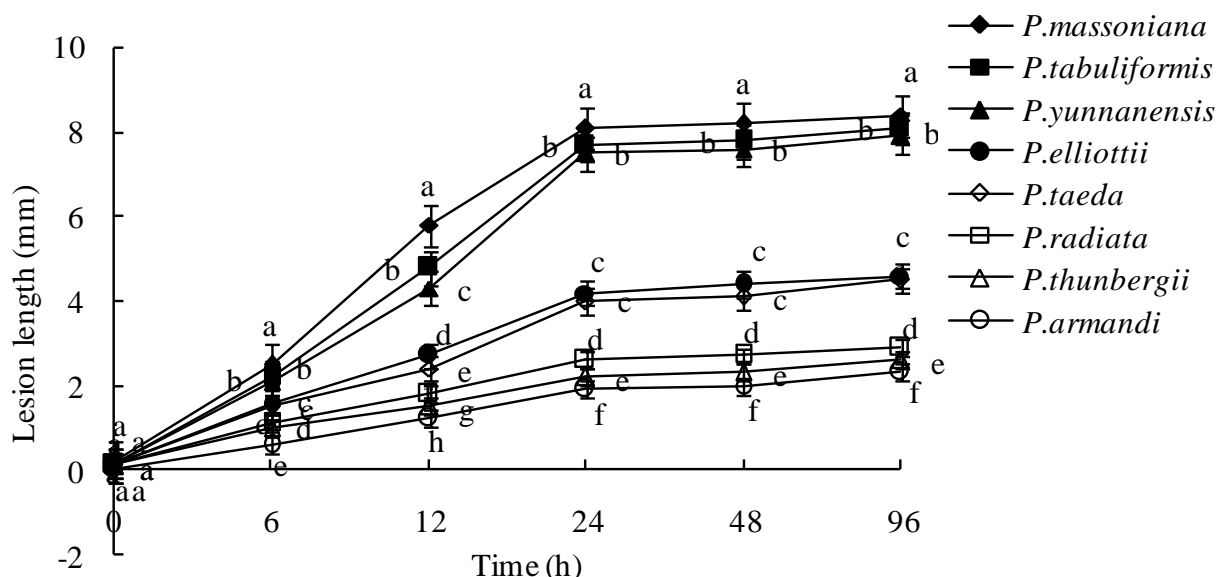


**Figure 4.** MDA content (relative percent) in pine needles. MDA content (relative percent, %) = [(MDA content in the toxin-treated group – MDA content in the control) / MDA content in the control]  $\times$  100%. Data in the same column followed by different lowercase letters indicate significant differences by the LSD test ( $P < 0.05$ ,  $n = 5$ ). LSD, Least significant difference; MDA, malondialdehyde.



**Figure 5.** Relative conductivities in pine needles. Relative conductivity (%) =  $(E_1 - E_2) / E_2 \times 100$ ;  $E_1$ , the initial conductivity with the needle sample treating by toxin;  $E_2$ , the initial conductivity with the needle samples treating by sterile distilled water. Data in the same column followed by different lowercase letters indicate significant differences by the LSD test ( $P < 0.05$ ,  $n = 5$ ). LSD, Least significant difference.





**Figure 6.** Lesion lengths of pine needles. Lesion lengths (mm) in the pine needles were measured after 0, 6, 12, 24, 48, and 96 h of toxin treatment. Data in the same column followed by different lowercase letters indicate significant differences by LSD test ( $P < 0.05$ ,  $n=5$ ). LSD, Least significant difference.

**Table 3.** Correlation coefficients among physiological indices and lesion lengths.

Test	Species							
	<i>P. massoniana</i>	<i>P. tabuliformis</i>	<i>P. yunnanensis</i>	<i>P. elliottii</i>	<i>P. taeda</i>	<i>P. radiata</i>	<i>P. thunbergii</i>	<i>P. armandi</i>
$O_2^-$ producing rate	0.877*	0.883*	0.801*	0.765*	0.639*	0.681*	0.592*	0.635*
MDA content	0.947*	0.971**	0.911**	0.904**	0.938**	0.770*	0.759*	0.750*
IUFA	0.847*	0.850*	0.840*	0.840*	0.918**	0.862*	0.936**	0.927**
Relative conductivity	0.946**	0.933**	0.973**	0.978**	0.985**	0.904**	0.897*	0.897*

MDA, malondialdehyde; IUFA, index of unsaturated fatty acids. Lesion lengths in 24 h were used for correlation analysis. Data followed by different letters indicate significant differences at  $P < 0.05$  by the LSD test. \*Significant correlations among physiological indices and lesion lengths. LSD, Least significant difference.

the rate of  $O_2^-$  production rapidly increased in the initial stage of toxin treatment. This result showed that all the pines were sensitive to the toxin. However, the rate of  $O_2^-$  production in *P. radiata*, *P. thunbergii* and *P. armandi* remained stable in later stages. This stability may be due to the stronger resistance of these three species than the others.

MDA is the product of lipid peroxidation and membrane damage. These phenomena result in physiological and biochemical disorders in related tissues, and could be regarded as signs of plasmalemma damage (Yuan et al., 2007). In this study, the MDA content increased sharply during Pf-toxin treatment. This result could confirm the occurrence of lipid peroxidation. However, after 12 h, the MDA content probably decreased because of the instability of MDA and the aging of cells (Ye et al., 2000). The degree of EL is also an important parameter in determining changes in plasmalemma permeability

(Zhang et al., 2008). In the EL experiment, relative conductivity initially increased, and then remained stable. These results suggest that all pine species had tolerance to the Pf-toxin, but the resistant pine species were more tolerant than the susceptible species.

Fatty acids are key nutrients associated with energy production and storage as well as gene regulation (Jump, 2004). Fatty acids are also the essential components of cell membrane phospholipids (Van der Vusse et al., 1992). Kasamo et al. (1992) indicated that the phase transformation of plasmalemma occurs with difficulty, and that the plasmalemma may modulate the degree of unsaturation to improve membrane fluidity. The IUFA of all the pine species changed after Pf-toxin treatment. The SFA contents in the pine needles of *P. armandi*, *P. thunbergii*, and *P. radiata* were lower than in those of *P. massoniana*, *P. tabuliformis*, and *P. yunnanensis*. In contrast, the USFA contents had opposite trends. These

results have indicated that the needles of *P. massoniana*, *P. tabuliformis*, and *P. yunnanensis* were more easily damaged by lipid peroxidation. Their unsaturated bonds were oxidized in partial fatty acids (Wang et al., 2006). The needles of *P. massoniana* were the most seriously damaged. Nevertheless, the changes in the SFA and USFA contents did not increase or decrease only in response to the superoxide anion radical (Ye et al., 2000). Subsequently, we found that the changes in USFA contents (C18:1, C18:2, and C18:3) were opposite to the SFA contents (C16:0 and C18:0). These results are consistent with previous studies on *P. elliotii* (Ye et al., 2000), *Pleurotus* sp. (Pedneault et al., 2007), kiwifruit (Antunes and Sfakiotakis, 2008), *P. tabuliformis* (Ma et al., 2010), and *Spiraea* sp. (Liu et al., 2011).

The results of the present study have indicated that lesion lengths in pine needles rapidly increased before 24 h of toxin treatment. This finding significantly correlated with the rate of O<sub>2</sub><sup>-</sup> production, MDA content, IUFA, and relative conductivity. Based on the study of Guo et al. (2005), we presumed that lesion lengths were visible symptoms of phytotoxin exposure. We also presumed its close relation to internal physiological indices.

## Conclusion

In conclusion, although phytotoxins may change plasmalemma permeability and damage cell tissues, the plasmalemma can self-adjust and recover. More importantly, our data reveals the resistance capabilities of different pine species. In the present study, certain pine species (*P. massoniana*, *P. tabuliformis*, and *P. yunnanensis*) whose plasmalemma permeability increased and with damaged cells and tissues have less resistance capabilities, while others (*P. armandi*, *P. elliotii*, *P. taeda*, *P. radiata*, and *P. thunbergii*). However, the activation of toxin degradation and the relief of toxicity in resistant species remain unclear. Nevertheless, the Pf-toxin could be used to select pine species resistant against pine needle blight. Further research on the permeation of the Pf-toxin into the plasmalemma and on its other molecular and cellular targets is necessary, as well as the signal transduction pathway involved in plant resistance needs to be investigated.

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