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Full Length Research Paper

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 ($C_5H_{11}O_5N$) 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_2^- 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 Williamas, 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 fusariose 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 ×*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^{-1}. 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 μg·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. elliottii*, *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 μg·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^{-1} potassium phosphate buffer (pH 7.8). The solution was then centrifuged at 10 000 ×*g* for 15 min. Subsequently, 0.5 ml of the supernatant was mixed with 0.5 ml of 65 mmol·l^{-1} potassium phosphate buffer (pH 7.8) and 1 ml of 10 mmol·l^{-1} hydroxylamine chloride. The homogenized mixture was warmed for 20 min at 25°C. About 1 ml of 58 mmol·l^{-1} p-aminobenzene sulfonic acid and 1 ml of 7 mmol·l^{-1} α-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 μmol·min^{-1}·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 ×*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 rewashed 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 $ml \cdot min^{-1}$), 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^{-1}, and was maintained for l0 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_{MDA} (µmol·l^{-1}) = 6.45 (OD_{532} – OD_{600}) – 0.56OD_{450}.

The MDA content was computed according to:

[MDA] (µmol·g^{-1}FW) = C_{MDA} × extract volume (ml) / 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:

Relative conductivity (%) = (E_1 – E_2) / E_2 × 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

O_2^- is the mono-negatron reduction product of O_2, and is the active oxygen species initially produced by an organism. The rate of 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 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 O_2^- production rate of *P. massoniana* was significantly higher than the others. For *P. elliottii* 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. elliottii* 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 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. elliottii*. 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

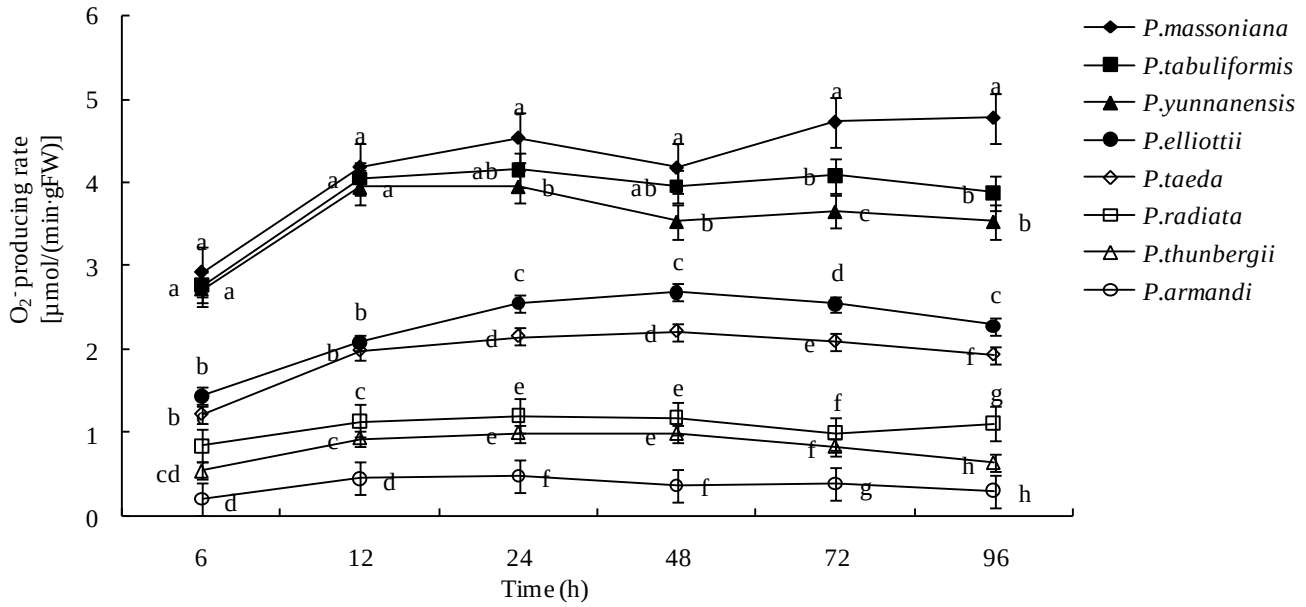

Figure 1. O_2^- production rate in the plasmalemma of pine needles treated with the Pf-toxin. O_2^- production rate was indirectly replaced with the absorbance amount at 530 nm (A_{530}), and A_{530} was converted to the concentration of [NO_2^-] according to the standard curve of nitrous acid colour reaction (Elstner and Heupel, 1976). O_2^- production rate (μmol·min^{-1}·g^{-1}FW) = ([NO_2^-] concentration × 2 × total volume of solution) / (warmed time × 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
P. massoniana	0	48.15	14.01	2.25	17.22	18.37	*P. tabuliformis*	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
P. yunnanensis	0	29.89	10.01	2.54	21.38	36.18	*P. elliottii*	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
P. taeda	0	27.54	9.61	3.49	25.86	33.50	*P. radiata*	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
P. thunbergii	24	15.33	4.74	11.03	28.16	40.74	*P. armandi*	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

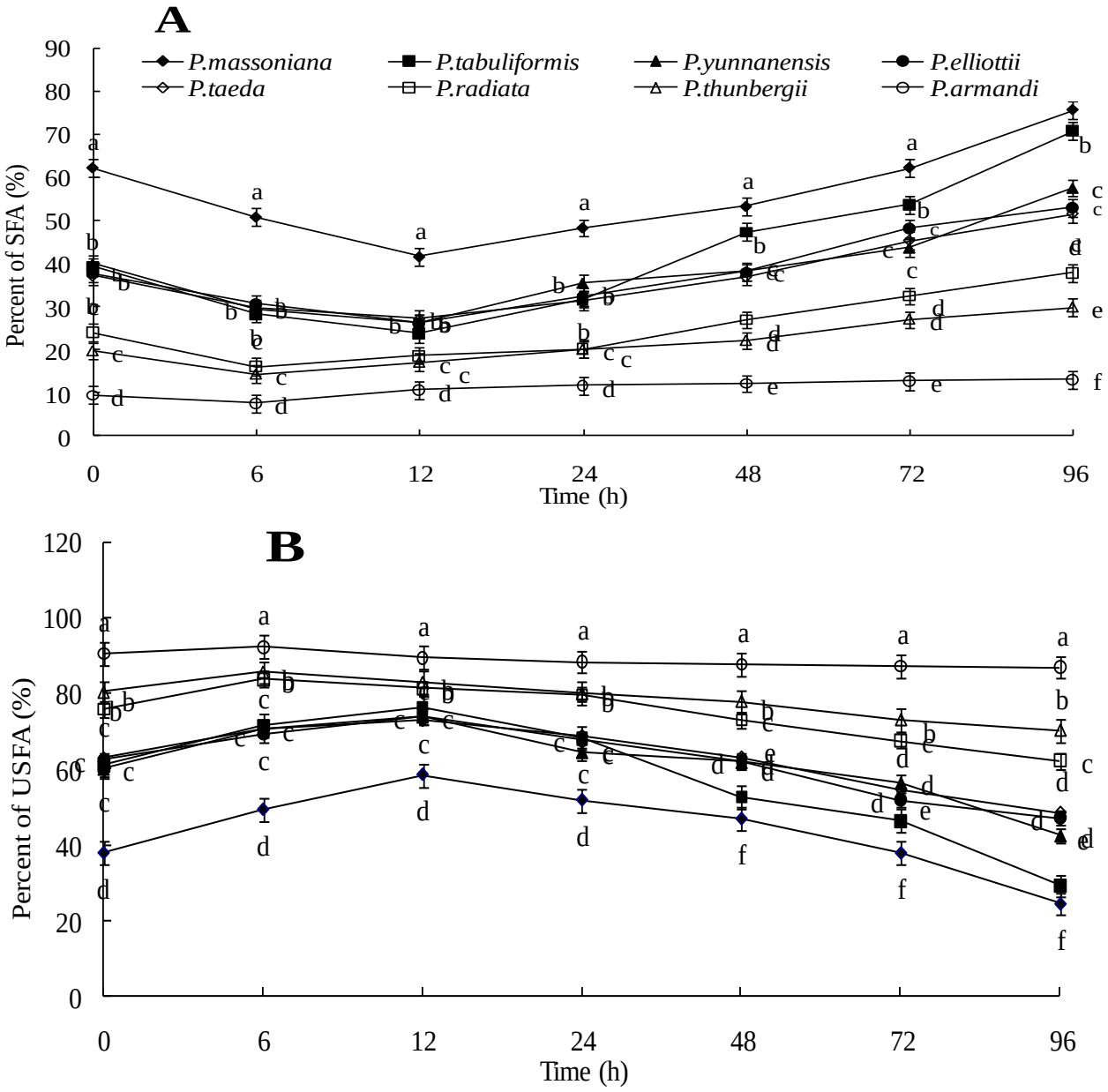

Figure 2. Percent saturated fatty acids (SFA) (a), and unsaturated fatty acids (USFA) (b), in the plasmalemma of pine needles treated with the Pf-toxin. The content of SFA was the sum of C16:0 and C18:0 content; the content of USFA was the sum of C18:1, C18:2 and C18:3 content. Percent SFA (%) = SFA 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.

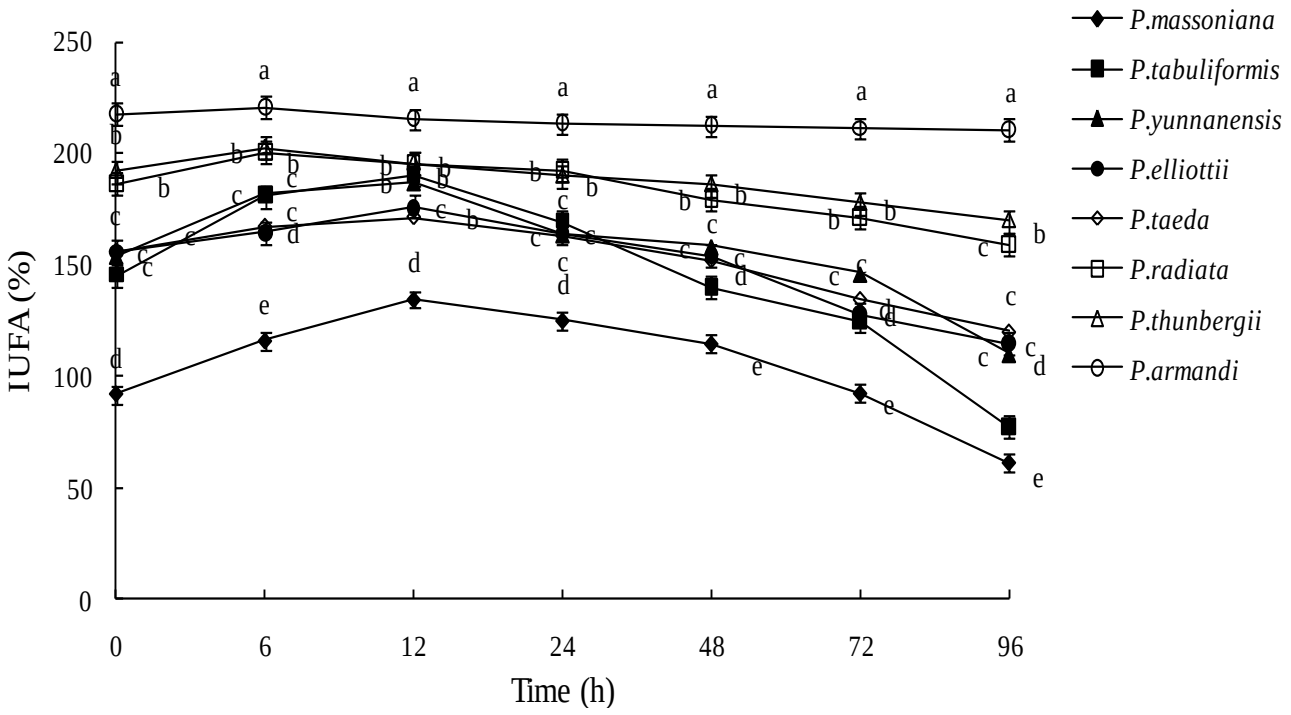

Figure 3. Index of unsaturated fatty acids (IUFA) in the plasmalemma of pine needles treated with the Pf-toxin. IUFA (%) =1×C18:1(%) + 2×C18:2(%) + 3×C18:3(%). Data in the same column followed by different lowercase letters indicate significant differences by the LSD test ($P < 0.05$, $n = 5$). LSD,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

Table 2. Malondialdehyde (MDA) content (μmol·g^{-1}FW) in the plasmalemma of pine needles.

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

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 (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 O_2^- startup. Similarly, in this study, the rate of O_2^- production increased with increased toxin treatments in eight species of pines in varying degrees. Moreover,

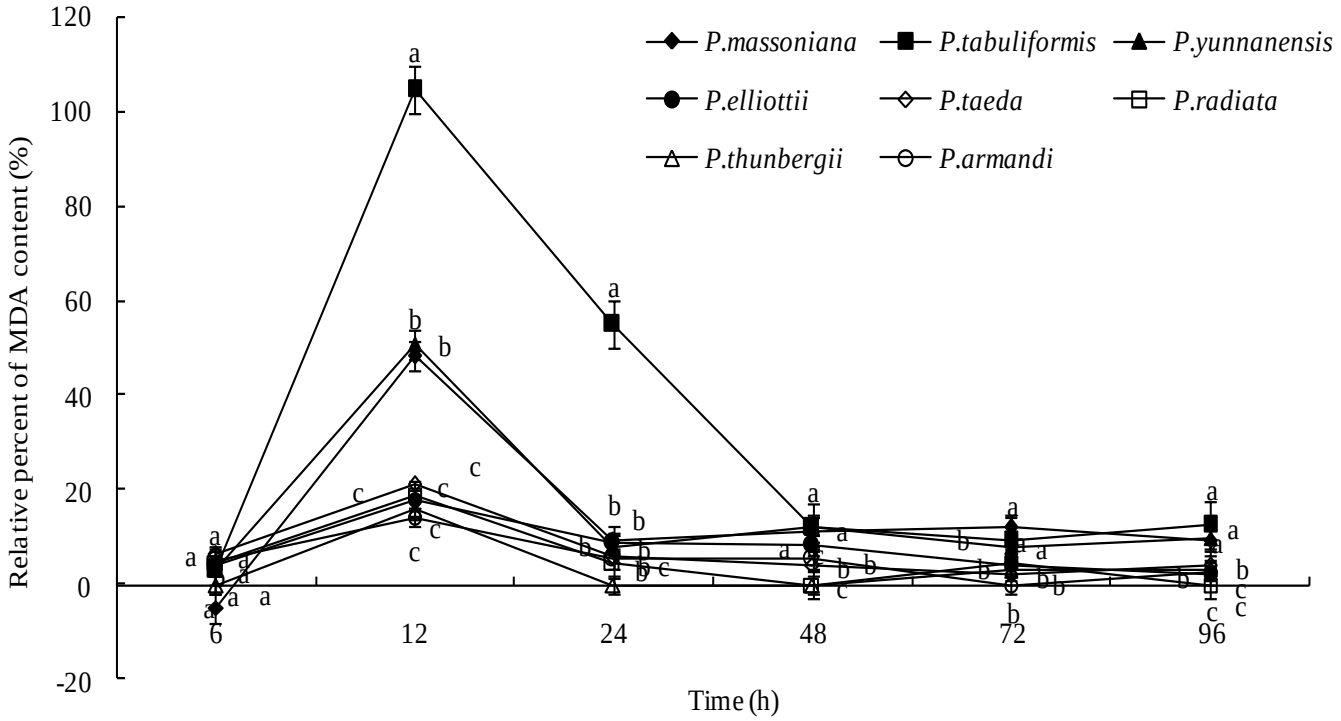

Figure 4. MDA content (relative percent) in pine needles. MDA content (relative percent, %) = [(MDA content in the toxin-treated group – MDA content in the control) / MDA content in the control] × 100%. Data in the same column followed by different lowercase letters indicate significant differences by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

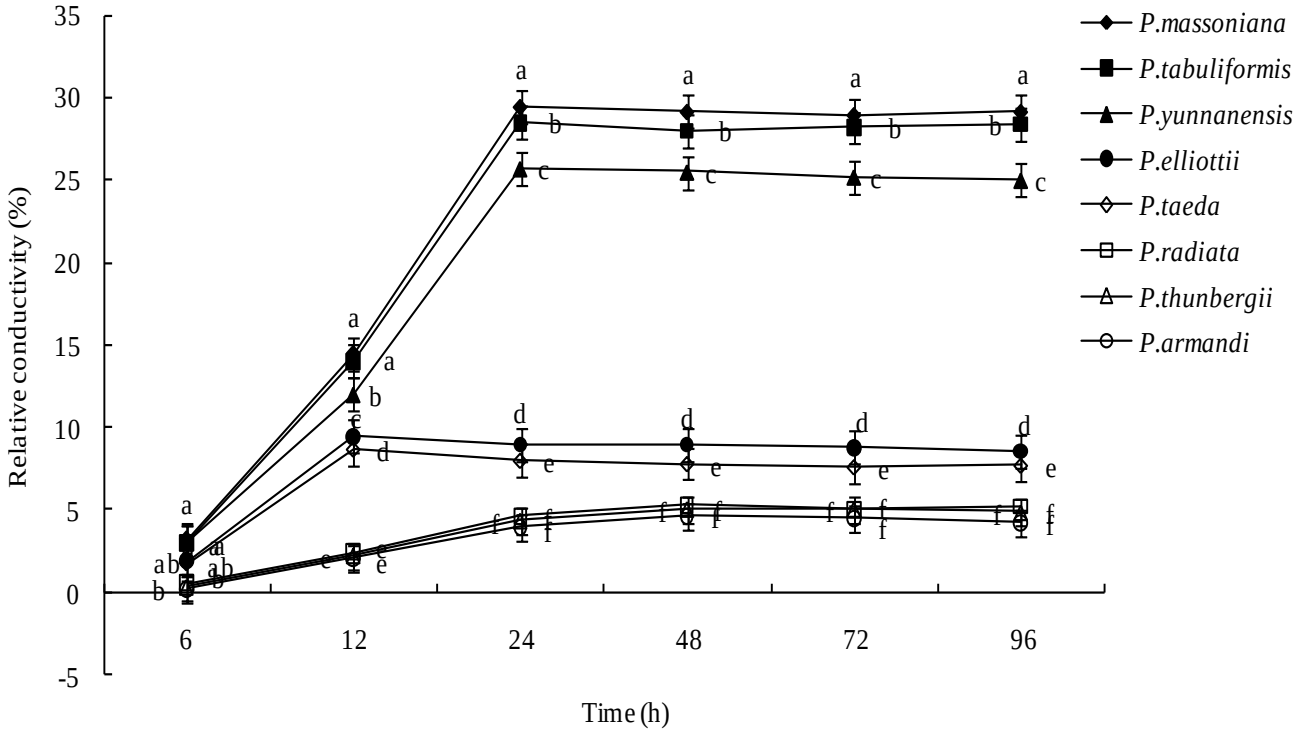

Figure 5. Relative conductivities in pine needles. Relative conductivity (%) = $(E_1-E_2)/E_2 \times 100$; E_1, the initial conductivity with the needle sample treating by toxin; E_2, the initial conductivity with the needle samples treating by sterile distilled water. Data in the same column followed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

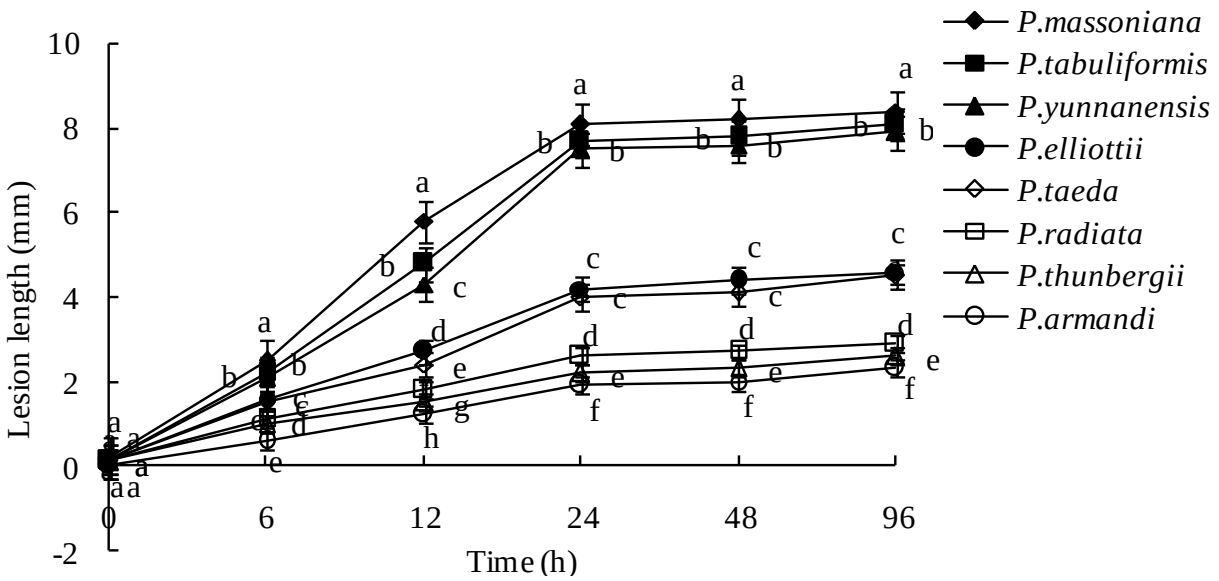

Figure 6. Lesion lengths of pine needles. Lesion lengths (mm) in the pine needles were measured after 0, 6, 12, 24, 48, and 96 h of toxin treatment. Data in the same column followed by different lowercase letters indicate significant differences by LSD test (P<0.05, n=5). LSD, Least significant difference.

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

Test	Species							
	P. massoniana	***P. tabuliformis***	***P. yunnanensis***	***P. elliottii***	***P. taeda***	***P. radiata***	***P. thunbergii***	***P. armandi***
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. elliottii* (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_2^- 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, elliottii*, *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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REFERENCES

Antunes MDC, Sfakiotakis EM (2008). Changes in fatty acid composition and electrolyte leakage of 'Hayward' kiwifruit during storage at different temperatures. Food Chem. 110: 891-896.

Cahill DA (1996). A quantitative bioassay for necrosis toxin from *Phytophthora cinnamomi* based on electrolyte leakage. Phytopathology, 86: 1360-1363.

Damann KE, Gardner JR, Scheffer RP (1974). An assay for *Helminthosporium victoriae* toxin based on induced leakage of electrolytes from oat tissue. Phytopathology, 64: 652-654.

Dubery IA, Smit F (1994). Phenylalanine ammonia-lyase from cotton (*Gossypium hirsutum*) hypocotyls: properties of the enzyme induced by a *Verticillium dahliae* phytotoxin. Biochem. Biophys. Acta. 1207: 24-30.

Elstner EF, Heupel A (1976). Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. Anal. Biochem. 70: 616-620.

Gechev TS, Breusegem FV, Stone JM, Denev I, Laloi C (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays, 28: 1091-1101.

Guo XM, Chen YF, Cao TW (2005). The effect of *Fusarium graminearum* crude toxin on membrane permeability of wheat varieties. J. Plant Genet. Resour. 6: 186-190.

Hartung W (1987). Effect of phytotoxins tension, HM T-toxin and HV-toxin on K+ efflux from unilamellar liposome. Plant Sci. 49: 9-13.

Heath RL, Packer L (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stochiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125: 189-198.

Heitefuss R, Williamas PH (Zhu et al. translation) (1991). Plant Pathology and Physiology. Beijing, China, China Agriculture Press.

Hidalgo OB, Matos AP, Cabral RS, Tussel1 RT, Arzola M, Santos R, Pérez MC (1998). Phytotoxic effect of culture filtrate from *Fusarium subglutinans* the causal agent of fusariose of pineapple (*Ananas comosus* (L.) Merr. Euphytica, 104: 73-77.

Ho SH, Koh L, Ma Y, Huang Y, Sim KY (1996). The oil of garlic, *Allium sativum* L. (Amaryllidaceae), as a potential grain protectant against Tribolium castaneum (Herbst) and *Sitophilus zeamais* Motsch. Postharvest Biol. Tech. 9: 41-48.

Holden JH (1984). *Helminthosporium maydis* T toxin increased membrance permeability to Ca2+ in susceptible corn mitochondria. Plant Physiol. 75: p. 225.

Huang Q, He JS (2000). Identification and biological characteristics of the pathogen of needle blight on *Pinus tabuliformis*. J. Sichuan For. Technol. 21: 28-30.

Jeewon R, Liew ECY, Hyde KD (2004). Phylogenetic evaluation of species nomenclature of Pestalotiopsis in relation to host association. Fungal Divers. 17: 39-55.

Jiang P, Wu XQ, Ye JR, Sheng MJ (2007). Screening of antagonistic microbes to two kinds of pine tree pathogens. J. Nanjing Forest. Univers. (Nat. Sci. Edit.) 31: 59-62.

Jump DB (2004). Fatty acid regulation of gene transcription. Crit. Rev. Clin. Lab. Sci. 41: 41-78.

Kasamo K, Kaglta F, Yamanishi H, Sakaki T (1992). Low temperature induced changes in the thermotmpic properties and fatty acid composition of the plasma membrane and tonoplest of cultured rice (*Oryza sativa* L.) cells. Plant Cell Physiol. 33: 609-616.

Keppler LD, Novaeky A (1987). The initiation of membrane lipid peroxidation during baeter-induced hypersensitive reation. Physiol. Plant Pathol. 30: 233-245.

Liang QX, Pan FY, Li DX (2002). Regularity of outbreak and control techniques of *Pinus massoniana* cercospora needle blight. J. Zhejiang Forest. Sci. Technol. 22: 64-65, 83.

Liu HM, Xin YQ, Lv GE, Zhu YT, Yan YQ (2011). Changes of membrane fatty acid in 2 species *Spiraea* L. under low-temperature and de-acclimation conditions. J. China Agric. Univ. 16: 52-57.

Lu T, Xie SY, Li BJ, Zhong ZW, Lin SC (2004). Evaluation on disease resistance and productivity of new sweet potato varieties with resistance to *Ralstonia solanacearum* and *Fusarium oxysporum*. Acta Agric. Jiangxi. 16(2): 33-37.

Luo MJ, Zhu TH (2002). Study on the property of crude extract of the toxin produced by *Pestalotia funereal*. J. Sichuan For. Technol. 23: 17-21.

Ma C, Dong WQ, Zheng CX (2010). Study on seasonal changes of the composition of membrane fatty acids of *Pinus tabulaeformis* Carr. J. Anhui Agric. Sci. 38: 10303-10305.

Manning VA, Ciuffetti LM (2005). Localization of Ptr ToxA produced by

Pyrenophora tritici-repentis reveals protein import into wheat mesophyll cells. Plant Cell. 17: 3203-3212.
Mehdy MC (1994). Active oxygen secies in pant dfense against pthogens. Plant Physiol. 105: 467-472.
Pan JZ, Feng T, Liu KZ (2010). Integrated control of the main diseases and pests on *Pinus tabuliformis*. Anhui Agric. Sci. Bull. 16: 200-201.
Park P, Ohno T, Nishimura S (1987). Leakage of sodium ions from plasma membrane modification, associated with permeability, in host cells treated with a host-specific toxin from a Japanese pear pathotype of Alternaria alternate. Can. J. Bot. 65: 330-339.
Pedneault K, Angers P, Avis TJ, Gosselin A, Tweddell RJ (2007). Fatty acid profiles of polar and non-polar lipids of *Pleurotus ostreatus* and *P. cornucopiae* var. 'citrino-pileatus' grown at different temperatures. Mycol. Res. 110: 1228-1234.
Potrich C, Bastiani H, Colin DA, Huck S, Prévost G, Serra MD (2009). The influence of membrane lipids in *Staphylococcus aureus* Gamma-Hemolysins pore formation. J. Membr. Biol. 227: 13-24.
Qiao TM, Zhu TH, Li FL (2006). Study on the biological control of pine needle blast with *Gliocladium virens* and *Bacillus firmus*. For. Sci. Technol. 31: 28-31.
Qiu DX, Tang SB, Wu JC (1980). A preliminary study of Pestalotia needle blight on *Pinus massoniana*. Sci. Silv. Sin. 16: 203-207.
Rasmussen JB, Kwon CY, Meinhardt SW (2004). Requirement of host signaling mechanisms for the action of Ptr ToxA in wheat. Eur. J. Plant Pathol. 110: 333-335.
Shah J (2005). Lipids, lipases, and lipid-modifying enzymes in plant disease resistance. Annu. Rev. Phytopathol. 43: 229-260.
Shimizu N, Hosogi N, Hyon GS (2006). Reactive oxygen species (ROS) generation and ROS-induced lipid peroxidation are associated with plasma membrane modifications in host cells in response to AK-toxin I from *Alternaria alternata* Japanese pear pathotype. J. Gen. Plant Pathol. 72: 6-15.
Su WA, Wang WY, Li JS (1980). The analytical technique of plant fat and fatty acids. Plant Physiol. Commun. 3: 54-60. (in Chinese)
Sutarman, Hadi S, Saefuddin A, Achmad, Suryani A (2004). Epidemiology of needle blight on Pinus merkusii seedlings incited by Pestalotia theae. J. Manaj. Hut. Trop. 1: 43-60.
Van der Vusse GJ, Glatz JFC, Stam HCG, Reneman RS (1992). Fatty acid nomeostasis in the normoxic and ischemic heart. Physiol. Rev. 72: 881-940.
Wang AG, Luo GH (1990). Quantitative relation between the reaction of hydroxylamine and superoxide anion radicals in plants. Plant Physiol. Communic. 6: 55-57.
Wang P, Zhang CJ, Chen GX, Wang J, Shi DW, Lv CG (2006). Effects of low temperature on lipid peroxidation and fatty acid composition of flag leaf in rice (*Oryza sativa* L.). Acta Agron. Sin. 32: 568-572.
Wu MS, Wei DF (1987). A preliminary study of occurrence law and control of Pestalotia needle blight on *Pinus taeda* and *Pinus elliottii.* Guizhou Forest. Sci. Technol. 1: 70-74.
Yang B, Ye JR, Bao H (2000). Studies on toxins of tree pathogens. For. Res. 3: 317-322.
Yang YL, Xiao LT, Hu XQ (2011). Study on the relationship between the toxin of *Phytophthora infestans* (Mont.) de Bary and resistance of potato. Agric. Sci. China, 10(2): 238-245.
Ye JR, Qiao GF, Bao H, Feng WZ (2000). Studied on the role machinisms of the brown spot needle blight fungus toxin to make host cell damages. Sci. Silv. Sin. 36: 82-86.
Yuan JY, Hou XL, Zhang CW, Ye F (2007). Active oxygen metabolism in the floral buds and leaves of the new cytoplasm male sterile (CMS) line and its maintainer line of non-heading Chinese cabbage. Front. Agric. China, 1: 47-51.
Zhang YL, Zhang M, Li F, Wang XF (2008). Programmed cell death of Ulmus pumila L. seeds during aging. Front. Forest. China, 3: 357-363.
Zhao SL, He PX (1993). Biology of pine red blight pathogen Pestalotiopsis funerea. Acta Phytopathol. Sin. 23: 41-47. (in Chinese)
Zhen XH, Li YZ (2004). Ultrastructural changes and location of β-1,3-glucanase in resistant and susceptible cotton callus cells in response to treatment with toxin of *Verticillium dahliae* and salicylic acid. J. Plant Physiol. 161: 1367-1377.
Zheng LL, Zhu TH (2006). Influence of cultivating conditions on toxin produced by *Pestalotiopsis funerea* Desm. J. Beijing For. Univ. 28: 115-118.
Zhu TH, Ye HZ, Luo MJ (2003). Isolation and purification of Pf-toxin *Pestalotiopsis funereal*. Acta Phytopathol. Sin. 33: 541-545.
Zhu TH, Luo MJ, Ye HZ (2005). The chemical composition of Pf-toxin from Pestalotiopsis funereal Istructure of pathogenic material-I. Mycosyst. 24: 112-115.