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ORIGINAL ARTICLE

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Rapid changes in induced non-volatile secondary metabolites in damaged *Pinus massoniana* Lamb.

Abstract Plants initiate the development of defense mechanisms as soon as pests start to cause damage to them. In order to have a thorough understanding of the physiological mechanisms of the *Pinus massoniana* self-defense mechanism, and to provide a theoretical foundation for an effective ecological management of this plant, levels of tannin, polyamine and phenolic acids were analyzed in undamaged (UDL), insect-damaged (IDL) and artificially-damaged (ADL) leaves at different times. Results show that, although the content of tannin significantly increased in IDL and ADL compared to UDL, its peaks appeared earlier in ADL than in IDL treatment. Tannin concentration substantially increased again 48 h after IDL treatment. On the other hand, the damage mode considerably affected putrescine and spermidine levels in leaves. Their concentrations in IDL plants remained higher than in UDL after a relatively long time (72 h), but spermine was barely detected in any of the samples. In general, total content of phenol acids significantly increased in damaged leaf treatments (ADL and IDL), with a higher level in IDL for most of the investigated phenolic acids, except for ferulic acid. Our study showed that, when damaged by insects, *Pinus massoniana* rapidly produces substances required in resistance induction to insects in order to insure its self-protection.

Keywords induced damage, non-volatile secondary metabolites, *Pinus massoniana*, rapid change

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1 Introduction

One of the most efficient defense strategies developed by plants against herbivorous insects is the induction of resistance to these insects. This phenomenon implies a mobilization of physiological, biochemical and morphological characteristics from plants. Among these characteristics, the induction of change in the contents of some non-volatile chemicals such as tannin, phenolic acids and polyamine appears to be the most important tool used in the defense mechanism of plants (Cai et al., 2003; Armengaud et al., 2004). Many studies over the last few years have demonstrated that the reaction of plants after damage by pests is quick, disjunctive, and flexible. The amount of some chemicals involved in the resistance to insects, such as tannin and phenolic acids, clearly increase, indicating that the reaction of plants can vary over time.

Dendrolimus punctatus Walker is a well-known pest of *Pinus massoniana* Lamb., reproducing on tree species in southern China. Its occurrence is cyclical. Ge et al. (1997) have reported that the damage to *P. massoniana* could induce pest-resistance and affect the growth and development of *D. punctatus*. Studies on pest resistance induction in *Pinus tabulaeformis* Carr. and its effect on the ingestion, survival rate and spawning amount of *Dendrolimus spectabilis* Butler have been conducted by Li et al. (2000). Wang et al. (2001) have analyzed the content of secondary metabolites and the variation of needles of *P. massoniana* using an artificial analogue-damaging test. Results show that the reaction of this tree after damage was a rapid, disjunctive and adjusted process. However, little information has been reported about systemic reactions of non-volatiles after damage by herbivores.

In order to understand thoroughly the physiological mechanisms of *P. massoniana* reactions and to provide a theoretical foundation for effective ecological management of this tree, we analyzed the changes of induced secondary metabolites such as tannin, phenols and polyamine over time after damage to *P. massoniana*.

2 Materials and methods

2.1 Experimental materials

Pinus massoniana trees grow in the mountainous area of the Jiangxi Agricultural University reservoir. The area is hilly. Larvae of *Dendrolimus punctatus* (at the third developmental stage) were provided by the Jiangxi Agricultural University (Jiangxi, China).

2.2 Coniferous leaf treatments

Three leaf treatments were used on three different (8-year-old and healthy) pine trees, and consisted of (1) insect-damaged leaves (IDL), (2) artificially-damaged leaves (ADL) and (3) undamaged (UDL) leaves. In the first treatment, leaves from two selected (8^{th}) lateral branches of the tree were subjected to an attack of *D. punctatus* larvae for 48 h, resulting in 25%–40% leaf damage, based on data from our previous studies (Ren et al., 2006). Larvae were applied to the branches using the net technique described by Li et al. (2002) and were immediately removed before harvesting the leaves. In the second treatment, leaves on the involved branches were manually cut with scissors, simulating the degree of leaf damage caused by the insects. Leaves from the third treatment were not damaged and were used as controls. The leaves involved were then separately and randomly sampled (3 g per sample) from trees after 1, 2, 4, 8, 24, 48 and 72 h, directly fixed in liquid nitrogen, and preserved at −80°C until further analyses.

2.3 Analytical methods

The quantification of phenolic acids was carried out according to published procedures (Peleg et al., 1991). Seven substances were detected using a HPLC (Agilent Company, USA) system with a ZORBAX Eclipse × DB-C_{18}, Analytical 4.6 × 250 mm 5-Micron. Column temperature was maintained at room temperature with a flow of 0.7 mL/min of a gradient programmed from 15/85 (0 min hold) to 100/0 (40 min hold), and a mixture of methanol (1.5%) and acetic acid in water. Detected length: 272 nm. The results were analyzed with ChromQuest software. The tannin content was estimated using a volumetric method according to a revised method by Terrill et al. (1992). Polyamine was analyzed by HPLC according to Liu J and Liu Y L (2004). 1,6-hexanediamine was added as an internal standard. Column: ZORBAX Eclipse × DB-C_{18}, Analytical 4.6 × 250 mm 5-Micron. Detected length: 230 nm. The flow rate was 0.5 mL/min and the gradient programmed from 30/37 (0 min hold) to 46/60 (40 min hold) to 100/0 (60 min hold), a mixture of acetonitrile and 1.5% acetic acid in water.

Samples in all analyses were replicated three times. Statistical analyses were performed using SPSS 10.0 (SPSS, Chicago, IL, USA).

3 Results

3.1 Rapid change in tannin content of *Pinus massoniana* needles

Compared to UDL, results showed a net increase in tannin content of *P. massoniana* needles over time after damage (IDL and ADL). The peak tannin levels of IDL appeared later than that of ADL (Fig. 1). The highest tannin content of ADL occurred at 1 h, then gradually decreased and reached its lowest level at 72 h. On the other hand, the tannin content of IDL was highest after 8 h, slowly decreasing until 48 h, before increasing again up to 72 h. The tannin content in the IDL of the conifer after 72 h was still higher than that in UDL. An F-test showed a significant difference ($p < 0.05$) among the three treatments, indicating their considerable effect on the tannin content in needles.

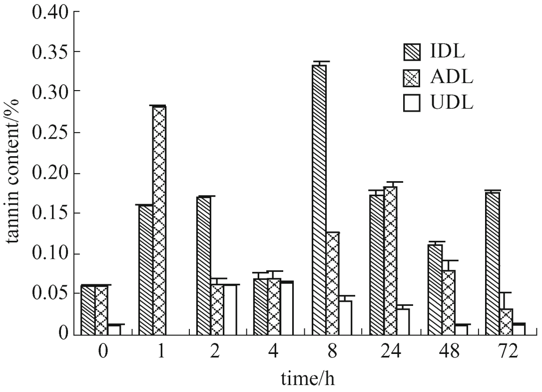

Fig. 1 Tannin content in needles of *P. massoniana*

3.2 Variation of polyamine in *P. massoniana* needles

Figure 2 shows that the putrescine level in needles with ADL treatment had its highest recorded value after 2 h, and the lowest value after 72 h compared to UDL. In IDL-treated plants, putrescine peaked after 4 h and remained higher than in UDL plants after 72 h. Spermidine content of both ADL and IDL increased markedly after 1 h, and was higher than in UDL at 72 h. An *F*-test showed a significant difference among the treatments in the putrescine content, but not in the content of spermidine ($p < 0.05$). Spermine was barely detected in any of the samples.

3.3 Rapid change of phenolic acid levels in *Pinus massoniana* needles over time.

The results presented in Table 1 show that the total phenolic acid content of *P. massoniana* needles in both ADL and IDL treatments were higher than in UDL at all sampling times. IDL displayed a significant increase in gallic

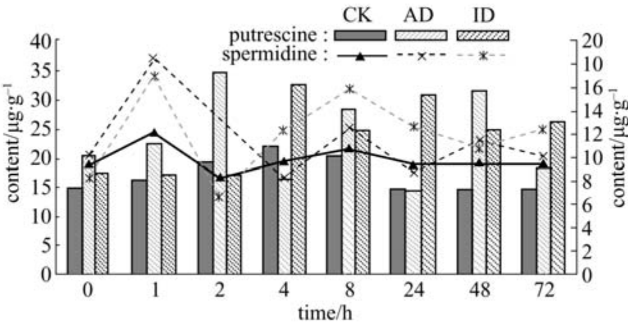

Fig. 2 Putrescine and spermidine content in needles of *P. massoniana*

acid, pyrocatechol, p-hydroxybenzoic acid, caffeic acid, ferulic acid and benzoic acid. At 72 h, phenolic acids in the IDL treatment were higher than in UDL except for ferulic acid, although they decreased over time. The results showed that changes in some non-volatile plant-resistant materials against insects were rapidly induced just after *P. massoniana* was damaged (Fig. 3). Results from *F*-tests showed a significant difference ($p < 0.05$) among the contents of gallic acid, pyrocatechol, p-hydroxybenzoic acid, caffeic acid and benzoic acid at the different treatments, but not for ferulic acid ($p > 0.05$). This indicates that different ways of damage had a significant effect on the content of the first five cited phenolic acids in *P. massoniana* needles.

Table 1 Change in total phenolic acid content in needles of *P. massoniana* (unit: $\mu g \cdot g^{-1}$)

time/h	treatment		
	UDL	IDL	ADL
0	20.70	23.97	28.90
1	22.41	42.18	136.51
2	48.56	228.87	165.40
4	38.86	267.29	91.53
8	30.95	206.90	51.16
24	36.20	91.28	143.29
48	20.70	336.36	145.92
72	20.70	143.22	103.46

4 Discussion

Tannin is one of the substances that mediate the defense mechanism of plants. Aromatic and hydroxyl groups of tannin molecules can combine with carboxyl groups of proteins to form stable crossing chains, which can inhibit the activity not only of complex proteins, but also of complex starches to cause herbivorous insect malnutrition. Further, tannin is toxic to some herbivorous insects and may destroy the intestinal cells of some insect larvae. Tannin can also combine and precipitate proteins in saliva, rendering plant texture coarse and bitter. All these chemical features result in the reduction of food consumption by plant-damaging insects. The adverse effect of tannin on insects consists mainly of disturbing the insects' nutritional system, such as food digestion in the intestines. Wang (1993) reported that the tannin content in *Solanum melongena* increased rapidly after the plant was damaged by *T. cinnabarinus*. The population of *T. cinnabarinus* decreased when the tannin content increased to a threshold value. In their research, Li et al. (1999) found that the tannin content in *P. tabulaeformis* needles after damage increased by 22.6% compared to that of UDL and that in 2-year old *P. massoniana* needles increased by 92.8%. The present study has revealed a net increase in tannin content in needles of *P. massoniana* over time after damage (IDL and ADL). Compared to UDL treatment, the highest tannin content in ADL occurred earlier than that in IDL, and increased again up to 72 h after damage, indicating that the tannin in *P. massoniana* could still inhibit pests by delaying induced resistance.

Polyamines have strong physiological activity. Increasingly, attention is being paid to their functions in the growth, development, ageing process and hardiness resistance of higher plants. However, it has been shown that some stress (such as moisture content, acidity, salinity, ions, low temperature, etc) highly stimulate plants' synthetic activity and the accumulation of polyamines, probably due to cytoplasm acidification. Currently, reports about the relationship between polyamines and plant resistance to insects have been few. Most polyamines have the effect of repelling insects (Yan, 2001). We have detected putrescine, spermidine and spermine in needles of ADL and IDL treatments. Putrescine showed a significant difference among the treatments, but not spermidine, which was hardly detected in any of the samples.

Putrescine is the most important product in the biosynthetic pathway of polyamine. Its synthesis in plants under stress conditions implies depletion of the amino acid pool, leading to malnutrition of herbivorous insects. Some reports have pointed out the use of polyamine as an animal growth-promoting agent. Cheng (1998) stated that a low content of polyamine could promote animal growth, whereas a high content was toxic. Further studies on the relationship between polyamine and plant resistance to insects and the mechanisms involved are required.

On the other hand, phenolic acids are secondary compounds playing an important role in plant resistance to insects during plant growth. The induction of these substances has been found in damaged plants and they have an inhibitory effect on insects. The accumulation of phenolic acids in gingko has prevented this tree from *Plutella xylostella* attacks. Furthermore, phenolic acid content has a negative correlation with the number of herbivorous insects. After *P. massoniana* damage by *D. punctatus*, the total phenolic acid content increased in the needles (Ge et al., 1997). Compared to the control treatment, the growth and development of larvae, which ate damaged

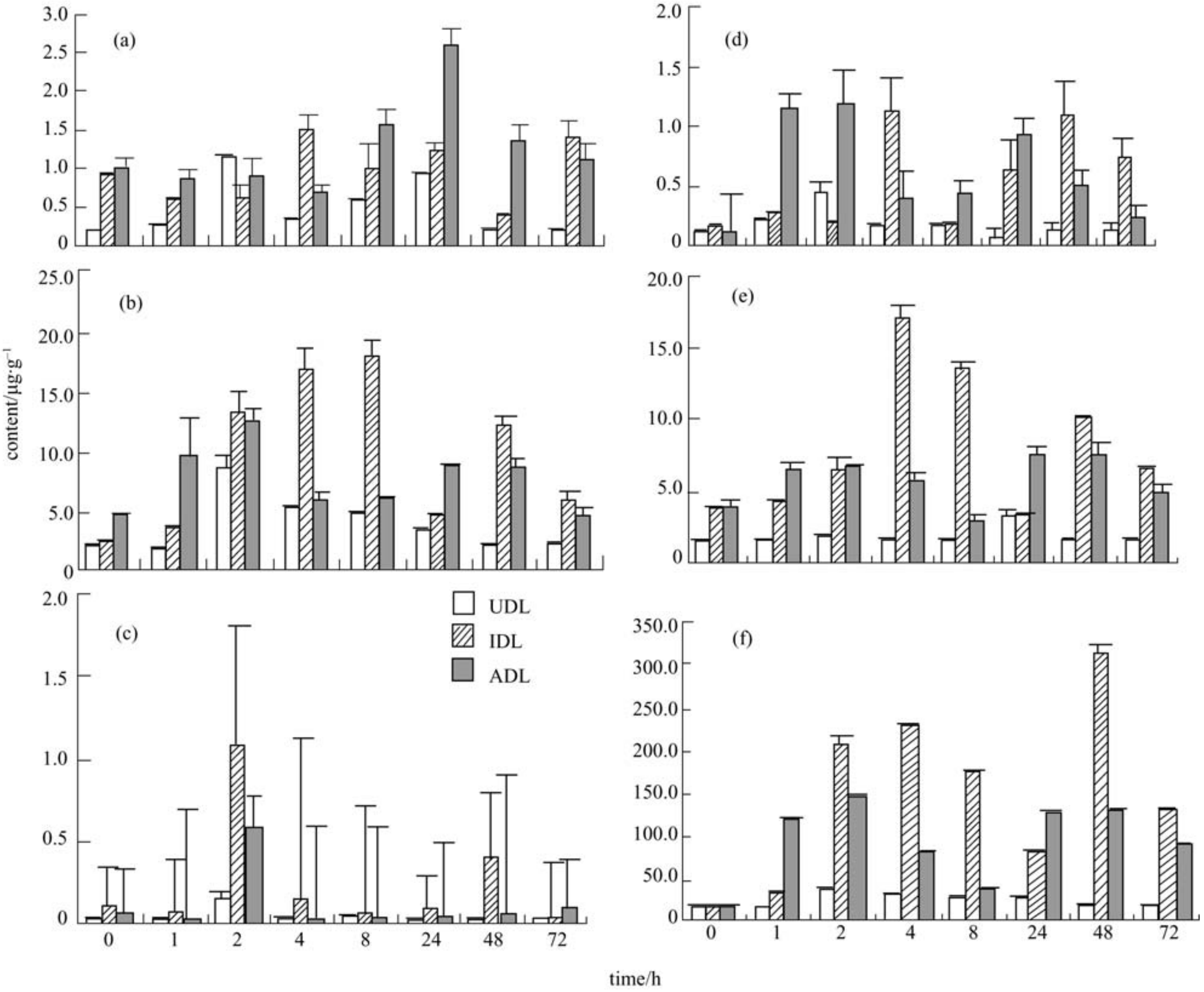

Fig. 3 Changes of total phenolic acids: gallic acid (a), p-hydroxybenzoic acid (b), ferulic acid (c), pyrocatechol (d), caffeic acid (e) and benzoic acid (f)

needles, were seriously inhibited. In addition, levels of four kinds of phenolic acids in needles of *P. sylvestris* var. *mongolica* Litv. and *P. tabulaeformis* were studied. Results showed that their levels in *P. sylvestris* var. *mongolica* were 0.58 times higher than that in *P. tabulaeformis*, and the caffeic acid level was 3.7 times higher. This is probably why *P. sylvestris* var. *mongolica* does not attract *D. spectabilis* Butler for its egg-laying (Li et al., 1999). When *Populus deltoides* leaves were exposed to MeJA or MeSA, the level of gallic acid, coumaric acid, caffeic acid, ferulic acid and benzoic acid increased. Gallic acid in leaves treated with MeJA peaked at 24 h, while a peak appeared after 12 d on leaves treated with MeSA (An et al., 2006).

The present trial screened out the content of gallic acid, pyrocatechol, p-hydroxybenzoic acid, caffeic acid, ferulic acid and benzoic acid. Until 72 h after treatment, the total phenolic acid content of *P. massoniana* needles in both ADL and IDL was significantly higher than in the control treatment (UDL) at all sampling times. IDL displayed a significant increase compared to the six different phenolic acids. The amounts of phenolic acids we extracted could properly induce the resistance of *P. massoniana* against insect attacks. Nevertheless, ferulic acid decreased over time, suggesting that synthesis from caffeic acid is small, or the precursor of lignin is produced more.

Plant defense induction in response to insects is a rather complex process (Karban and Baldwin, 1997). The induced resistance, which normally acts together with the constitutive resistance of a tree, is an energy-consuming process. Plants need a stable precursor from basic metabolism as well as a supply of enzymes and energy to form secondary compounds (Agrawal, 1998). Over its lifespan, a plant needs to ensure its growth and defense. Induced resistance is a way for plants to reduce resource distribution during its defense operation. Competition for resources occurs in various ways. It is impossible for a plant to consume much energy during defense processes

(Zhu et al., 2005). Instead, it should consume more energy during its growth and development. This is an economic strategy, feasible when the plant needs to engage its defense process. After plants are damaged by pests, the content of secondary chemicals fluctuates, first increasing and gradually decreasing. This indicates that the composition of secondary chemicals requires a lot of energy and materials, and that the synthesis of the primary metabolite can be affected. The reaction of plants is rapid, disconnected and flexible, but not sustained and controlled. It changes over time to emergency responses.

Induced resistance in plants shows a complex and flexible adaptation mechanism (Koricheva, 2002). Although much work has already been done, further investigation is needed on the status and coordinating functions of different chemicals in plants during a chemically-induced defense. Further research on the mechanism of rapidly induced resistance would lay the foundation for delaying induced resistance, which would have special significance and utility in studying the relationship between forest pests and forest trees.

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