ASSOCIATION BETWEEN ENZYME ACTIVITY LEVELS IN *EUCALYPTUS* CLONES AND THEIR SUSCEPTIBILITY TO THE GALL WASP, *LEPTOCYBE INVASA*, IN SOUTH CHINA

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CHANG RL, ARNOLD RJ & ZHOU XD. 2012. Association between enzyme activity levels in *Eucalyptus* clones and their susceptibility to the gall wasp, *Leptocybe invasa*, in South China. The activity levels of five defensive enzymes, namely, peroxidase (POD), polyphenol oxidase (PPO), superoxide dismutase (SOD), phenylalanine ammonia-lyase (PAL) and catalase (CAT) were assessed in shoots of four commercial *Eucalyptus* clones (DH201-2, DH32-29, U6, GL9) at ages of 13, 29 and 38 months. Levels were also assessed in healthy and damaged shoots of clone DH201-2 at 15 months, shortly after it was severely attacked by the shoot gall wasp *Leptocybe invasa*. Foliar levels of these five enzymes did not show any significant increase with age. The level of CAT was found to be significantly higher at ages 29 and 38 months in clone GL9, a clone relatively resistant to *L. invasa*, than in the three other clones. Clone DH201-2, which was relatively susceptible to *L. invasa*, had non-significantly lower CAT levels than the rest of the clones at all three ages examined. The levels of the other four enzymes showed no obvious differences between clones and overall activity levels of the five enzymes did not show any clear association with the qualitative ranking susceptibility of the clones to *L. invasa*. In 15-month-old DH201-2 trees, marked increases in the levels of CAT, SOD and PPO were observed in shoots attacked by *L. invasa* compared with shoots from healthy trees of the same age, while levels of PAL and POD decreased compared with those in shoots from healthy trees.

Keywords: Defence systems, insect attacks, pest resistance

CHANG RL, ARNOLD RJ & ZHOU XD. 2012. Hubungan antara aktiviti enzim klon *Eucalyptus* dan kerentanan terhadap serangan penyengat puru, *Leptocybe invasa*, di selatan China. Aras aktiviti lima enzim pertahanan iaitu peroksidase (POD), polifenol oksidase (PPO), superoksida dismutase (SOD), fenilalanin ammonia-liase (PAL) and katalase (CAT) dinilai dalam pucuk empat klon *Eucalyptus* komersial (DH201-2, DH32-29, U6, GL9) yang berusia 13 bulan, 29 bulan dan 38 bulan. Aras enzim turut dinilai dalam pucuk yang sihat serta yang rosak bagi klon DH201-2 yang berusia 15 bulan tidak lama selepas ia diserang penyengat puru, *Leptocybe invasa*. Aras kelima-lima enzim daun tidak menunjukkan peningkatan signifikan dengan usia. Aras CAT lebih tinggi pada klon GL9 yang berusia 29 and 38 bulan. Klon GL9 secara relatifnya lebih rintang terhadap *L. invasa* berbanding ketiga-tiga klon yang lain. Klon DH201-2 yang agak rentan terhadap *L. invasa* mempunyai kandungan CAT yang lebih rendah berbanding klon lain yang dikaji. Aras enzim empat lagi enzim tidak menunjukkan perbezaan ketara antara klon. Secara keseluruhannya, aras aktiviti kelima-lima enzim tidak menunjukkan sebarang hubungan yang jelas antara tahap kerentanan kualitatif klon terhadap *L. invasa*. Pokok DH201-2 yang berusia 15 bulan menunjukkan peningkatan ketara dalam aras CAT, SOD dan PPO bagi pucuk yang diserang *L. invasa* berbanding pucuk pokok sihat yang sama usianya. Aras PAL and POD pula menurun berbanding pucuk pokok sihat.

INTRODUCTION

The group of trees commonly known as eucalypts now includes three genera, namely, *Angophora*, *Corymbia* and *Eucalyptus* within the family Myrtaceae (Hill & Johnson 1995, Brooker 2000). Within this group there are about 950 tree species and these dominate landscapes throughout much of Australia (Wang 2010).

To date, more than 20 mil ha of eucalypt plantations have been established worldwide, with the largest areas being located in Brazil, China, India, South Africa and Australia. Eucalypts are of great commercial importance in these countries and are used as sources of construction materials, fibre and fuelwood.

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In many places, eucalypts are also used in plantings for environmental protection including as shelterbelts and windbreaks surrounding cultivated and residential areas (Mendel et al. 2004). In southern provinces of China such as Guangdong, Guangxi, Hainan, Fujian and Yunnan, more than 3 mil ha of plantations of these species have been established to meet some of the raw material needs of the country's rapidly growing economy.

Pests threaten the sustainability and economic viability of eucalypt plantations. In 1980, only 53 insect pest species were recorded in eucalypt plantations in China but by 1991, this number had increased to more than 206 (Pang 2001). By 2001 there were at least 282 insect pests on eucalypts in China and these represented 59 families and 10 orders (Pang 2001). Since then, there have been further rapid increases in the number of insect pests on eucalypts in this country. By 2009, 106 species representing 63 families and 16 orders had been recorded from eucalypt plantations in Fujian alone (Yao 2009).

A shoot gall wasp, *Leptocybe invasa*, was discovered on eucalypts in the Middle East and Mediterranean regions in 2000 (Mendel et al. 2004). It caused severe injury to *Eucalyptus camaldulensis* (red river gum) and a range of other eucalypt species by inducing galls on rapidly growing shoots. It was first found in mainland China in Guangxi Zhuang Autonomous Region in 2007 (Tang et al. 2008). Soon after, it was also recorded in some neighbouring provinces, including Hainan and Guangdong (Chang & Zhou 2010). Results of surveys indicated that a common Chinese commercial eucalypt clone, DH201-2, was particularly susceptible to *L. invasa* and often sustained serious damage while two other common Chinese clones, DH32-29 and GL9, appeared relatively resistant.

It is known that defence systems of plants to insect attacks can be activated by prior insect attack (Gomes et al. 2005). One type of compound known to be associated with such plant responses are peroxidases (POD). These exist as isoenzymes with diverse expression profiles (Rajeswari & Paliwal 2008) and are known to participate in various physiological processes (Kawano 2003). They are related to lignification, suberisation and auxin catabolism—all of which increase the hardness of tissues—and to the production of quinones and active oxygen, which possess antibiotic properties (Hiraga et al. 2001, Ralph et al. 2004).

Polyphenol oxidase (PPO) is responsible for the oxidative catalysis of phenols to quinones, which become complexed with proteins, thus decreasing the nutritional quality of food, making protein digestion difficult (Mohammadi & Kazemi 2002). Another such enzyme, catalase (CAT), is a tetrameric heme-containing enzyme that catalyses the dissociation of hydrogen peroxide to oxygen and water. It plays a central role in protecting cells from the toxic effects of activated oxygen (Halliwell 1974). Another enzyme, phenylalanine ammonia-lyase (PAL), is related to the synthesis of phenolic compounds which are known to have deterrent, toxic and antinutritional properties to some insect pests (Appel 1993). Yet another enzyme, superoxide dismutase (SOD), catalyses the destruction of O^{2-} free radicals. By doing so, the enzyme helps to protect oxygen-metabolising cells against harmful effects of superoxide free radicals (Petkau et al. 1975).

In order to examine possible causes of the differences in resistance and/or susceptibility to *L. invasa* among some of the current Chinese commercial eucalypt clones, the activity levels of the above five enzymes, reputed to have defensive roles against predation by insects such as *L. invasa*, were assessed in a group of four of the more widely planted commercial eucalypt clones in south China.

MATERIALS AND METHODS

Materials

The four clones selected for this study were DH201-2 (*E. grandis* × *E. camaldulensis*), DH32-29 (*E. urophylla* × *E. grandis*), U6 (*E. urophylla* × *E. tereticornis*) and GL9 (*E. grandis* × *E. urophylla*). According to our observation, these four clones had a range of susceptibility to *L. invasa*. Clone DH201-2 was particularly susceptible and often sustained serious damage, U6 was often attacked in the nursery and considered relatively susceptible though less so than DH201-2 while DH32-29 and GL9 so far seemed to be relatively resistant.

One nursery and two clonal field trials were selected to provide samples of appropriate ages from these target clones for this study. The nursery and one of the field trials were located in the South China Nursery and Experimental Field Station near Zhanjiang in the far west of Guangdong province (21° 16' N, 110° 6' E, 92 m above sea level (asl), mean annual rainfall 1567 mm, mean

annual temperature 23.1 °C). Samples from the nursery and one of the field trials were 13 and 29 months old respectively. The second field trial, which provided 38-month-old trees, was located in Shiling Forest Farm, also located in the far west of Guangdong province (21° 37' N, 110° 6' E, 41 m asl, mean annual rainfall 1644 mm, mean annual temperature 22.9 °C).

As *L. invasa* mainly infests rapidly growing shoots of eucalypts, young shoots (comprising shoots and young leaves) were collected from the uppermost parts of the crowns from five trees of each of the four eucalypt clones from each of the three ages sampled in this study. Trees sampled from each clone at each age were selected to ensure they showed no sign of attack from *L. invasa* or other insect pests.

In order to compare enzyme levels between healthy trees (i.e. pest free) and those attacked by *L. invasa*, five 13-month-old DH201-2 trees from the nursery (that had not been sampled) were placed in insect-proof cages and kept along side another five trees of the same clone (that also had not been sampled) but that were left unprotected. Within the space of only two months, an outbreak of *L. invasa* occurred at the nursery—fortuitous for this study but not so for general propagation of quality eucalypt tree stocks. By the age of 15 months the five unprotected trees of this clone showed symptoms of cork tissue in the shoots at points where eggs of *L. invasa* insects had been inserted. Subsequently, samples were collected from five healthy and five attacked trees.

After collection, shoot samples were cut into very small pieces (≤ 4.0 mm) and the five samples from each five-tree group (clone–age combination) were mixed together into one composite sample. Each of these five-tree composite samples were treated with liquid nitrogen and then stored in an ultra low temperature refrigerator (-40 °C).

Methods

CAT extraction and activity assay

A 0.4 g lot was measured from each five-tree composite shoot sample. This was homogenised in 2.0 ml of 50 mM cold phosphate buffer (pH 7.0), containing 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was then collected and stored at -20 °C.

CAT activity was determined according to Hao et al. (2004). The reaction mixture contained 1.5 ml phosphate buffer (pH 7.0), 1.0 ml distilled water, 20 µl of the supernatant and 0.3 ml H_2O_2. Changes in the absorbance at 240 nm were measured for 4 min.

PPO extraction and activity assay

A 0.4 g lot was also measured from each five-tree composite shoot sample and homogenised in 2.0 ml of 0.2 M cold phosphate buffer (pH 6.5) containing 1% PVP. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and stored at -20 °C.

PPO activity was determined according to Gao (2000). The reaction mixture contained 2.0 ml of 0.2 M phosphate buffer (pH 6.5), 1.0 ml of 0.1 M o-dihydroxybenzene and 100 µl of the supernatant and was incubated for 10 min at 37 °C. Then, the reaction was stopped by adding 2.0 ml of 20% trichloroacetic acid. The inactive enzyme was used as contrast with absorbance read at 525 nm.

POD extraction and activity assay

Method of extraction to obtain the supernatant was the same as for CAT. POD activity was determined according to Hammerschmidt et al. (1982). The reaction mixture contained 2.0 ml of 0.2 M phosphate buffer (pH 7.0) comprising 0.25% (v/v) guaiacol, 1.0 ml of 0.2% H_2O_2 and 20 µl of the supernatant, and the reaction was run for 5 min at 30 °C. Absorbance value was read at 470 nm.

PAL extraction and activity assay

A 0.4 g lot was measured from each five-tree composite shoot sample. This was homogenised in 2.0 ml of 0.1 M cold borate buffer (pH 8.8) containing 1 mM EDTA, 5 mM β-mercaptoethanol and 1% PVP, 1% (v/v) glycerol. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and stored at -20 °C.

The reaction mixture contained 2.0 ml of 0.1 M borate buffer (pH 8.8), 0.1 ml of 0.2 M L-phenylalanine and 0.1 ml of the supernatant, and was incubated for 30 min at 30 °C. The activity was determined at 290 nm (Hahlbrock & Ragg 1975, Koike & Nanbu 1997).

SOD extraction and activity assay

The method of extraction was also similar to that for CAT. SOD activity was determined according to El-Moshaty et al. (1993). The reaction mixture comprised 2.0 ml of 0.2 M phosphate buffer (pH 7.0) containing 13 mM Met, 0.075 mM NBT, 0.002 mM lactochrome and 0.1 mM EDTA mixed with 0.02 ml supernatant. This mixture was incubated under 4 kLux light for 10 min at 25 °C. Darkness was used to stop the reaction and absorbance was determined at 560 nm. There was no enzyme used for contrast.

Data analysis

All extractions and determinations were conducted in triplicates. Analyses of data were carried out by SPSS Statistics 17.0 (2011). Duncan's multiple range tests were used to determine the statistical significance of differences between means.

RESULTS

Enzyme activity of 13-month-old eucalypts

The mean enzyme activity levels of the five enzymes in the four eucalypt clones at age 13 months are shown in Figure 1. There were significant differences ($p \leq 0.05$) in the levels of CAT, SOD, PAL, POD and PPO activities between the four clones sampled, with key differences being between clone DH32-29 and the other three clones for CAT activity levels; between clone U6 and DH32-29 for SOD activity levels; between clone GL9 and clones U6 and DH32-29 for PAL activity levels; and between clone DH201-2 and the other three clones for POD activity levels. Also, for PPO activity levels clone U6 differed significantly from clones DH201-2 and GL9, and clone DH32-29 differed significantly from clone DH201-2.

Enzyme activity of 29-month-old eucalypts

It was clear that activity levels of both CAT and PAL in 29-month-old GL9 were substantially and significantly higher than the levels in the other three clones (Figure 2). For PAL, the activity level in clone DH32-29 was also significantly higher than U6 and DH201-2. For POD, the activity levels were significantly greater in clone DH201-2 than in clones U6 and DH32-29, while for SOD, only clones DH32-29 and U6 were significantly different. The activity levels of PPO were low and similar between the four clones.

Enzyme activity of 38-month-old eucalypts

Mean activity levels of the five enzymes in the four eucalypt clones studied at age 38 months are shown in Figure 3. The levels of both CAT

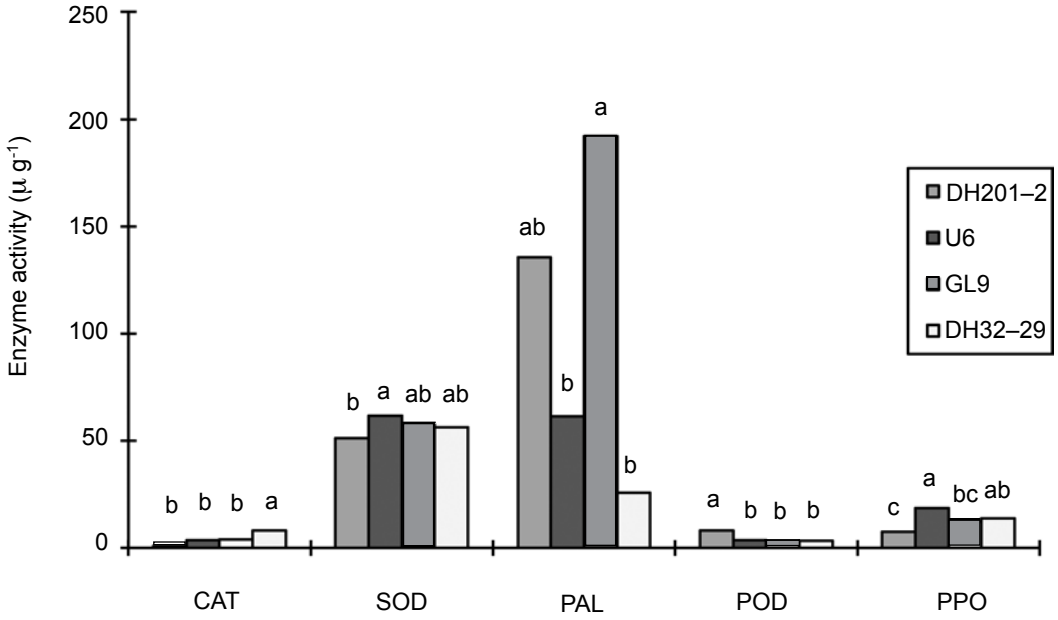

Figure 1 Activity levels of enzymes in four Chinese commercial eucalypt clones at age 13 months; different letters above the bars indicate significant difference ($p \leq 0.05$); CAT = catalase, SOD = superoxide dismutase, PAL = phenylalanine ammonia-lyase, POD = peroxidase, PPO = polyphenol oxidase

and SOD activities were found to be significantly higher in clone GL9 than in the rest of the clones, with the exception of SOD level in clone U6. Clone DH32-29 also showed significantly higher CAT activity level than both clones U6 and DH201-2. The highest level of POD activity was found in clone DH201-2, i.e. similar to the patterns observed between clones for this enzyme in both 13- and 29-month-old trees. The levels of PPO activity were highest in clone DH32-29 and were significantly different from the other three clones. Despite apparent marked variation in PAL activity levels between clones at this age, these differences were not significant.

Enzyme activity of three plantation ages

The levels of CAT activity were significantly higher in 29- than 13-month-old trees (Figure 4), but the levels in 38-month-old trees were intermediate and did not differ significantly between ages. The levels of SOD activity followed the same pattern across ages as CAT. In contrast, the levels of PAL activity were significantly lower in the 29-month-old trees than in either the younger (13 months) or older (38 months) trees, and POD activity followed the same pattern as PAL. There were no significant differences observed in POD activity levels between the different ages.

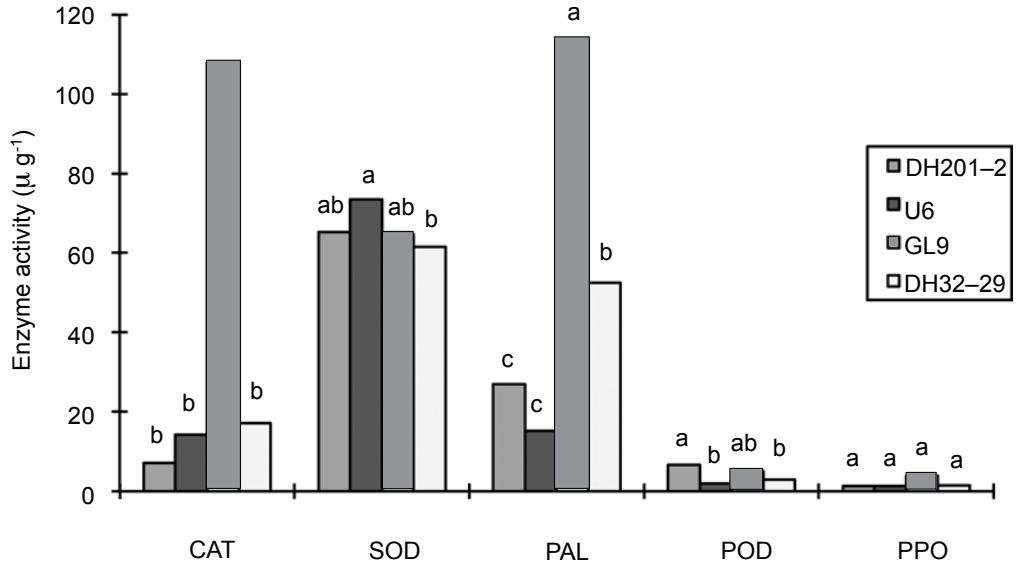

Figure 2 Activity levels of enzymes in four Chinese commercial eucalypt clones at age 29 months; different letters above the bars indicate significant difference ($p \leq 0.05$); for details of enzymes, see Figure 1

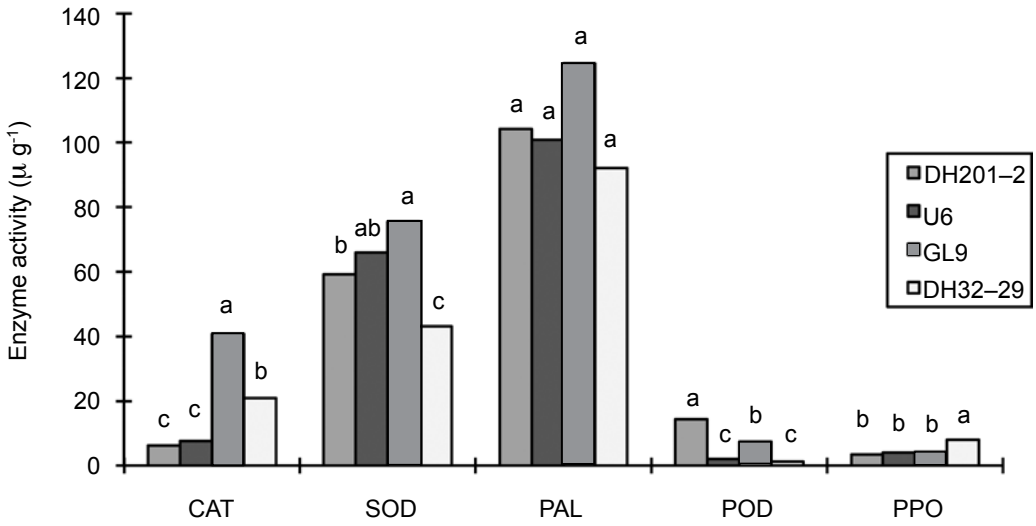

Figure 3 Activity levels of enzymes in four Chinese commercial eucalypt clones at age 38 months; different letters above the bars indicate significant difference ($p \leq 0.05$); for details of enzymes, see Figure 1

Enzyme activity in four commercial eucalypt clones

The highest levels of both CAT and PAL activities were found in the clone GL9, significantly greater than those in all other three clones for these two enzymes (Figure 5). The level of SOD activity was significantly greater in clone U6 than in both DH201-2 and DH32-29. Similarly, the level of SOD activity in GL9 was greater than in DH32-39. For POD activity, DH201-2 had the highest level followed by GL9. There was no significant difference in level of PPO activity between clones.

Comparison of enzyme activities between healthy and attacked trees of DH201-2

The activities of CAT, SOD and PPO were significantly higher in attacked trees than in healthy trees, with SOD having the highest value (Figure 6). However, the levels of both PAL and POD were significantly lower in the attacked than in the healthy material.

DISCUSSION

Previous studies have shown that *L. invasa* mainly damaged nursery seedlings and young (1–3

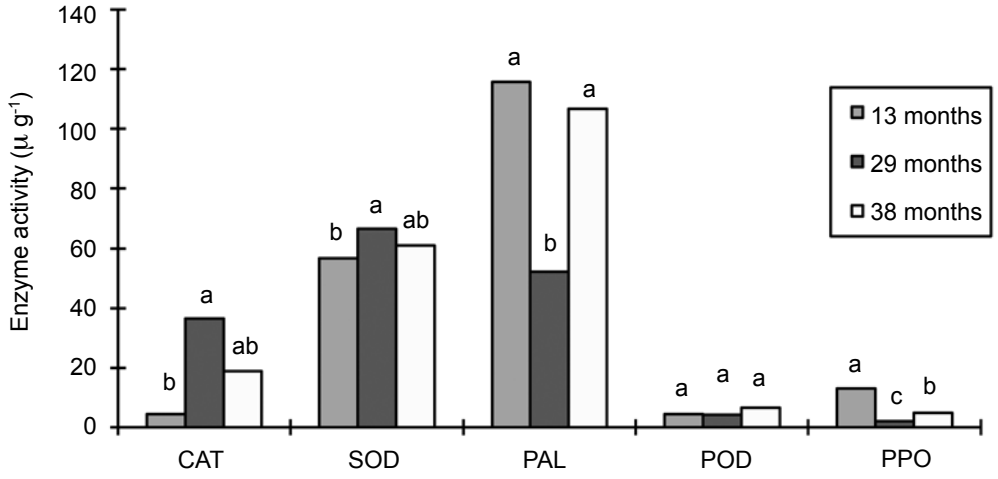

Figure 4 Activity levels of enzymes in four clones of eucalypts at three different plantation ages; different letters above the bars indicate significant difference ($p \leq 0.05$); for details of enzymes, see Figure 1

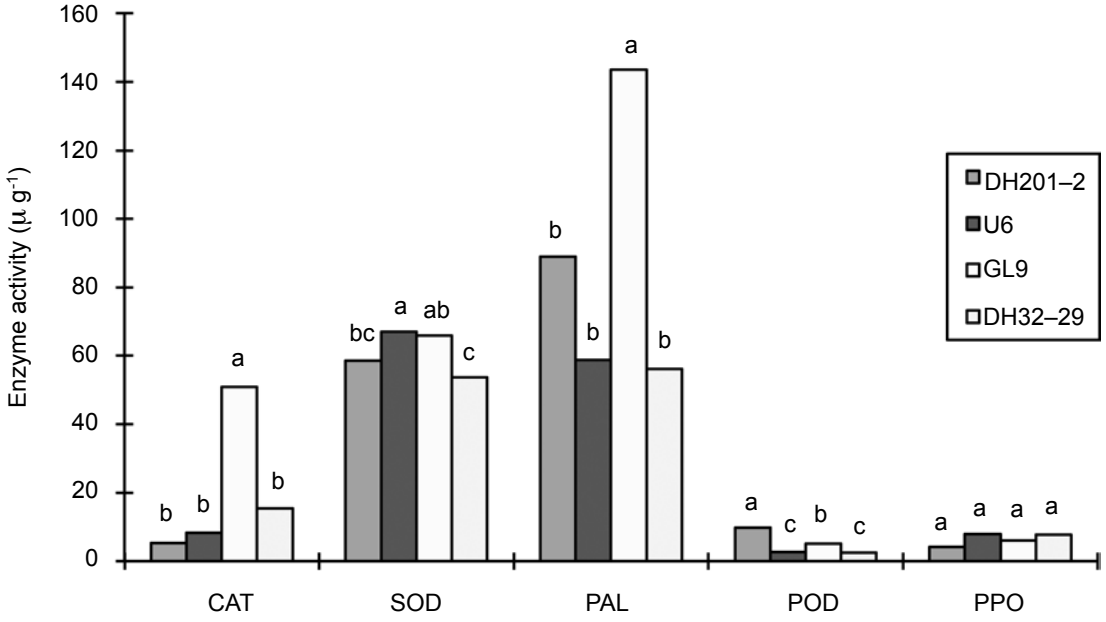

Figure 5 Activity levels of enzymes in four Chinese commercial eucalypt clones averaged across the three ages; different letters above the bars indicate significant difference ($p \leq 0.05$); for details of enzymes, see Figure 1

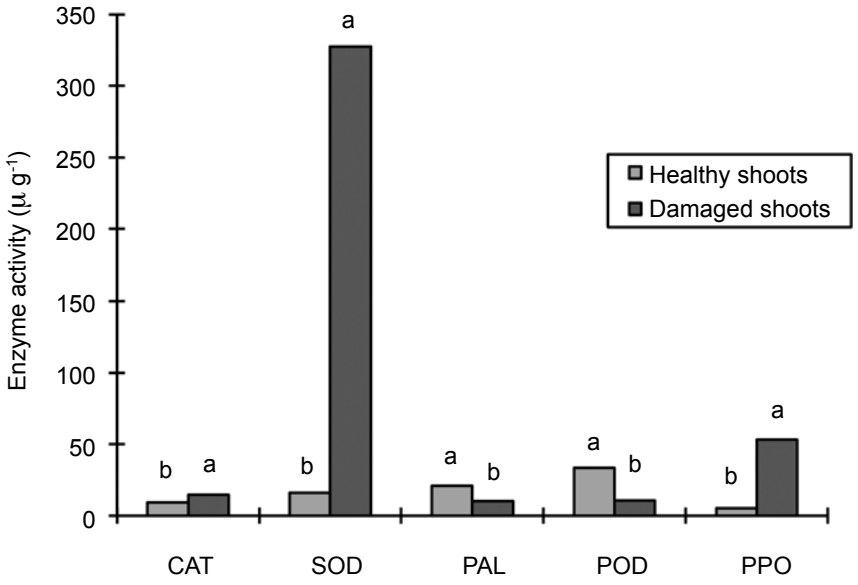

Figure 6 Levels of enzyme activities of shoots from healthy trees and those of shoots attacked by *Leptocybe invasa* at age 15 months; different letters above the bars indicate significant difference ($p \leq 0.05$); for details of enzymes, see Figure 1

years old) plantations of eucalypts (Mutitu et al. 2005, Nyeko 2005, Nyeko et al. 2009). This is why this current study was limited to examining enzyme activity levels in 13- to 38-month-old trees. However, this study found that the activity levels of the five enzymes did not show any regular increase or decrease with plantation age. For example, high levels of CAT activity were found in the 29-month-old trees but much lower levels in all the clones at age 13 months and in three of the four clones at age 38 months.

It was clear that the levels of the five enzymes studied here showed no evident association with *L. invasa* resistance or susceptibility. The resistant clones (GL9 and DH32-29) did not show significantly higher activity levels of CAT, PAL and PPO enzymes than the other two clones (U6 and DH201-2) at any of the three ages examined. For POD enzyme activity, the highest levels were found in one of the susceptible clones (DH201-2) while the other susceptible clone (U6) did not differ significantly from the two more resistant clones (GL9 and DH32-29) for activity levels of this enzyme at ages 13 and 19 months. Furthermore, there were no patterns of SOD enzyme activity levels among the clones across different ages. It was still possible that some or all of the enzymes played a role in determining susceptibility and/or resistance. It was likely that there were other more important factors involved such as physical property of the leaf (Chang et al. 2008) and secondary metabolites of the plant (Jiang et al. 2009). Indeed, previous studies have concluded that insect resistance cannot generally be determined by only one or limited number of enzymes (Sha et al. 2004, Shi et al. 2008).

Insect damage to plants can induce transcription of a number of genes responsible for controlling productions of some enzymes such as PPO (Haruta et al. 2001). Indeed, the activity levels of all five enzymes studied here did change significantly in clone DH201-2 due to attack by *L. invasa* whereby CAT, SOD and PPO increased and PAL and POD decreased (Figure 6). These findings were in concurrence with findings by Yang et al. (2007) who reported increased levels of CAT activity in leaves of *E. urophylla* after damage by *Dappula tertia* but contrast to their results for POD. In another study, higher levels of POD activity were also reported in crabapple leaves infested by carmine spider mites (*Tetranychus viennensis*) and the levels increased rapidly as damage time or pest densities increased (Jia et al. 2004). In cowpeas, POD activity levels were also found to increase significantly after damage by carmine spider mites, as were levels of SOD, PPO and PAL (Li et al. 2003, Li et al. 2004). Activity levels of POD and CAT enzymes in *Bischofia javanica* leaves attacked by *Coloana cinerea* had a significant increase compared with healthy leaves (Qin et al. 2009). In contrast, the authors reported that SOD activity decreased significantly in attacked compared with healthy leaves. These results for

CAT and SOD concured with our study but not for POD where our study showed lower values in attacked shoots compared with healthy shoots. These contrasting results between studies seem to suggest that enzymes respond differently to different insects.

CONCLUSIONS

Our study revealed that activity levels of the five enzymes—POD, PPO, SOD, PAL and CAT—did not show any clear association with qualitative ranking of the susceptibility of the four study clones to *L. invasa*. In the 15-month-old DH201-2, significant differences were found in activity levels of all five enzymes between damaged and healthy tree shoots. Thus, it was not possible to draw any definitive conclusion about possible association or relationship between the enzymes studied and resistance or susceptibility to *L. invasa* in eucalypts. What the results do suggest is that a larger study seems warranted to track enzyme activity levels in trees before and after attack in a much broader sample of clones.

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