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ORIGINAL ARTICLE

Effect of associated fungi on the immunocompetence of red turpentine beetle larvae, *Dendroctonus valens* (Coleoptera: Curculionidae: Scolytinae)

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Abstract *Dendroctonus*–fungus symbioses are often considered as the ideal model systems to study the development and maintenance of ectosymbioses, and diverse interactions, including antagonism, commensalism and mutualism, have been documented between these organisms. The red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae) is a pine-killing invasive beetle in northern China. Fungi species *Ophiostoma minus*, *Leptographium sinoprocerum*, *L. terebrantis* and *L. procerum* were associated with this bark beetle. Antagonistic interactions between *D. valens* and its associated fungi, such as *O. minus* and *L. sinoprocerum*, have been demonstrated, but the underlying causes of this phenomenon are unknown. Here, we first found the two tested fungi species retarded the net weight gain of *D. valens* larvae after completing 3-day feeding on their media. Furthermore, we provide direct evidence indicating the effect of associated fungi on the immunocompetence of *D. valens* larvae to explain the documented antagonism. Our results showed that the activity of phenoloxidase and total phenoloxidase in *D. valens* larvae were significantly upregulated by two strains of associated fungi, *O. minus* and *L. sinoprocerum* as compared with the controls. The phenoloxidase ratio increased significantly in the larvae which had fed for 3 days on media inoculated with *O. minus*. Because insect immune defenses are costly to be deployed, these results could be explored as one of the underlying mechanisms of the documented antagonism.

Key words bark beetle, ecological immunology, fungi, phenoloxidase, symbiosis

Introduction

Dendroctonus bark beetles are a major mortality agent of conifers. These beetles carry a variety of associated fungi, either within or outside of their mycangia (Paine *et al*., 1997). Symbioses among *Dendroctonus* beetles and fungi vary greatly in type and include antagonisms, and possibly commensalisms and mutualisms, and can range from obligate to facultative (Six & Wingfield, 2011). For example, *Ophiostoma ranaculosum* Hausner and *Entomocorticium* sp., mycangial fungi carried by the southern pine beetle *D. frontalis*, are inoculated into the phloem during construction of the egg galleries. Adults carrying these fungi have increased egg production (Goldhammer *et al*., 1990), and fungal development significantly increases larval development and survival (Bridges, 1983; Ayres *et al*., 2000). *Ophiostoma minus* is a bluestain fungus introduced into the phloem from the surface of attacking southern pine beetles or their phoretic mites (Hofstetter *et al*., 2006). During the early stages of tree colonization, the relationship between *D. frontalis* and *O. minus* can be

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categorized as mutualistic, as the pathogenicity of the fungus aids the beetles to overcome tree defenses (Paine *et al*., 1997). However, later in the colonization process, *O. minus* competes with the mycangial fungi and becomes highly antagonistic to the beetle (Six & Klepzig, 2004). Although it has been documented that some fungi had strong negative effects on bark beetle development and survival, the underlying cause of antagonism is not known (Six & Wingfield, 2011).

The red turpentine beetle (RTB), *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae), is generally considered as a secondary pest in its native range of North America. Since its introduction to China in the early 1980s, *D. valens* has become an aggressive killer of pines (Yan *et al*., 2005). The primary host in China is *Pinus tabulaeformis*, and over 0.5 million ha have been affected (Miao *et al*., 2001). Ten species of fungi have been identified as associates of *D. valens* in North America: *Leptographium terebrantis*, *L. procerum*, *L. wingfieldii*, *Grosmannia wageneri*, *G. clavigera*, *G. piceiperda*, *Ophiostoma ips*, *O. piliferum*, *Graphium* sp. and *Ceratosystiopsis collifera* (Wingfield, 1983; Klepzig *et al*., 1991). Of the fungal associates identified to date for *D. valens* in China, only *L. procerum* and *O. ips* are common to both North America and China. The other fungi that have been isolated from *D. valens* or its galleries in China are *L. pini-densiflorae*, *L. truncatum*, *L. sinoprocerum*, *Hyalorhinocladiella pinicola*, *Ophiostoma flocossum*, *O. minus*, *O. piceae*, *O. abietinum*, and an undescribed taxon close to *O. rectangulosporium* (Lu *et al*., 2008, 2009). Little is known about the symbiotic relationships between *D. valens* and its fungi in either North America or China, and it has not be determined if *O. minus* or the other fungal associates found exclusively in China are implicated in the observed differences in the aggressive tree-killing behavior by *D. valens* between the two continents.

The pathogens and associated fungi species of RTB might affect its immune responses in nature. Immunocompetence is the ability of an organism to mount an immune defense against pathogens, and it is usually estimated by measuring one or more components of the immune system (Adamo *et al*., 2001). The prophenoloxidase (proPO) cascade is one of the major immune responses in insects (Kan *et al*., 2008). Once an insect is infected or injured, proPO in the hemolymph is activated to phenoloxidase (PO). PO is an oxidoreductase that catalyzes the oxidation of phenols to quinones, which then polymerize nonenzymatically to melanin (Schmid-Hempel, 2005). The quinones as well as melanin are toxic to microorganisms (Nappi & Ottaviani, 2000). PO is thus frequently used to estimate immune function in insects. PO activity also frequently correlates with host resistance (Adamo *et al*., 2001) and can be measured through the enzyme kinetics of PO using hemolymph or tissue (Schmid-Hempel, 2005). The activation of immune defense in insects may come at the expense of other physiological functions such as reproduction (McKean & Nunney, 2001; Ahmed *et al*., 2002). Conversely, mating-induced reduction of immunocompetence has also been documented in several species (McKean & Nunney, 2001).

Shi and Sun (2010) examined the immunocompetence of the life stages of *D. valens*. PO and total PO activity generally were higher in larvae and pupae than in adults, while the PO ratio (PO activity/total PO activity) was the lowest for pupae. As antagonistic interactions between *D. valens* and its associated fungi have been demonstrated (Wang *et al*., 2012), we studied whether the immunocompetence of *D. valens* larvae was affected by its associated fungi, *L. sinoprocerum* and *O. minus*. PO activity and total PO activity were assayed as the indicators of physiological immunocompetence. The objective was to explore the physiological mechanisms underlying the influence of fungi on *D. valens* and discover whether the influence is positive or negative.

Materials and methods

Specimen collection and preparation

Fourth instar larvae of *D. valens* were collected from freshly cut stumps in a *Pinus tabulaeformis* stand at Tunlanchuan forest farm (37°48′N, 111°44′E; average elevation 1 400 m), Gujiao City, Shanxi Province, China, in mid-September, 2009. The larvae were placed into plastic 18 × 12 × 10 cm microwave boxes containing artificial diet (100 g phloem powder of Chinese pine, 2 g vitamin C, 10 g agar, 2 g methylparaben, 1 g sorbic acid, 12 drops linolic acid, 200 mL distilled water) and then transferred to a climate incubator at 20°C, L : D = 0 : 24.

Two species of fungi, *O. minus* (CMW26254) and *L. sinoprocerum* (MUCL 46352), were used to test the effects of fungi on the immunocompetence of *D. valens* larvae. Artificial media (water 400 mL, phloem 15 g, agar 15 g) were prepared. The ground phloem and the agar–water mixture were autoclaved separately for 30 min at 126°C and 0.14 MPa of pressure, then mixed together in a 500 mL conical flask. Fifteen milliliters of the mixture were poured into a Petri dish (90 mm diameter by 15 mm high). After cooling and solidifying, fungi were inoculated on the surface of the media and incubated at 25°C and relative humidity (RH) 70% in darkness for 35 days. Although the tested fungi had different growth rates,

all the media were fully covered by fungi after 35 days. Subsequently, 3 cm circles of the media were cut by a glass tube (3 cm inner diameter), and the discs were transferred into a six-well cell culture plate (Costar®, Corning Incorporated, Lowell, MA, USA). One fourth instar *D. valens* larva was placed on each disc and allowed to feed for 3 days. Larvae fed on artificial media without fungi were used as controls. Twenty larvae each were used for both fungus/media combinations and for the control. The body weight of each larva was measured by an analytical balance (METTLER TOLEDO, AL204, Shanghai, China) and recorded one by one.

PO activity

After the 3-day feeding period, the larvae were removed and their cuticles sterilized with 95% ethanol. Each larva cuticle was torn open with two sterilized forceps, and hemolymph collected by putting the dissected larva into a 0.5 mL microcentrifuge tube containing 500 μL cold phosphate buffer saline (distilled water 500 mL, NaCl 4 g, KCl 100 mg, Na_2HPO_4 720 mg, K_2HPO_4 720 mg, pH 7.2). All hemolymph samples were mixed individually by a vortexer for several seconds and stored at -20°C for 72 h and an additional 24 h at 4°C. The activity of naturally activated PO enzymes only (hereafter "PO activity") and the sum total activity of the PO, including the additional activity of the proenzymes ("total PO activity") in each hemolymph sample were measured using a spectrophotometric assay (Cornet *et al*., 2009). PO activity was quantified without further activation, while total PO activity required the activation of the proenzymes into PO with trypsin. After centrifugation (4°C, 9 300 × *g*, Sigma, Osterode am Harz, Germany, 1-15PK Centrifuge), 30 μL of the supernatant were mixed with either 110 μL of ultrapure water to measure PO activity only, or 110 μL of trypsin solution (Amrexco, Solon, OH, USA, 2 mg/mL of ultrapure water), 30 μL phosphate buffer saline, and 30 μL *L*-Dopa (Acros Organic, Morris Plains, NJ, USA, 4 mg/mL ultrapure water) as a substrate. The reaction was allowed to proceed at 30°C in a microplate reader (VersaMax, Molecular Devices Corp., Sunnyvale, CA, USA) for 30 min. Absorbance readings were taken every 30 s at 492 nm. Enzyme activity was measured as the slope (V_{max} value) of the reaction curve during the linear phase of the reaction. For each individual, we performed two independent measurements and determined an average V_{max} for the two reactions. The PO ratio was calculated by PO activity/total PO activity. Because PO often existed in the hemolymph as the proenzyme, PO ratio was calculated to evaluate the investment in the proenzyme system of *D. valens* larvae (Shi & Sun, 2010).

Data analysis

Normality of the data was tested by One-Sample Kolmogorov-Smirnov Test and the results indicated the data were normally distributed. General linear model (GLM) multivariate was used to analyze differences in PO, total PO and PO ratio among different treatments. To account for differences in hemolymph volume among larvae, fungal species was selected as the fixed factor, with net weight gain of each larva as the covariate. GLM multivariate revealed that larval net weight gain, a surrogate for hemolymph volume, did not significantly affect the immunocompetence of larvae (Table 1). The data analysis was run again with analysis of variance (ANOVA) to detect the effects of fungi associates. If a significant *F*-test statistic ($P < 0.05$) was obtained from ANOVA, differences of least squares means were used as the multiple comparison procedure for determining cohort group differences. All tests were performed with the statistical software SPSS for windows, v 11.5 (SPSS Inc., Chicago, IL, USA).

Results

First, our results indicated two tested fungal associates of RTB significantly decreased the net weight gain of larvae after exposure to their media for 3 days (ANOVA: $F_{2,50} = 3.532$, $P < 0.05$). Compared with the control, the larvae had the least increment in their weight gain after exposure for 3 days in the media of *O. minus* (Fig. 1).

GLM multivariate revealed that larval net weight gain, a surrogate for hemolymph volume, did not significantly affect the immunocompetence of larvae (Table 1). Because tested fungi species had various performances on the growth of RTB larvae in each treatment, ANOVA was run again to detect the exact effects of associated fungi on the RTB larvae's immune response in our experiments.

Seven larvae died prior to completing 3 days of feeding, thus they were not tested for PO and total PO activity. Two larvae died in the media of *O. minus*, one larva in the media of *L. sinoprocerum* but four larvae in the control. Both PO activity and total PO activity were significantly up-regulated in *D. valens* larvae fed on media inoculated with one of two species of associated fungi when compared to the control group (PO activity: $F_{2,50} = 8.95$, $P < 0.001$; total PO activity: $F_{2,50} = 6.821$, $P < 0.05$). Immunocompetence was the highest for larvae fed on the media with *O. minus* (V_{max} mean PO = 609.43 ± 55.76; and V_{max} mean total PO = 671.49 ± 46.08) (Fig. 2A). The means for PO activity and total PO activity were 2.2 and 1.8 times higher than those of the controls, respectively.

Table 1 Analysis of general linear method multivariate on the effects of larval net weight gain and fungal associates on red turpentine beetle immunocompetence.

Source	Dependent variable	Type III sum of squares	df	Mean square	*F*	Significance
Corrected model	Total PO	0.821†	3	0.274	5.873	0.002*
	PO	0.647‡	3	0.216	4.514	0.007*
	PO ratio	0.246§	3	0.082	2.800	0.050
Intercept	Total PO	2.749	1	2.749	59.000	0.000*
	PO	4.040	1	4.040	84.508	0.000*
	PO ratio	9.576	1	9.576	326.727	0.000*
Net weight gain	Total PO	0.002	1	0.002	0.053	0.820
	PO	0.007	1	0.007	0.137	0.713
	PO ratio	0.000	1	0.000	0.000	0.994
Fungal associates	Total PO	0.748	2	0.374	8.025	0.001*
	PO	0.606	2	0.303	6.338	0.004*
	PO ratio	0.228	2	0.114	3.892	0.027*
Error	Total PO	2.283	49	0.047		
	PO	2.342	49	0.048		
	PO ratio	1.436	49	0.029		
Total	Total PO	13.246	53			
	PO	18.401	53			
	PO ratio	34.318	53			
Corrected total	Total PO	3.103	52			
	PO	2.990	52			
	PO ratio	1.682	52			

†$r^2 = 0.264$ (adjusted $r^2 = 0.219$);
‡$r^2 = 0.217$ (adjusted $r^2 = 0.169$);
§$r^2 = 0.146$ (adjusted $r^2 = 0.094$);
*Significant at $P < 0.05$.
PO, phenoloxidase.

Immunocompetence of the larvae fed on the media with *L. sinoprocerum* was also increased significantly, with both PO and total PO activity being 1.4 times higher than the controls. PO ratio for the larvae fed on media with *O. minus* was 88.7% ± 4.0%, which was significantly higher than the ratios for larvae fed on media with *L. sinoprocerum* (75.9% ± 5.4%) and the controls (72.9% ± 3.2%, Fig. 2B).

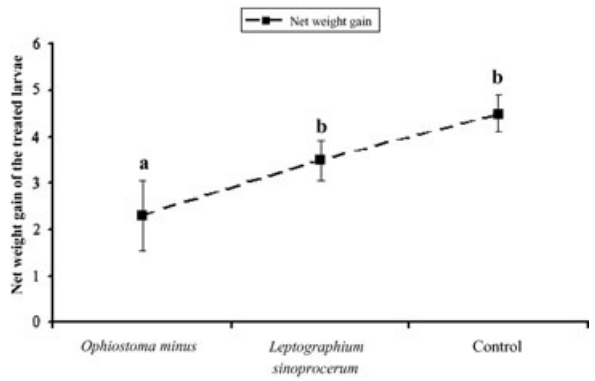

Fig. 1 Effect of two associated fungal species, *Ophiostoma minus* and *Leptographium sinoprocerum*, on the net weight gain (unit: mg) of *Dendroctonus valens* larvae.

Discussion

Activation of immune defenses can involve trade-offs and hence affect insect fitness (Rolff & Siva-Jothy, 2003). For example, Bascuñán-García *et al*. (2010) found the activation of immune responses markedly impaired growth, female reproduction and survival in the house cricket *Acheta domesticus*. In this study, feeding on media inoculated with either *O. minus* or *L. sinoprocerum* significantly upregulated the PO activity and total PO activity of *D. valens* larvae, indicating that the proPO cascade of the larvae had been activated. Cytotoxic byproducts (e.g. phenols) produced in response to the activation of the proPO cascade travel through the open hemocoel to attack a suspected pathogen, but are also believed to attack the

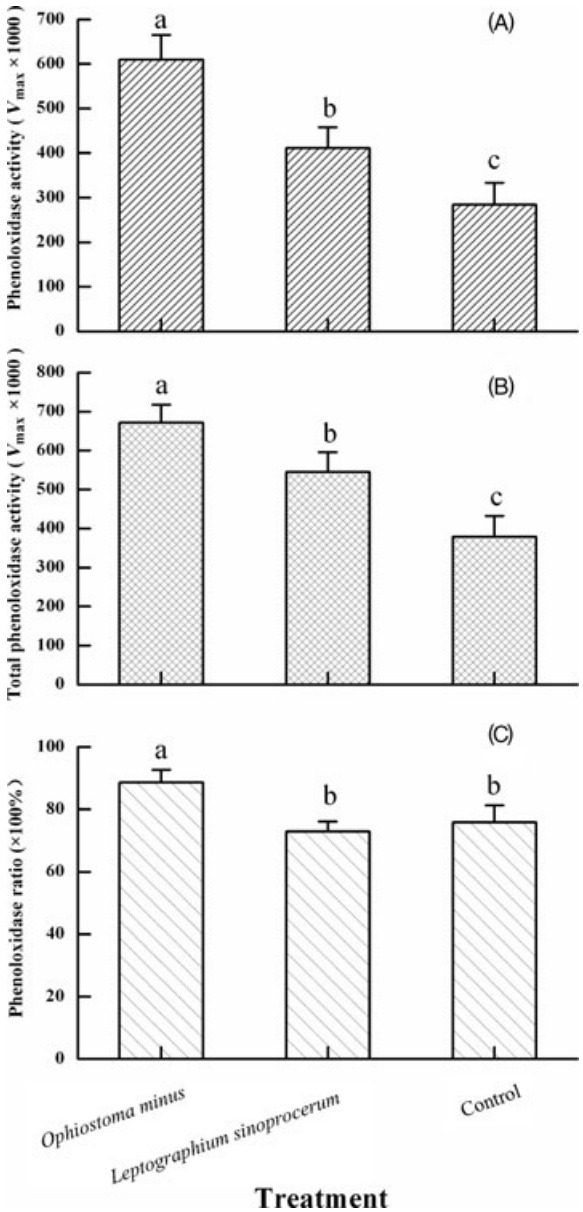

Fig. 2 Effect of two associated fungal species, *Ophiostoma minus* and *Leptographium sinoprocerum*, on the phenoloxidase (PO) activity (A), total PO activity (PO + prophenoloxidase, B) and PO ratio (C) of *Dendroctonus valens* larvae. Bars represent the mean for each treatment. Different letters above the standard error bars indicate significant differences between treatments ($P < 0.05$).

"self" (Nappi & Ottaviani, 2000). Thus the activation may impair the fitness of the larvae. Fungal infections have been shown to detrimentally affect insect growth (Dean *et al*., 2002). In a previous study with *D. valens*, Wang *et al*. (2012) found reductions in feeding activity and weight gain in larvae after exposure to *O. minus* and *L. sinoprocerum*. In this study, we also demonstrated *O. minus* and *L. sinoprocerum* decreased the net weight gain of RTB larvae after feeding for 3 days in their media. Because the initiation of immune responses often occur at the expense of growth and development, the significant upregulation in the PO activity and total PO activity of *D. valens* larvae in the present study may have been a major factor in the reduced weight gains. Pathogens can affect key metabolic routes important for weight gain, reproduction and so on, and immune responses are often involved in these effects (Vilcinskas & Götz, 2008).

It is well known that recognition of nonself is the first step in mounting immune responses of insect innate immune systems through detecting the pathogen-associated molecular patterns (PAMPs). However, the two fungi used in this experiment are not known pathogens of *D. valens*, and do not spread through the hemocoel as entomopathogenic fungal species do in other insects. Therefore, the trigger which activated the proPO cascades in the *D. valens* larvae requires further study. Hosts may modulate their immune response by measuring a combination of signals from pathogens and damaged tissue (Lazzaro & Rolff, 2011). Fungal metabolites released into the media and ingested by the larvae could have initiated the observed immune response. The fungi also may have altered the nutritional value of the phloem media. Wang *et al*. (2012) determined that fungal infection decreased the sugar and nitrogen content of the media. Therefore, it is unlikely that the increased PO activity observed in larvae fed on media plus fungus was due to an improved diet.

Any negative effects of the fungi on larval growth might be countered if the fungi assist the beetles in overcoming tree defenses and providing a suitable environment for offspring development. Although our results indicate the potential for an antagonistic relationship between the fungi and the beetle, the overall interaction between the two is still unknown. The role of these fungi in the successful colonization of hosts must be considered

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Disclosure

The authors declare that we have no conflict of interest.

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