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SCIENTIFIC NOTE

Order of Inoculation Affects the Success of Co-Invading Entomopathogenic Fungi

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Abstract
The effect of order of inoculation of *Pandora blunckii* and *Zoophthora radicans* co-infecting *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) was investigated. After co-inoculation, the proportion of larvae infected by either species was greatly reduced compared to when they were inoculated singly. The order of inoculation influenced the final outcome; the isolate inoculated last always killed more larvae than the isolate inoculated first.

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (DBM), is the primary insect pest of cruciferous crops worldwide (Talekar & Shelton 1993, Verkerk & Wright 1996). The entomopathogenic fungi *Pandora blunckii* and *Zoophthora radicans* (Entomophthoromycotina: Entomophthorales) are commonly recorded regulating field populations of DBM (Riethmacher & Krans 1994, Velasco-Silva *et al* 2000). Studies on competitive interactions between these species can help in the prediction of what might happen when one species is released in the field for biological control and interacts with other species already present.

Our previous research with these two species showed a significant effect of conidial concentration on outcomes of co-infection (Guzmán-Franco *et al* 2009). However, the effect of the order of inoculation was not studied, and it is considered an important factor affecting interactions between other entomopathogenic fungi (Thomas *et al* 2003). Here, we consider the effect of the order of inoculation on interactions between *Z. radicans* and *P. bluckii* in a Mexican DBM population.

One *P. blunckii* isolate (NW449) from Guanajuato, Mexico, and one *Z. radicans* isolate (NW250) from Cameron Highlands, Malaysia were used. Both were obtained from infected DBM larvae. Groups of 15 early third instars of DBM were placed in 30-mm Petri dish bases each containing a disk of a broccoli leaf embedded in 1.5% water agar (2 mL) and exposed to conidia from actively sporulating plugs of *Z. radicans*, *P. blunckii*, or both species. A 10-mm diameter glass coverslip was placed at the center of each cabbage leaf during inoculation to estimate the concentration of conidia. Identity of conidia in dual inoculations was determined based on morphology. Actively sporulating plugs were prepared by placing 9-mm diameter plugs of each isolate in a Petri dish at high humidity and 22°C in darkness for 18 h prior to experimentation.

When *Z. radicans* was inoculated first, two groups of larvae were inoculated with *Z. radicans* conidia for 60 min.

Of the two groups, one was then immediately inoculated with *P. blunckii* conidia for a further 60 min. The first group was a positive control for the *Z. radicans* isolate. A third group of 15 larvae was inoculated only with *P. blunckii* as a positive control for *P. blunckii* and a fourth group of larvae was maintained under the same inoculation conditions for 120 min, but with no fungal inoculum (control treatment). Simultaneously, the complete experiment was done with the order of inoculation of the two species reversed. All treated larvae were incubated in ventilated cylindrical plastic cages of 15 cm diameter containing broccoli leaves as food at 22°C in darkness for 5 days. To determine the cause of death [attributed to *Z. radicans*, *P. blunckii*, a combination of both pathogens (dual-infected), or to an unknown cause of mortality], each dead larva was placed in the base of a 30-mm diameter Petri dish containing sterile damped Whatman no.1 filter paper and incubated in an inverted position at 22°C for 24 h. The inverted position allowed the collection of conidia on a coverslip placed beneath the cadaver; each coverslip was assessed microscopically to identify the fungal species based on conidial morphology. The presence or absence of resting spores inside each dead larva was also recorded. Where neither conidia nor resting spores were present, the larva was attributed to the "mortality due to unknown causes" category. The complete experiment was repeated on three separate occasions.

Each isolate combination was analysed separately. Data were analysed using logistic regression in GenStat v. 8.1. (Payne *et al* 2005), with each cause of death as a response variable and their interaction with the order of inoculation included. The effect of conidial concentration of each isolate at inoculation was not included as an experimental factor; however, its effect on the results was analysed to account for any potential effects of differences in conidial concentration between treatments and replicates.

The proportion of larvae with sporulation attributable to *P. blunckii* was significantly influenced by the order of inoculation $\left(\chi_1^2 = 18.14, P < 0.001\right)$; when *P. blunckii* was inoculated first, the proportion of larvae sporulating with *P. blunckii* conidia was greatly reduced (less than 0.1) compared with when it was inoculated alone (Fig 1a). When *Z. radicans* was inoculated first, the proportion of larvae sporulating with *P. blunckii* conidia was significantly greater than when *P. blunckii* was inoculated alone (Fig 1a). Differences in conidial concentration between *Z. radicans* (average of 279.9 conidia/mm^2) and *P. blunckii* (average of 181.4 conidia/mm^2) at inoculation may have been a contributory factor; however, no significant interaction between conidia concentration of *P. blunckii* $\left(\chi_2^2 = 0.757, P = 0.685\right)$or *Z. radicans* $\left(\chi_2^2 = 0.299, P = 0.861\right)$ and the proportion of larvae sporulating with *P. blunckii* conidia was found.

The greatest proportion of larvae sporulating with *Z. radicans* conidia occurred when the *P. blunckii* isolate was inoculated first (Fig 1b), showing a significant interaction with the order of inoculation $\left(\chi_2^2 = 5.483, P = 0.019\right)$. Again, there was no significant interaction between conidial concentration of *P. blunckii* $\left(\chi_2^2 = 0.164, P = 0.921\right)$ or *Z. radicans* $\left(\chi_2^2 = 2.928, P = 0.231\right)$ at inoculation and the proportion of larvae sporulating with *Z. radicans* conidia. Larvae sporulating with conidia from both species only occurred in the dual-inoculated treatment where *P. blunckii* was inoculated first

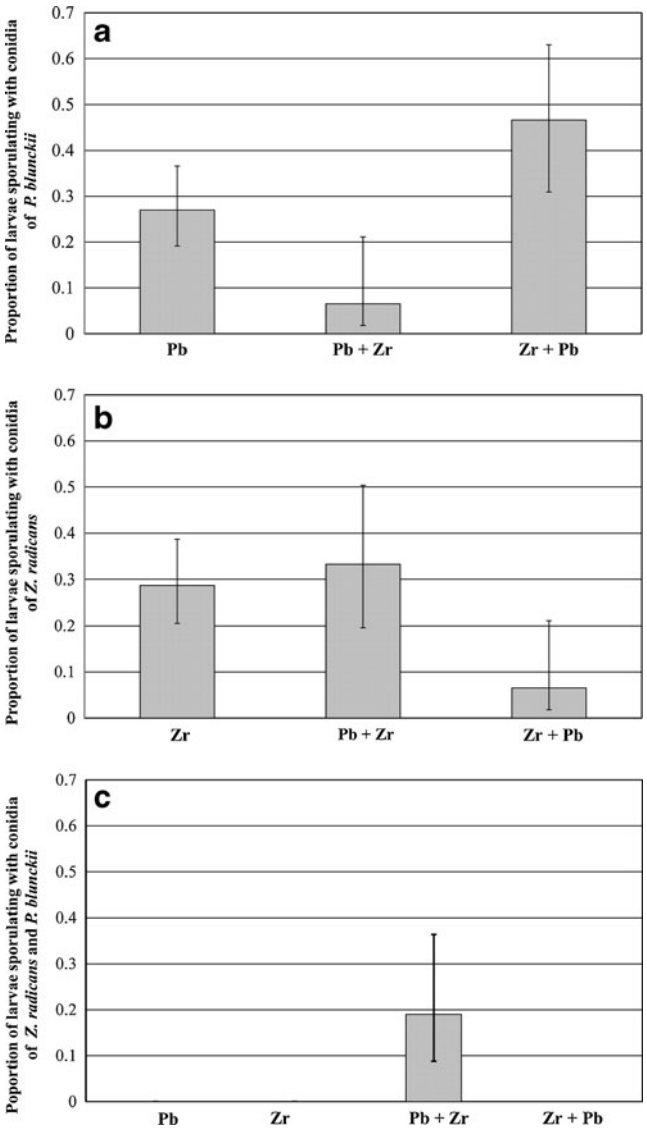

Fig 1 Proportion of infected *Plutella xylostella* larvae. **a** Infection caused by the *Pandora blunckii* isolate alone and in combination with *Zoophthora radicans* where the order of inoculation was modified. **b** Infection caused by the *Zoophthora radicans* isolate alone and in combination with *Pandora blunckii* where the order of inoculation was modified. **c** Infection caused by both fungal species (*Zoophthora radicans* and *Pandora blunckii*). *Pb* = *Pandora blunckii*, *Zr* = *Zoophthora radicans*. *Error bars* represent 95% confidence intervals back-transformed from logistic scale. In dual-inoculated treatments, the order of isolate codes in legends represents the order of inoculation.

and at relatively low conidial concentrations (Fig 1c). Most dead larvae only sporulated with the conidia of one species, even when they had been inoculated with both. The most interesting result from consecutive inoculations was that the isolate inoculated last was always responsible for the greatest proportion of successfully infected larvae (i.e., resulting in sporulation) (Fig 1), suggesting that the interaction may have been mediated by the insect's immune system (Cox 2001). It is possible that the challenge to the host by the first pathogen was so costly to the host in terms of energy that while invasion of the first pathogen was reduced, the immune system was sufficiently impaired that it could no longer prevent invasion by the second pathogen. Similar results have been recorded for virulent (*M. anisopliae*) and avirulent (*Aspergillus flavus*) pathogens in ant populations (Hughes & Boomsma 2004). In conclusion, our results showed that the outcome of sequential inoculation is affected by the order of inoculation, even when the temporal separation between inoculation events is very short. This knowledge will lead to a better understanding of the role of co-infections in the ecology of fungal isolates and improve the design of microbial control programs.

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