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Competition between isolates of *Zoophthora radicans* co-infecting *Plutella xylostella* populations

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ABSTRACT

Interactions between *Zoophthora radicans* isolates were studied *in vitro* and *in vivo* during infection of *Plutella xylostella* larvae. We distinguished between isolates within infected hosts using PCR-RFLP. Isolates obtained from *P. xylostella* larvae (NW386 and NW250) were more virulent than isolates from other insect hosts. Isolate NW250 was most virulent at 27 °C and isolate NW386 was most virulent at 22 °C. *In vitro* growth of all isolates except NW386 was affected by the presence of other isolates. During *in vivo* interactions between NW250 and NW386, the isolate with the greatest conidial concentration at inoculation infected more larvae than its competitor. Dual infected larvae were only found in treatments where inoculation concentrations of conidia were high for both isolates. Where concentrations of conidia at inoculation were low for both isolates, only NW250 caused successful infection. The implications of these results for the ecology of *Z. radicans* are discussed.

1. Introduction

The diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), is a severe pest of brassica crops with a worldwide distribution (Talekar and Shelton, 1993). The use of chemical insecticides remains the principal control strategy, and its intensive use has encouraged the rapid development of insecticide resistance in many regions (Tabashnik, 1994; Ferré and van Rie, 2002). As a consequence, *P. xylostella* is considered amongst the 20 most resistant arthropods in the world (Mota-Sanchez et al., 2002). These facts demonstrate that alternative control approaches, such as microbial control, are urgently needed. Different pathogens have been suggested for the microbial control of *P. xylostella* (Pell et al., 2001), and amongst them, the entomopathogenic fungus *Zoophthora radicans* (Bref.) Batko is one of the most promising. This is an entomomophthoralean fungus that has been reported causing natural epizootics in a number of different insect species including *P. xylostella* (McGuire et al., 1987b; Vandenberg and Soper, 1987; Galaini-Wraight et al., 1991; Riethmacher et al., 1992).

When selecting a species or isolate for microbial control, virulence against target and non-target hosts, and field persistence are always considered (Posada and Vega, 2005). However, basic and fundamental studies on the potential interactions that could occur between the released species and other pathogen species or con-specific isolates in the environment are less well studied. These interactions are important because, in combination with variable abiotic conditions, they could change the performance of the released isolate by influencing its ability to multiply and survive when infecting a susceptible host. This has been observed previously with *Z. radicans* and *P. blunckii* co-infecting *P. xylostella* (Guzmán-Franco et al., 2009, 2011). Studies on intraspecific interactions between isolates of the same species of fungus are particularly scarce, even though their importance is well accepted (Read and Taylor, 2001; de Roode et al., 2005). While intraspecific interactions have been studied for the fungus *Beauveria bassiana* infecting locusts (Quesada-Moragaa and Vey, 2003) there have been no studies with entomophthoralean species of fungi.

One reason for the lack of studies on the ecology of dual infections caused by con-specific isolates is the difficulty in distinguishing between two or more isolates infecting one host when the conidia have the same size and morphology. However, this problem can be overcome using molecular techniques (Enkerli and Widmer, 2010). Recent studies have demonstrated that *Z. radicans* is comprised of genetically diverse lineages that can be separated using molecular techniques (Guzmán-Franco et al., 2008a) and this represents an important model system for the study of interactions between isolates of the same species and how they may influence

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the success of a microbial control agent. Here we report the first study on interactions between genetically distinguishable isolates of *Z. radicans* infecting the same *P. xylostella* host. Using the results from a series of virulence, temperature and *in vitro* interaction studies on six isolates of *Z. radicans,* we selected two isolates for a further experiment to quantify their *in vivo* interactions during co-infection of *P. xylostella* and the influence of temperature on this interaction. Isolates were distinguished from each other using PCR-RFLP; each isolate produced a different RFLP banding pattern (Guzmán-Franco et al., 2008a) allowing us to categorise infected larvae with respect to whether they were infected by one of either of the two different isolates of *Z. radicans* or by both isolates.

2. Materials and methods

2.1. Insects

The *P. xylostella* colony was maintained in acrylic cages (50 × 35 × 60 cm) at 22 °C (16:8 LD) in the insectary of the Colegio de Postgraduados. The original colony came from Guanajuato state and had been maintained at the Instituto de Investigaciones Forestales Agricolas y Pecuarias (INIFAP) as a permanent colony. The colony was maintained on broccoli plants var. Waltham.

2.2. Isolates

Six *Z. radicans* isolates were used. Isolates NW379, NW386 and NW353 were obtained from infected *P. xylostella* larvae in Guanajuato state, Mexico. Isolate NW250 was obtained from infected *P. xylostella* larvae in Malaysia. Isolate ARSEF6003 was obtained from an infected *Trichoplusia ni* Hubner (Lepidoptera: Noctuidae) larva in Mexico and isolate ARSEF1699 from a *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Crambidae) larva from the Philippines. All isolates were grown in 90 mm diameter triple vented Petri dishes containing 24 mL SEMA medium (Sabouraud dextrose agar supplemented with egg yolk and milk; Wilding and Brobyn, 1980). Cultures were incubated at 22 °C in darkness for 15 days before use. All isolates were sub-cultured no more than three times after retrieval from liquid nitrogen.

2.3. Dose response assays and interactions with temperature

The dose response study was partitioned into two separate experiments. First, the virulence of all six isolates against *P. xylostella* larvae was estimated at 22 °C. Then, the virulence of four isolates was estimated at 15, 22 and 27 °C.

2.3.1. Virulence estimated at 22 °C

Groups of five fungal plugs (9 cm diameter) were cut from the growing edge of 15-day-old cultures of each isolate and placed into the lids of 50 mm diameter Petri dishes containing damp filter paper. These were placed into a plastic box containing moistened tissue paper (to ensure a high humidity and encourage sporulation) at 22 °C in darkness for 18 h prior to experimentation.

Early third instar larvae were placed in groups of 15 onto 45 mm diameter broccoli leaf disks embedded abaxial side uppermost in 1.5% water-agar (5 mL) in the bases of 50 mm diameter Petri dishes. A 10 mm diameter glass cover slip was placed in the centre of each leaf disk. For inoculation, the lids were replaced with the lids containing the sporulating fungal plugs. The larvae remained feeding on the leaf while they were inoculated and did not come into direct contact with the inoculum.

To obtain a range of doses, different batches of 15 larvae were exposed to the fungus for 5, 10, 25, 35, 45 and 60 min. Exposing insects to actively sporulating plugs of mycelium for different periods of time to achieve different inoculation doses is a standard technique for evaluating entomophthoralean fungi (e.g., Pell et al., 1993; Guzmán-Franco et al., 2009). Although, the different exposure times used did produce different conidia concentrations there was not always a direct relationship between longer exposure times and higher conidia concentration (Table 1). For this reason, in the analysis, the different conidia concentrations were sorted in ascending order regardless of the exposure time required to achieve each concentration. Control larvae were maintained under the same conditions for 60 min without any inoculum. During inoculation, the lids containing the fungal plugs were rotated to ensure an even deposition of conidia.

Table 1
These data show the variation in conidia concentration (con/mm^2) achieved within each exposure time and for the six *Zoophthora radicans* isolates used in the dose–response assays.

Isolate	Replicate	Inoculation times (min)					
		5	10	25	35	45	60
NW379	R1	12	67	116	197	119	114
	R2	31	101	226	233	235	180
	R3	140	167	409	130	201	180
NW386	R1	119	77	220	347	384	145
	R2	16	32	53	42	110	175
	R3	16	23	32	172	64	389
NW250	R1	9	8	125	69	101	162
	R2	27	31	84	116	291	146
	R3	52	121	235	279	173	219
ARSEF6003	R1	62	41	16	111	140	184
	R2	13	23	55	87	145	235
	R3	4	6	5	40	53	145
ARSEF1699	R1	83	179	370	382	187	615
	R2	17	199	231	258	305	236
	R3	151	211	260	350	118	459
NW353	R1	15	28	30	45	70	161
	R2	25	79	53	133	97	114
	R3	11	25	15	65	143	108

After inoculation, each batch of larvae was transferred to ventilated transparent polystyrene cylindrical containers (15 cm diameter × 10 cm) containing fresh broccoli leaves. All containers were incubated at 22 °C and mortality recorded daily for 5 days. Coverslips from the inoculation chambers were removed, fixed with 10% cotton blue in lactophenol on glass slides and the conidial concentrations estimated by counting conidia from 10 fields of view with a 1 mm^2 eyepiece graticule using the 10× microscope objective.

To score larval mortality due to successful infection by any isolate, evidence of external sporulation to produce conidia was recorded. However, because resting spores were known to be produced by some of the isolates (Guzmán-Franco et al., 2009), the presence of resting spores inside all the cadavers from all treatments were also recorded microscopically. For each isolate, 315 larvae were used for all inoculation times including the control (seven treatments) and three replicates, which represents a total of 1890 larvae for all six isolates. All treatments were carried out on the same day, and the complete experiment was repeated on three occasions (replicates).

2.3.1.1. Statistical analysis. The mortality data from the bioassays were analysed using a generalized linear model (GLM) with binomial error and probit link in the statistical package GenStat v. 8.0 (Payne et al., 2005). That is, the numbers of infected larvae were assumed to follow a binomial distribution with sample sizes equal to the number of larvae tested. Before comparing amongst isolates, a parallel model analysis was done for each isolate separately to determine whether data for all replicates could be combined in each case. First, a single line was fitted to data from all the repli-