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Direct plantlet inoculation with soil or insect-associated fungi may control cabbage root fly maggots

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ABSTRACT

A potential *Delia radicum* biological control strategy involving cauliflower plantlet inoculation with various fungi was investigated in a series of laboratory and glasshouse experiments. In addition to entomopathogenic fungi, fungi with a high rhizosphere competence and fungi with the ability to survive as saprotrophs in soil were tested. The following fungal species were evaluated in the experiments: *Trichoderma atroviride*, *T. koningiopsis*, *T. gamsii*, *Beauveria bassiana*, *Metharhizium anisopliae*, *M. brunneum* and *Clonostachys solani*. A commercial carbosulfan-based insecticide was used as a positive control. Additionally, two commercial products, one based on *B. bassiana* (Naturalis) and one on *Bacillus thuringiensis* (Delfin) were used as reference biocontrol agents. The aims were (i) to assess the pathogenicity of the selected fungal isolates to *Delia radicum*, (ii) to evaluate the fungal isolates' rhizosphere competence, with the emphasis on the persistence of the original inoculum on the growing roots, (iii) to assess possible endophytic plant tissue colonization, and (iv) to evaluate potential plant growth stimulating effects of the added inoculi. Significant pathogenicity of tested fungi against *Delia radicum* was confirmed in *in vitro* and glasshouse experiments. All tested fungi persisted on cauliflower rhizoplane. More importantly, the added fungi were found on thoroughly washed roots outside the original point of inoculation. This provided us with evidence that our tested fungi could be transferred via or grow with the elongating roots. In addition to colonizing the rhizoplane, some fungi were found inside the plant root or stem tissue, thus exhibiting endophytic characteristics. The importance of fungal ecology as a criterion in appropriate biological control agent selection is discussed.

1. Introduction

The cabbage root fly (CRF) *Delia radicum* (L.) and turnip root fly *Delia floralis* (Fallén) belong to the most important pests on Brassicaceous crops in Europe (Klingen et al., 2002). Several strategies have been developed to control these pests. They are based either on insecticide treatments (Straub, 1988; Yildrim and Hoy, 2003) or alternative methods (Dosdall et al., 1996; Dosdall, 1999; Finch and Collier, 1984; Nawrocka, 1996; Städler and Schöni, 1990; Yildrim and Hoy, 2003).

Entomopathogenic fungi kill dipterous insects after they are ingested by their insect host or through infection via external contact (Lacey et al., 2009; Thomas and Read, 2007). Both mechanisms imply that an effective biological control agent should live in juxtaposition of either the crop or the insect pest (Bruck et al., 2005; Singh et al., 2011). Several papers report the possibility of controlling *Delia* sp. larvae using entomopathogenic fungi (Bruck et al., 2005; Klingen et al., 2002; Vänninen et al., 1999). However, according to our knowledge, no entomopathogenic fungi-based biopesticides are currently registered for the control of CRF.

Because CRF maggots are soil dwelling (Harris and Svec; 1966; Söndgerath and Müller-Pietralla, 1996), any fungal biocontrol agent should be well-adapted to the soil (Abdul-Wahid and Elbanna, 2012; Harman et al., 2004; Verma et al., 2007) or the rhizosphere (Bruck, 2005; Meyling and Eilenberg, 2007; Pava-Ripoll et al., 2011; St Leger, 2008). However, solely entomopathogenic fungi were investigated until now as biological control agents against CRF (Bruck, 2005; Bruck et al., 2005; Klingen et al., 2002; Vänninen et al., 1999).

The focus of this research was to investigate a CRF biological control strategy involving young plantlet inoculation with various fungi in a glasshouse experiment. In addition to commonly known

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entomopathogens (Bruck, 2005; Meyling and Eilenberg, 2007), highly rhizosphere competent fungi (Bruck, 2005; Pava-Ripoll et al., 2011; St Leger, 2008), and fungi with the ability to survive as saprotrophs in soil (Abdul-Wahid and Elbanna, 2012; Harman et al., 2004; Verma et al., 2007), previously proven to significantly increase CRF mortality (Razinger et al., 2013), were used. We hypothesized that different ecological characteristics of the various fungal isolates will have an effect on their ability to colonize and persist in the rhizosphere, thereby offering dissimilar protection from the attack of ground dwelling CRF maggots. The aims were to (a) assess the pathogenicity of the selected fungal isolates to CRF in laboratory *in vitro* and glasshouse experiments, (b) assess the rhizosphere competence of the tested fungi, especially the persistence of the original inoculum on the extending roots, (c) evaluate potential endophytic traits and (d) evaluate potential plant-growth stimulating effects of the added inoculi to the cauliflower plants. Additionally, to assess the presence and potential influence of endogenous soil microorganisms and micro-biota, the glasshouse tests were performed in sterile and non-sterile soil.

2. Materials and methods

2.1. Delia radicum rearing

CRF were reared according to protocols kindly provided by Dr. M. Hommes (Julius Kühn Institute, Braunschweig, Germany) and Harris and Svec (1966) with slight modifications. The flies were kept in an environmental chamber with the following light and temperature regime: 18 h light at 20 °C and 6 h dark at 18 °C. Relative humidity was 77 ± 3%. The flies were fed glucose, honey, soy meal, powdered milk, brewers' yeast and water. Larvae were fed rutabaga. Eggs were regularly removed from the egg deposition vessels by flotation allowing the retrieval of fresh, less than two days old eggs for the experiments.

2.2. Fungi collection and growing

Seven fungal species were used in the *in vitro* and glasshouse bioassays. The fungal strains were isolated from various substrata in Slovenia. The isolates are kept in the mycological collection of the Agricultural Institute of Slovenia (Table 1).

All fungal isolates were grown on potato dextrose agar (PDA, Sigma–Aldrich Chemie GmbH, Steinheim, Germany), except *Trichoderma* spp. which sporulated faster and more abundantly on 1.5% malt extract agar (MEA, Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Fungal cultures were incubated at 24 ± 1 °C in darkness for 14 d. Conidial suspensions were prepared by scraping cultures with a sterile objective glass and transferred to 50 ml centrifuge tubes (Vitaris AG, Baar, Switzerland) in a laminar flow chamber. To the tubes 10 ml of 0.05% Tween 80 (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) was added. The tube was vigorously vortexed for 30 s and shaken on an orbital shaker (650 RPM, 30 min). This produced a suspension of approximately 10^8 conidia ml^{-1}. Conidial viability was assessed by plating 100 µl of a 10-fold dilution of the original suspensions onto MEA. The plates were incubated at 24 ± 1 °C. After 24 h they were covered with cover slips (20 × 40 mm) and observed under a microscope. Conidia were considered viable if the germination tube was at least twice as long as the diameter of the conidium. Test suspensions of the desired concentrations were prepared after the viability assessment of the spores using a haemocytometer (Faust Laborbedarf AG, Schaffhausen, Switzerland).

2.3. In vitro pathogenicity screening

The *in vitro* entomopathogenicity tests were designed to evaluate the effect of the various fungal isolates against CRF eggs or larvae. The experimental system consisted of a 100 ml PVC dish, into which sterile filter paper (9 cm in diameter) (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was placed. The filter paper was moistened with 1.5 ml of sterile demineralized water. On each filter paper a slice of rutabaga that had been surface sterilized with 70% ethanol, peeled and cut into pieces of approximately 5 × 20 × 35 mm in a laminar flow chamber, was placed. Five CRF eggs were transferred to the test dish using a small piece of sterile filter paper placed 35 mm from the rutabaga slices. A volume of 50 µl of 1×10^8 viable conidia ml^{-1} was directly applied to CRF eggs. The test vessels were sealed with two layers of parafilm and put into an environmental chamber set at 20 °C, 80% RH, without illumination. The insecticide 'Marshal 25 CS' (Maag Agro, Dielsdorf, Switzerland), based on carbosulfan (24.5% active ingredient) was used as a positive control, at recommended concentration of 0.1% (v/v). Additionally, Naturalis (Andermatt biocontrol AG, Grossdietwil, Switzerland, based on *Beauveria bassiana* ATCC 74040) and Delfin (Andermatt biocontrol AG, Grossdietwil, Switzerland, based on *Bacillus thuringiensis* var. *kurstaki*) were used as reference biocontrol agents, at recommended concentrations of 0.1 and 0.05% (v/v), respectively. Tween 80 (0.05%) was used as the negative control. In order to check the state of the experiments and the effects of potentially rotting rutabaga pieces, a so-called 'zero' control was made lacking CRF eggs. Five replicates per treatment with five eggs per individual test dish were made. The experiments were evaluated after 14 days for egg hatching rate, and after 35 days for counting pupae. Rutabaga was replaced after 14 days.

2.4. Glasshouse experiments

Cauliflower (*Brassica oleracea* L. var. *botrytis* subvar. *cultiflora* 'Neckerperle'; COOP, Switzerland) seeds were sown in industrial seeding trays consisting of 77 100 ml compartments filled with commercially available planting substrate (Floragard, Germany). After 17 days young plantlets were inoculated by pipetting 2 ml of 1.15×10^7 viable conidia (test isolates) or 2.3×10^7 CFU or infective units (Naturalis and Delfin), onto the germination substrate. The insecticide Marshal 25 CS was not used in glasshouse experiments. Twenty-four hours after inoculations, the young plants were transplanted into 2 l plastic pots containing either

Table 1
List of fungal isolates used in the study. All isolates originated from Slovenia.

Id number	Genus	Species	Host organism/isolated from
1154	*Metarhizium*	*anisopliae*	Soil
1868	*Metarhizium*	*brunneum*	*Agriotes* sp. imago
1174	*Beauveria*	*bassiana*	Soil
1828	*Clonostachys*	*solani f. nigrovirens*	Potato tuber
1873	*Trichoderma*	*atroviride*	Maize kernels infected by *Fusarium* spp.
1874	*Trichoderma*	*koningiopsis*	Maize kernels infected by *Fusarium* spp.
1876	*Trichoderma*	*gamsii*	Maize kernels infected by *Fusarium* spp.