Isolation and identification of entomopathogenic fungi and their evaluation against Tribolium confusum, Sitophilus zeamais, *and* Rhyzopertha dominica *in stored maize*

P. Barra, L. Rosso, A. Nesci & M. Etcheverry

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ORIGINAL PAPER

Isolation and identification of entomopathogenic fungi and their evaluation against *Tribolium confusum*, *Sitophilus zeamais*, and *Rhyzopertha dominica* in stored maize

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Abstract The objective of this study was to isolate and identify native entomopathogenic fungi from different components of maize agroecosystem, and evaluate their virulence against *Tribolium confusum*, *Rhyzopertha dominica*, and *Sitophilus zeamais*, three insect pest vectors of aflatoxigenic fungi. *Paecilomyces* and *Metarhizium* were the most abundant genera isolated from the soil. Identification of fungal cultures by DNA extraction, amplification, and sequencing showed that all isolates macro- and micromorphological identified as *P. lilacinus* were *Purpureocillium lilacinum.* The isolate JQ926223 showed the lowest LT_{50} for *T. confusum* (4, 66 days) and *R. dominica* (9, 38 days), and the isolate JQ926212 demonstrated similar LT_{50} for the three insects evaluated between the range of 11, 7 to 14, 95 days. Maximum mortality rate was observed for the isolate JQ926223. The isolates of *Purpureocillium lilacinum* JQ926223 and JQ926212 may be considered good candidates for biologic control in the ecosystem of stored maize.

Keywords Entomopathogenic fungi · Maize agroecosystem · Stored pest · LT_{50} · Mortality

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P. Barra · A. Nesci (✉) · M. Etcheverry
Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ruta Nacional 36, km 601, Río Cuarto, Córdoba, Argentina
e-mail: anesci@exa.unrc.edu.ar

L. Rosso
Istituto per la Protezione delle Piante, Consiglio Nazionale delle Ricerche (CNR), Bari, Italy

Introduction

Maize (*Zea mays* L.) is one of the main crops in Argentina. Over the sixty percent of maize produced in this country is exported (INAI 2009). To maintain or increase this percentage, maize producers and stockpilers must assure the importer countries good quality grains and free of contaminants. The saprophytic activity of different fungus, like species of the genus *Aspergillus*, is the main cause of degradation of agricultural products before and after harvest. Fungi of this genus can grow in a wide range of environmental conditions; therefore, preharvest, harvest, and storage conditions are ideal (Payne 1998). Potentially toxigenic *Aspergillus* section *Flavi* strains were extensively distributed in non-rhizospheric soil, debris, and insects of maize agroecosystem (Nesci and Etcheverry 2002). Moreover, *Aspergillus* section *Flavi* was constantly recovering from soil under different tillage practices (Nesci et al. 2006). The agroecosystem of stored grain has its own dynamic which depends on environmental, biologic, and substrate-specific factors. The interaction between the substrate, the biologic, and the abiotic factors may favor the invasion of *Aspergillus* species and lead to the concomitant production of mycotoxins called aflatoxins. Aflatoxins, especially aflatoxin B_1 (AFB_1), are considered the most carcinogenic, mutagenic, and teratogenic substance found naturally in foods and feeds (IARC 1993).

Insects are involved in the colonization of grains. They can provide sites for fungal infection through the damage that can result in grain. It is therefore essential to understand that in this biologic system, insects are a common problem. *Sitophilus zeamais* (Motschulsky), *Rhyzopertha dominica* (Fabricius), and *Tribolium confusum* (Jacquelin du Val) cause significant damage to stored maize (Mejía 2007). Damage may be direct, such as weight loss, reduced

germination, and reduced nutritional value of grain. Indirect damage can be heat and moisture migration, reservoir of disease, and distribution of microorganisms (White 1995). Previous studies show that certain insects that attack stored grains have the ability to disperse toxigenic *Aspergillus flavus* in those grains (Nesci et al. 2011a, b).

Knowing that many insects, besides damaging the grain, are vectors of *Aspergillus* section *Flavi* which facilitates increased levels of aflatoxins, integrated control strategies are necessary. Currently, most of the post-harvest management of insect pests is made with synthetic chemical insecticides. However, the use of synthetic insecticides allowed in argentine grains for export is very limited (Casini and Santajuliana 2012). In recent times, the research is aimed at finding ways to prevent the entry of xenobiotics in the food chain (Jayashree and Subramanyam 1999). Therefore, the search for natural methods of crop protection is still valid despite the fact that the market offers a wide variety of products. There are biologic agents with natural potential to protect crops. This derives from the intrinsic richness of species and their struggle for survival (Stoll 1989). Entomopathogenic fungi are natural enemies of a wide range of insects and some species are used as microbial bio pesticides (Tanada and Kaya 1993; James and Elzen 2001). Entomopathogenic fungi are distributed in a wide range of habitats including aquatic forest, agricultural, pasture, desert, and urban habitats (Sánchez-Pena 1990; Lacey et al. 1996; Chandler et al. 1997). The selection of virulent isolates adapted to local components of the agroecosystem is one of the most important aspects in the development of mycoinsecticides (Cortez-Madrigal et al. 2003). Soil is considered an excellent environmental shelter for entomopathogenic fungi since it is protected from UV radiation and other adverse abiotic and biotic influences (Keller and Zimmermann 1989). Isolation of entomopathogenic fungi involves soil sampling since that is their natural habitat (Asensio et al. 2003). It is also interesting to compare the diversity of entomopathogenic fungi in different components of the same ecosystem to obtain greater variety of antagonistic microorganisms (Cross et al. 1999; Bidochka et al. 1998; Asensio et al. 2003).

Numerous studies show that isolates of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin are potential microbial control agents against some stored product pests (Tanya and Doberski 1984; Adane et al. 1996; Hidalgo et al. 1998; Dal Bello et al. 2001; Ekesi et al. 2001; Padin et al. 2002; Khashaveh et al. 2008). Nevertheless, there is a constant search for other fungal biologic control agents, including nematophagous fungi like *Paecilomyces lilacinus* (Thom) Samson (Fiedler and Sosnowska 2007). *P. lilacinus* is a soil-inhabiting fungus that has shown promising results for use as an effective biocontrol agent (Morgan-Jones et al. 1984; Jatala 1986; Dube and Smart 1987; Khan et al. 2006).

The objective of this study was to isolate and identify native entomopathogenic fungi from different components of maize agroecosystem, and evaluate their virulence against *Tribolium confusum*, *Rhyzopertha dominica,* and *Sitophilus zeamais*, three insect pest vectors of aflatoxigenic fungi.

Materials and methods

Sampling site

Soil samples were collected from the University of Río Cuarto Experimental Field Station in Río Cuarto, Córdoba, Argentina (30°57′S latitude, 64°50′W longitude, 562 m altitude) during the maize growing season (2009–2010). Soil consisted of a sandy loam texture (pH 6.1 in water 1:1 v/v, 1.4 % organic matter, 86 ppm of nitrates).

Collection procedures

Sampling was performed during the first 20 days of development of the maize crop. Fifty samples of non-rhizospheric soil were collected. Each of the samples was taken from the top 3 cm of soil at different places within the field. The samples were taken in double diagonal section at 4 m intervals. Samples were individually placed in plastic bags and stored at −20 °C until analysis.

From 50 soil samples, 25 samples were selected randomly and dried at 50 °C during 24 h. Dried samples were passed through a testing sieve (2-mm mesh size) and the debris separated from the soil. The debris were placed in plastic bags and stored at −20 °C until use.

Isolation and identification of entomopathogenic fungi

Insect baiting method

Cultures of one strain of the confused flour beetle *Tribolium confusum* (Jacquelin du Val) were obtained from the Department of Agricultural Zoology, Faculty of Agronomy, University of Buenos Aires, Argentina. Mixed-sex adults 1–3 weeks old were used in the test. Insects were reared on a diet of wheat flour, corn starch, and yeast (10:10:1.5) in plastic containers containing 200 g of the mixture. Insects were reared at 27 ± 1 °C and 70 ± 5 % relative humidity (R.H.) and photoperiod of 12:12 h light:dark cycle. These insects were used to entomopathogenic fungi isolation. Three types of substrates were used; twenty five dried soil samples (250 g each) without debris,

10 not-dried soil samples (250 g each) with debris, and 5 maize grain samples (500 g each). Maize grains were harvested from plants grown in the same experimental maize field in the growing season 2009–2010. All samples were weighed in plastic jars of 500-ml capacity. Twenty adults of insects were placed per jar. All jars were placed in a chamber with controlled conditions 27 ± 1 °C and 70 ± 5 % relative humidity (R.H.) and incubated during 15 days. After 15 days, all samples were frozen at −20 °C to kill insects. Then, the soil and maize samples were sieved to recover the insects. Recovery insects were surface-disinfected using 1 % sodium hypochlorite for 1 min. The disinfected agent was eliminated and insects were washed two times in sterile distilled water (5 min each time). Insects were finally plated directly on semi-selective isolation media (SM) (maize meal 17 g l^{-1}, ClNa 17.5 g l^{-1}, rose bengal 75 mg l^{-1}, triton X-100 0.3 ml l^{-1}, chloramphenicol 50 mg l^{-1}, streptomycin 0.1 ml l^{-1}, cycloheximide 2.5 ml l^{-1}, and agar-agar 15 g l^{-1}, 1,000 ml) and incubated at 25 °C during 10 days. After the incubation period, colonized insects were analyzed. The colonies isolated from insects, suspected to be entomopathogenic fungi, were subcultured on Sabouraud Dextrose Agar (SDA) (trypticase 5 g l^{-1}, peptone beef 5 g l^{-1}, glucose 20 g l^{-1}, and agar–agar 15 g l^{-1}, 1,000 ml) and incubated at 25 °C during 10 days. The control treatment consisted of taking 80 insects from containers in which they were kept, and frozen at −20 °C to kill them. Then, the insects were surface disinfected and finally plated directly and incubated as described above. After microscopic observation, genus was assigned according to Samson (1974), Samson et al. (1988), Humber (2005) and Luangsa-ard et al. (2011).

Soil samples

The soil plating method was adapted from Garrido-Jurado et al. (2011). Enumeration of fungal propagules was carried out on solid medium, by the surface spread method, by blending 10 g of soil from each of the 25 samples randomly selected from a total of 50 samples with 90 ml 0.1 % peptone water solution. Serial dilutions from each sample and 0.1 ml aliquots were inoculated in SM medium and incubated at 25 °C for 13 days. Fungal count was expressed as $\log_{10}$ per g of soil. The fungi were subcultured to SDA medium to obtain pure cultures and identified by microscopic characteristics as described above.

Debris samples

The experience was conducted according to the methodology proposed by Nesci and Etcheverry (2002) with some modifications. Twenty five pieces of debris from each of the 25 samples were surface disinfected using 1 % sodium hypochlorite for 1 min. The disinfected agent was eliminated and debris pieces were washed two times in sterile distilled water (5 min each time). Debris pieces were finally plated directly on SM medium and incubated at 25 °C for 10 days. After the incubation period, colonized debris was analyzed. Count of possible entomopathogenic fungi was expressed as percentage of contaminated pieces. The fungi were subcultured and identified as described above.

Extraction of DNA from fungi grown in culture

The fungal cultures isolated in the previous assays were maintained in sterile soil (Abreu et al. 2003). All fungal cultures were grown on PDA medium for 7 days at 25 °C. Mycelial biomass was extracted for DNA analyses according to the procedure of Passone et al. (2010) with same modifications. An aliquot of 100 mg mycelium of each fungal isolate was transferred into microtubes. The mycelium was vortexed for 1 min in the presence of 300 µl of sterile water plus 700 µl of extraction buffer (100 mM Tris–HCl, 2 % CTAB, 1.4 mM NaCl) and glass beads (425–600 µm diameters) to favor the disruption of fungal material. After incubation at 65 °C for 60 min, 500 µl of chloroform was added to the sample, homogenized and centrifuged for 10 min at 13,000 rpm. The aqueous phase was recovered and 500 µl of chloroform was added. The sample was homogenized and centrifuged again for 10 min at 13,000 rpm. The aqueous phase was recovered and precipitated with 2 volumes of precipitation buffer (14 mM CTAB, 40 mM NaCl, pH 8). After incubation at room temperature for 1 h, the sample was homogenized and centrifuged for 5 min at 13,000 rpm. The sample was homogenized by inversion in presence of NaCl (1 M) 350 µl and chloroform 350 µl and centrifuged for 5 min at 13,000 rpm. Chloroform phase was recovered and precipitated with 0.6 volumes of isopropanol at −20 °C. After incubation at room temperature for 20 min, it was centrifuged for 10 min at 13,000 rpm and the aqueous phase was discarded. Finally, the DNA pellet was washed with 70 % ethanol and suspended in 25 µl of nuclease-free H_2O. Polymerase chain reaction (PCR; TECHNE TC-512, Barloworld, Scientific Ltd., UK) was conducted in 50 µl reactions with the following concentrations: 5 U μl^{-1} of Invitrogen (Brazil) *Taq* DNA polymerase, 5× Invitrogen Buffer, 2 mM of dNTPs Invitrogen, 1.5 mM Mg^{2+}, and 3 pmol μl^{-1} of each primer. The primers used were the fungus-specific forward primer EF1T (ATGGGTAAGGACAAGAC) and the reverse primer EF2T (GGAAGTACCAGTGATCATGTT) (O'Donnell et al. 1998) manufactured by Invitrogen Custom Primers (Carlsbad, CA). PCR was carried out by the following protocol: initial

denaturation at 94 °C for 1 min, followed by 31 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The reaction was held at 4 °C. The presence of PCR products was confirmed by gel electrophoresis on agarose gel.

Purification, sequencing, and identification of fungal DNA

Purification of PCR products was conducted using DNA Wizard Clean-Up Kit (product A9282, Promega, Madison, WI). To estimate the concentration of DNA needed for sequencing, purified PCR products were compared with Invitrogen's Low DNA Mass Ladder. All samples were sequenced using an Applied Biosystem ABI 3730 sequencer (Applied Biosystem). Each PCR product was sequenced in the forward and reverse directions, and consensus sequences were created by means of the BioEdit program version 7.0.9.0. (Thompson et al. 1994) (http://www.mbio.ncsu.edu/RNasaP/info/programs/BIOEDIT/bioedit.html).

Lethal time and percentage of mortality

The lethal time (LT_{50}), the number of days until 50 % of insects were dead, was determined for each of the 20 fungal suspensions. For this purpose, subsamples of maize grains of 500 g were put into plastic jars and then 20 adults of each insect pest *Tribolium confusum*, *Sitophilus zeamais*, and *Rhyzopertha dominica* treated by immersion for 30 s with 600 µl of 10^7 spores ml^{-1} suspensions of each entomopathogenic fungi (Goettel and Inglis 1997) were introduced in each jar (Padín et al. 1997). All jars were placed in a chamber with controlled conditions (27 ± 1 °C, 70 ± 5 % R.H., with photoperiod of 12:12 h light:dark cycle) (Wicklow et al. 1998). Mortality was analyzed during 20 days and compared with the untreated control samples. All dead insects were placed directly on plates containing water agar medium (agar–agar 15 g l^{-1}, distilled water 1,000 ml), which were incubated at 25 °C for 7 days to confirm that the inoculated fungus was the causal agent of the death of insects. The lethal time (LT_{50}) for each of the fungal isolates was analyzed by probit analysis.

Statistical analysis

Analysis of variance was performed on LT_{50} and mortality percentage using SigmaStat for windows version 3.0 (SPSS, Chicago, IL, USA). To establish differences, Holm-Sidak Method was performed.

Results

Isolating entomopathogenic fungi by the insect bait method

The total number of insects bait plated on semi-selective isolation medium were 800: 200 prior contact with 10 not-dried soil samples with debris, 500 prior contact with 25 dried soil samples without debris, and 100 prior contact with 5 maize grain samples. Table 1 shows the percentage of samples with fungal contamination, in which insects were in contact. In 90 % of soil samples and in 100 % of grain samples, insects showed fungal contamination. However, all insects placed in maize grains were colonized by *Fusarium.*

Samples of not-dried and dried soil used to place insects bait showed between 5–25 and 5–45 %, respectively, of fungal contamination. The highest percentages of infection were detected in dried soil samples. Only insects of 4 samples were completely free of fungal contamination.

Figure 1 shows the frequency of different fungal genera isolated from insect bait. They were *Penicillium*, *Fusarium*, and *Paecilomyces*. Genus *Penicillium* was isolated with more frequency reaching 56.3 % of infection. The 40.5 % of fungal isolates were identified as species from genera *Fusarium*; only 3.2 % were identified as species from genera known to include potential entomopathogenic species, such as *Paecilomyces.*

Entomopathogenic fungi isolated from soil and debris samples

Our results showed that micobiota population was different in soils and debris samples. Densities of filamentous fungi in soil samples varied between 2×10^2 and 1.1×10^4 ufc g^{-1} (Fig. 2a). Mycoflora analysis showed that the 64 % of total fungal population isolated was confirmed as fungus from possible entomopathogenic genera. Figure 2b shows that the main entomopathogenic genus isolated was *Paecilomyces*, comprising 75 % of the total isolates, while

Table 1 Fungal contaminations with potential entomopathogenic fungi from insects bait prior contact with not-dried soil samples, dried soil samples and maize grain, and percentage of debris contaminated

	% Infection (±ES)	
Substrate	Bait insects	Debris
Dried soil	21 ± 2.4	15.2 ± 3.7
Not-dried soil	10.5 ± 2.8	NE
Maize grain	100 ± 0	NE

NE not evaluated

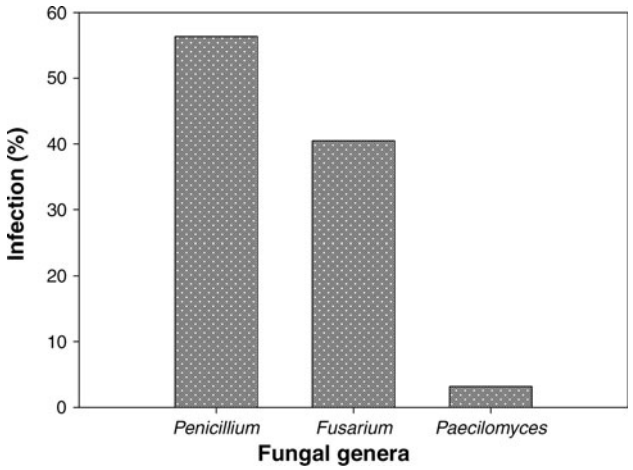

Fig. 1 Frequency of different fungal genera isolated from insects bait

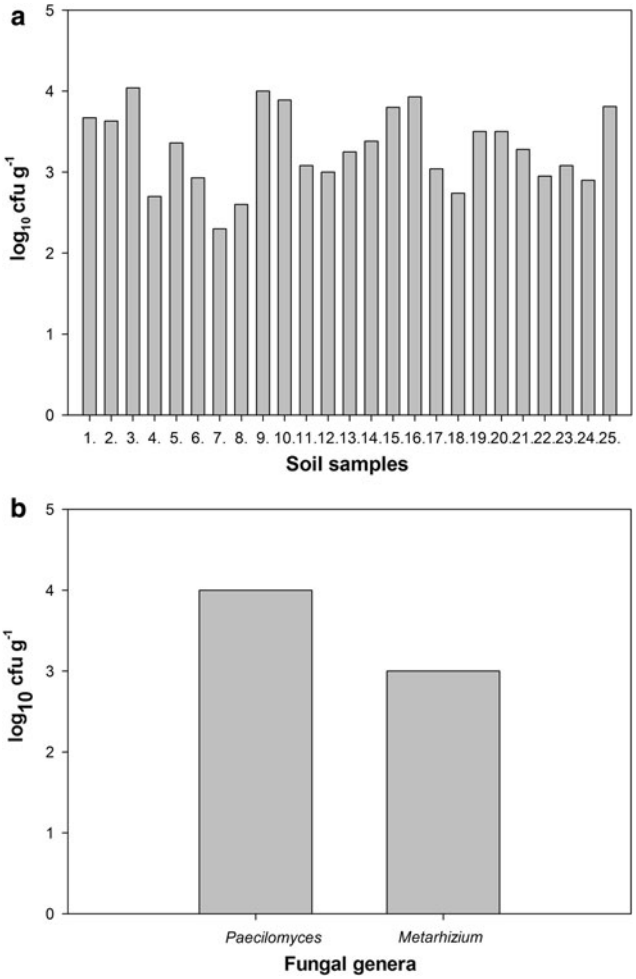

Fig. 2 **a** Incidence of potential entomopathogenic fungi in soil samples. **b** Incidence of different fungal genera isolates from soil samples

the remaining 25 % corresponded to isolates belonging to the genus *Metarhizium.* The counts were 4 and 3 log for *Paecilomyces* and *Metarhizium,* respectively.

Micobiota analysis of 25 debris samples showed that 40 % of samples (10 samples) were contaminated with fungi with percentage of debris contaminated between 4 and 36 % (Table 1). All fungi isolated were identified as *Fusarium* and *Penicillium*, none of interest in this study.

Identification of entomopathogenic fungus

The 35 *Paecilomyces* isolates were identified as *Paecilomyces lilacinus*. These isolates showed colonies on MEA of fast growing, attaining a diameter of 20–37 mm after 7 days at 25 °C. Colonies consisting of a basal felt with or without floccose aerial overgrowth, the color is white at first and becoming vinaceous, reverse mostly in shades of purple or sometimes uncolored. Conidiophores are erect with roughened thick walls around 3-µm wide consisting of verticillate branches with two to four phialides. The phialides measure 8 × 2.1 µm and have a swollen basal portion tapering into a short neck. Conidia in divergent chains are ellipsoidal to fusiform, hyaline, measuring 2.8 × 2.4 µm. No or restricted growth was observed at 37 °C with colonies of 2–3 mm of diameter.

All these isolates were selected for DNA analysis. Seventy-seven cultures isolates provided clean sequence results. All culture isolates, when sequenced and compared with those in GenBank, were identified as *Purpureocillium lilacinum* (Table 2). Fifteen isolates showed a 99 % homology to the BLAST identities for sequences from *P. lilacinum*. Nine isolates are identical to the BLAST identities and 3 isolates showed homologies of 98, 97, and 95 %.

Determination of lethal time (LT_{50})

The mortality in control treatment was low (<10 %) and no mycosis was detected on any insects. LT_{50} values for 20 isolates of *P. lilacinum* against *T. confusum* varied from 4.66 to 17.41 days (Table 3). However, LT_{50} values against *S. zeamais* and *R. dominica* were higher with values ranging between 11.13 to >62.5 days and 9.38 to 48.38 days, respectively. The isolate JQ926223 showed the lowest LT_{50} for *T. confusum* and *R. dominica*, but the highest LT_{50} for *S. zeamais*. The isolate JQ926212 demonstrated similar LT_{50} for the three insects evaluated with a range of 11.7–14.95 days. Maximum mortality rates, 20 days after fungus application for *T. confusum* and *R. dominica*, were observed for the isolate JQ926223 (90 and 65 %, respectively). The isolate JQ926212 showed a mortality rate of 45, 50, and 45 % for *T. confusum*, *S. zeamais,* and *R. dominica*, respectively. Fungal infection with *P. lilacinum* was confirmed for all dead insects in each treatment.

Table 2 Homology of translation elongation factor 1 alpha gene sequences from *Purpureocillium lilacinum* isolates with sequences in GenBank. Sequence informations and BLAST results are shown for closest accessions found in GenBank

GenBank accession N°	Culture strain	Sequence length	N° BP used in BLAST	BLAST results		
				Sequence homology *P. lilacinum* strain	Max identity (%)	Coverage (%)
JQ926202	46S	587	364	CBS 74988	100	62
JQ926203	56S	539	346	CBS 74988	99	64
JQ926204	68S	584	364	CBS 74988	99	62
JQ926205	61S	590	364	CBS 74988	100	61
JQ926206	64S	590	364	CBS 74988	100	61
JQ926207	66S	578	364	CBS 74988	99	62
JQ926208	14S	587	364	CBS 74988	99	62
JQ926209	62S	581	364	CBS 74988	99	62
JQ926210	32S	557	364	CBS 74988	99	65
JQ926211	44S	590	364	CBS 74988	100	61
JQ926212	50S	573	351	DTO 30H4	99	61
JQ926213	42S	586	361	DTO 149F4	100	61
JQ926214	38S	586	361	DTO 149F4	100	61
JQ926215	20S	525	364	CBS 74988	100	69
JQ926216	77S	590	364	CBS 74988	99	61
JQ926217	90S	578	364	CBS 74988	100	62
JQ926218	29S	587	364	CBS 74988	99	62
JQ926219	84S	590	364	CBS 74988	99	61
JQ926220	01S	590	364	CBS 74988	99	61
JQ926221	57S	590	364	CBS 74988	99	61
JQ926222	39S	590	364	CBS 74988	98	61
JQ926223	79S	590	364	CBS 74988	99	61
JQ926224	25S	580	355	CBS 74988	99	61
JQ926225	81S	590	364	CBS 74988	100	61
JQ926226	52S	396	227	CBS 74988	95	57
JQ926227	82S	325	154	DTO 149F4	97	47
JQ926228	22S	509	364	CBS 74988	99	71

Discussion

The methods used to isolate entomopathogenic fungi using bait insects (*T. confusum*) on three types of substrates, plating soil, and plating debris showed differences in growing potentially entomopathogenic fungi on semi-selective isolation media plates. Zimmermann (1986) suggests that the bait method as a standard isolation of entomopathogenic fungi is using the larvae highly susceptible *Galleria mellonella* (Linnaeus) (Lepidopera: Pyralidae). However, other studies showed that entomopathogenic strains more virulent were isolated more frequently from larval and adult stages of the insect to be controlled (Prior 1991; Klingen and Haukeland 2006) than with *G. mellonella*. All bait insects placed in maize kernels were contaminated with *Fusarium*. While bait insects placed in not-dried and dried soil showed contamination with *Penicillium*, *Fusarium*, and *Paecilomyces*. The lowest percentage corresponded to potentially entomopathogenic fungal colonies. No entomopathogenic fungus of interest was isolated from debris samples. In contrast, the higher number of possible entomopathogenic fungi like *Paecilomyces* and *Metarhizium* were obtained from soil samples. Thus, data suggest that these types of fungus are not distributed equally in different components of maize agroecosystem. Our results are similar to Demirci and Denizhan (2010) who while evaluating a potential biocontrol agent on apple rust mite showed that much of the fungi isolated from mite cadavers were saprophytic or common contaminants such as *Rhizopus* spp. and *Penicillium* spp., and *Paecilomyces* was the only one genera isolated known to be entomopathogenic.

Soil is the natural habitat of entomopathogenic fungi (Hajek 1997) and studies have shown differences in the relative abundance present in different soils like the agricultural use and soils adjacent to crops and forest

Table 3 LT_{50} and mortality percentage for *Tribolium confusum*, *Sitophilus zeamais* and *Rhyzopertha dominica* adults exposed during 20 days to 20 isolates of *P. lilacinum*

Isolates	*Tribolium confusum*		*Sitophilus zeamais*		*Rhyzopertha dominica*	
	LT_{50} (days)	Mortality (%)	LT_{50} (days)	Mortality (%)	LT_{50} (days)	Mortality (%)
JQ926202	17.41b	35h	11.13a	50a	12.23b	55c
JQ926203	14.8b	35h	62.5h	10h	37.6d	20h
JQ926204	16.79b	35h	>i	15g	43.18f	20h
JQ926205	12.4b	40g	37.92e	30d	20.34b	40e
JQ926206	6.81a	65c	26.62d	25e	15.47b	45d
JQ926207	8.36a	55d	18.16c	35c	26.04b	25g
JQ926208	8.47a	55d	53.3f	30d	22.9b	40e
JQ926210	14.41b	35h	>i	15g	14.49b	45d
JQ926211	15.48b	35h	37.75e	25e	18.24b	40e
JQ926212	11.7b	45f	13.99b	50a	14.95b	45d
JQ926214	5.58a	75b	36.58e	15g	21.9b	40e
JQ926216	10.7b	50e	52.75f	25e	21.6b	30f
JQ926217	9.89b	50e	59.33g	20f	17.58b	40e
JQ926218	12.11b	45f	>i	10h	48.38g	20h
JQ926220	7.81a	65c	19.47c	30d	11.75b	60b
JQ926222	8.19a	35h	51.16f	30d	25.1b	30f
JQ926223	4.66a	90a	>i	10h	9.38a	65a
JQ926225	10.79b	55d	27.58d	35c	40.43e	15i
JQ926226	5.75a	75b	>i	15g	32.16c	30f
JQ926228	12.75b	40g	38.21e	40b	23.48b	40e

>62.5 days

Values within a column followed by the same letter(s) are not significantly different at $P = 0.05$

(Bidochka et al. 1998; Keller et al. 2003). Many of the species belonging to the Phylum Ascomycota, such as *Beauveria* spp., *Metarhizium anisopliae*, and *Paecilomyces* spp., develop in the soil part of their life cycle in which are outside the insect hosts (Keller and Zimmermann 1989). Jenkins and Grzywacz (2000) proposed that there are competing microorganisms that produce active metabolites that may affect the viability of fungal propagules of interest. In a previous study in which evaluated soil fungal populations of fields for the cultivation of maize in Río Cuarto, Córdoba, Argentina, it was found that the predominant genera were *Aspergillus*, *Fusarium*, and *Penicillium* (Nesci et al. 2006). Pereira et al. (2010, 2011) found that the count of *Fusarium* spp., obtained from the same field, where we take the different samples for this study, was in the order of 4 and 2 log per g of maize and soil, respectively. It is possible that the high incidence of the genus *Fusarium* and *Penicillium* in different components of maize agroecosystem have influenced the colonization of the insects bait. Most *Fusarium* species are saprophytic and relatively abundant members of soil microbiota (Leslie and Summerell 2006). Many species are known as plant, insects, and humans pathogens, and there are species of *Fusarium* pathogenic for insects and non-pathogenic for plants (Majumbar et al. 2008). Some species isolated from insect larvae and adults were reported as pathogens of these insects (Claydon and Grove 1984; Sur et al. 1999) and in other studies as an opportunistic pathogen for insects in soil (Ali-Shtayeh et al. 2002; Sun Bing et al. 2008; Abdullah and Mohamed Amin 2009).

Tuininga et al. (2009) analyzing different field samples showed important deficiencies in environmental conditions needed to maintain entomopathogenic fungi in dry leaf litter. Probably, something similar occurs with our debris samples in which no fungi of interest for this study were detected. In agreement with other authors (Asensio et al. 2003) in our study, we confirm that soil is an important reservoir of entomopathogenic fungi, potential antagonist for controlling insect pests.

Paecilomyces belongs to the family of filamentous fungi, like *Aspergillus*, *Penicillium*, and *Fusarium* (Humber 1997), is present in soil and decaying organic matter, and is usually recognized as an infectious agent in animals and insects (Luangsa-ard et al. 2011). In this genus, there are nematophagous species like *Paecilomyces fumosoroseus* (Wize) (Hoodle 2011; Rodriguez Dos Santos and del Pozo Núñez 2003) and *Paecilomyces lilacinus* (Borisov and Ushchekov 1997; Gökce and Er 2005; Fiedler and

Sosnowska 2007). *P. lilacinus* was evaluated as biologic control agents against maize weevil (*Sitophilus zeamais*) (Ahmed 2010). *P. lilacinus* can be a human pathogen (Takayasu et al. 1977), but there is a large differentiation in host infection between strains of this fungus (Fiedler and Sosnowska 2007). This fungus has been found as the causal agent of infections in patients with compromised immune systems (Luangsa-ard et al. 2011). In our study, according to macro- and micromorphological characteristics, *Paecilomyces* isolates were identified as *P. lilacinus*. These characteristics are consistent with those described by Samson (1974), Samson et al. (1988), and Humber (2005). Identification of fungal cultures by DNA extraction, amplification, and sequencing showed that all isolates macro- and micromorphologically identified as *P. lilacinus* were *Purpureocillium lilacinum*. Luangsa-ard et al. (2011) show that species previously assigned to *Paecilomyces* like *P. lilacinus* was accommodated in the genus *Purpureocillium*.

In vitro assessment of potential entomopathogenic fungi against insect pests is one essential step in the selection of virulent strains before large scale application. Results of this study indicated that there was variability in the virulence of the 21 isolates of *P. lilacinum* against the three insect pest evaluated. Only one isolate, JQ926212, showed a similar virulence activity against the three insects assayed. Similar lethality levels were observed with different isolates of *M. anisopliae* against thrips (Sánchez-Pena et al. 2011). Rodriguez-Kabana et al. (1984) established significant differences in pathogenicity among different strains of *Paecilomyces lilacinus* against the nematode *Meloidogyne arenaria* (Chitwood). Adane et al. (1996) demonstrated that several isolates of *B. bassiana*, obtained from different coleopetan insects, against *S. zeamais* showed significant differences with respect to virulence. The isolate JQ926223 used in our study showed the highest mortality against *T. confusum* and *R. dominica*. Similar results with high mortality rates were observed with *M. anisopliae* against *Sitophilus granaries* (Linnaeus) (Khashaveh et al. 2008). Golnaz et al. (2011) showed that one isolate of *B. bassiana* had a LT_{50} of 10.45 days against *S. granarius*, similar to the average LT_{50} (10.23 days) of the 21 isolates of *P. lilacinum* against *T. confusum* evaluated in this study.

In conclusion, this study shows that the soil destined for cultivation of maize is an important reservoir of entomopathogenic fungi. The combination of methods, macro- and microscopic identification and DNA extraction and sequencing, are necessary to characterizing these fungi. The native soil *P. lilacinum* JQ926223 and JQ926212 will be evaluated as biologic control agents in the ecosystem of stored maize. Enhancement of the efficacy of different entomopathogens may still be required for successful use (Hallsworth and Magan 1994). These studies should be coupled with host-pathogen assays in the presence of biotic and abiotic stress factors. Major parameters for fungal growth, such as water requirements and humidity, should be examined in depth for their effect. Assays with induced changes in water stress are currently taking place for the above-mentioned isolated entomopathogenic fungi. In addition, sensitivity and tolerance assays should test the compatibility of theses entomopathogenic fungi with natural fungicides. This last point could contribute to an integrated management to reduce aflatoxins in stored grains.

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Conflict of interest The authors declare that they have no conflict of interest.

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