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Efficacy of Various Entomopathogenic Fungi Strains as Biocontrol Agents for Control of *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae)

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ABSTRACT
Cowpea seed beetle, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae), is considered an important bruchid pest in cowpea seed storages. The used pesticides against this pest have caused the occurrence of resistant populations and direct toxicity to the users. The objective of this study was to evaluate the mortality effects of six entomopathogenic fungi isolates obtained from ARSEF (USA) collection [*Paecilomyces farinosus* (2538), *Isaria fumosorosea* (4501), *Isaria farinosa* (3580), *Beauveria bassiana* (4984), *Lecanicillium muscarium* (972) and *Lecanicillium muscarium* (5128)] against *C. maculatus* adults under laboratory conditions (26±2 °C, 70±5% RH and 16h light: 8h dark). The isolates were cultivated in Potato Dextrose Agar (PDA, Oxoid, CM0139) medium at 26±2 ºC in dark conditions for two weeks before using them as control agents. Spore suspensions of the isolates were prepared at two different concentrations ($1x10^5$ and $1x10^7$) and mixed with Tween 20 (0.04%). Each concentration was replicated three times and the mortality rates were observed on the 2nd, 4th, 6th, 8th and 10th day of incubations. As a commercial control, a Mycotal extraction of *L. muscarium* and as a negative control, Tween 20+sterile water was used. Six entomopathogenic fungal isolates at both conidial concentrations yielded high mortalities (from 62.6% to 100%) of *C. maculatus* adults. These results illustrated that tested fungi strains led to significant mortalities on *C. maculatus* adults in all the treatments as compared to the controls. Consequently, these fungi strains were regarded as an encouraging alternative method to control the population of *C. maculatus* adults in the stored cowpea grains.

Keywords: Entomopathogenic fungus, Biological control, Cowpea seed beetle, Bruchid

1. Introduction

Legume plants are very important source of protein for human feeding in undeveloped countries. Among them, cowpea (*Vigna unguiculata* (L.) Walp.: Fabaceae) is grown for its green beans and seeds by farmers especially in tropical and subtropical regions of the world. It is also cultivated all over the world for animal feed (Ofuya & Akhidue 2005). Due to their prosperous source of nutritious elements, the fresh and green crusts of cowpea are consumed as vegetable. In addition, the leaf, branch and stem parts of the plant are used as fresh animal food for livestocks (Remya 2007).

Cowpea seed beetle (*Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae)) is one of the most destructive pests on cowpea and other legumes growing in tropical and sub-tropical countries, both in fresh green crusts in fields and in stored seeds (Singh & Van Emden 1979). The adults are not harmful. But, the larvae of this pest feed on cowpea (*V. unguiculata* (L.)), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik.), soybean (*Glycine max* Mer.) and haricot beans (*Phaseolus vulgaris* L.) (Mahfuz & Khalequzzaman 2007). The female adults of *C. maculatus* lay their eggs in the fresh cowpeas before the reaping in the field. The larvae from these eggs bore into the cowpea seeds, develop by feeding the embryo of the seeds and mature just about in a month in storage conditions (Fox & Tatar 1994). Therefore, the larvae can lead to both quantitative (due to grain weight loss caused by larvae feeding) and qualitative (due to product alterations such as loss of nutritious and aesthetic values, resulting increased levels of losses in the grain mass) damages on cowpea grains (Moina et al. 1998). This pest requires great care due to the potentials of severe damages mentioned above.

In the past, many commercial and persistent insecticides such as Phosphine, Methyl bromide, Deltamethrin and Malathion were largely used to control this pest in stored cowpea. But, these chemicals pose risks such as food and environmental

pollution, toxicities to non-target organisms, pest resistance, pesticide residues, direct toxicities to users and ozone depletion (Arthur 1996; Isman 2006; Khashaveh et al. 2011). Therefore, health authorities are reluctant to allow chemical insecticides due to their residues on stored grains. New alternative control strategies are required, because the commercial insecticides have quite toxic impacts on environment and human health. The growing research on biological protectant alternatives has revealed the positive role of microbial insecticides (Sheeba et al. 2001). One of the most promising and environment-friendly alternatives for pest control is entomopathogenic fungi. They are among the first entomopathogenic organisms used against harmful arthropods. Entomopathogenic fungi cause disease by infecting to insects or other arthropods and subsequently provoke rapid declines in large populations of their arthropod hosts. They have garnered the most interest to research for utilizing as microbial insecticides. For these reasons, their roles are crucial on biological control of hazardous insects because of environmentally safe and very low toxicity to mammalians (Cox & Wilkin 1996). The use of entomopathogenic fungi for biological control of the pests is an attractive alternative to classical pesticides, because these beneficial fungi are very friendly control agents against a wide range of organisms, and have no detrimental effects on environment (Khetan 2001; Sevim et al. 2013). Therefore, entomopathogenic fungi could be a viable alternative method to control this pest. Up to now, there are many studies with the use of entomopathogenic fungi to control insect pests, but very little focused on using fungi as control agents against stored product pests (Ferroni 1977; Serale & Doberski 1984; Moina et al. 1998; Sheeba et al. 2001; Gökçe & Er 2005; Sevim et al. 2010; Shifa Vanmathi et al. 2011; Muştu et al. 2014; Reddy et al. 2014; Erler & Ateş 2015; Komaki et al. 2017; Usanmaz Bozhüyük et al. 2018; Bjornson & Elkabir 2019).

The main objective of the present study was to investigate the efficacies of various entomopathogenic fungi isolates [(*Paecilomyces farinosus* (2538), *Isaria fumosorosea* (4501), *I. farinosa* (3580), *Beauveria bassiana* (4984), *Lecanicillium muscarium* (972) and *L. muscarium* (5128))] for the control of the adults of important and destructive storage pest; *C. maculatus*, under laboratory conditions.

2. Material and Methods

2.1. Storage pest insect

Callosobruchus maculatus (F.) (Coleoptera: Bruchidae) adults were collected from private store houses in Muğla, Turkey and kept on cowpea (*Vigna unguiculata* (L.) seeds. After, a certain amount of cowpea seeds was purchased from a local market and maintained at a freezer in two days at -15 ˚C in order to prevent any arthropod pests prior to in the bioassay. Then, *C. maculatus* adults were reared in 1 L jars containing cowpea seeds. The cultures were maintained in the dark conditions in a growth chamber set at 26±2 ˚C and 70±5% RH without exposure to any insecticide for several generations. Newly emerged adults (three days-old, mixed males and females) were used for subsequent experiments. All experimental procedures were carried out under the same conditions as the cultures. Experiments were carried out in three replicates with 25 adults of this pest in each Petri dish. Sufficient amount (1 seed/1 insect) of cowpea seeds were placed in each Petri dish for the adult insects during entomopathogenic tests.

2. 2. Entomopathogenic fungi isolates and preparation

The entomopathogenic fungi strains [(*Paecilomyces farinosus* (2538), *Isaria fumosorosea* (4501), *I. farinosa* (3580), *Beauveria bassiana* (4984), *Lecanicillium muscarium* (972) and *L. muscarium* (5128)] were obtained from an entomopathogenic fungi collection (ARSEF, USA). Another *L. muscarium* isolate, used as commercial control, was obtained from a commercial product (Mycotal, Koppert, NL). Fungal isolates were cultivated in the Potato Dextrose Agar (PDA, Oxoid, CM0139) medium at 26±2 ºC in dark for two weeks and used as spray source on the storage pests. Harvested conidia from 14-day-old cultures grown on PDA plates were thoroughly mixed with the carrier in screw capped bottles in 3 mL distilled sterile water. Spore solutions of entomopathogenic fungi isolates were prepared at $1x10^5$ and $1x10^7$ concentrations and mixed with Tween 20 (0.04%). The suspension was sieved and 1 mL of prepared suspension was sprayed on each replicate of 25 beetles in each Petri dishes. The sprayed Petri dishes were then incubated in an incubator at 26±2 ºC and the dead beetles were counted in each 48 h. For evaluating the conidial viability, the spores of different isolates were saved in the suspension of distilled sterile water and Tween 20, checked by light microscopy (Olympus BH2) after 2, 4, 6, 8 and 10 days.

2. 3. Bioassays

In order to test the toxicity of six entomopathogenic fungi, the applications were carried by adding 1×10^5 and 1×10^7 conidia to 1 mL in 9 cm diameter sterile Petri dishes with two layers of drying paper. Then, 25 newly emerged adults of *C. maculatus* were collected with an aspirator and placed in each Petri dish. Separately, sufficient amount of cowpea seeds (1 seed/1 insect) were added to each Petri dish. The prepared entomopathogenic fungal suspensions were sprayed on the adults and seeds contained in Petri dishes and incubated at 26±2 ºC. Each assay was repeated three times for each dose and exposure time combination. Mycotal extraction of *L. lecanii* was utilized as commercial control and distilled sterile water with Tween 20 as negative control in the study. After these treatments, the alive and dead *C. maculatus* adult individuals were counted in every 48 h for 10 days (Table 1).

Table 1- Percent mortalities of *Callosobruchus maculatus* (Fab.) adults inoculated with two different conidial concentrations ($1x10^5$ and $1x10^7$) of six entomopathogenic fungi isolates

Treatment Entomopathogenic fungi	*Dose*	*Callobruchus maculatus (Fabricius)*				
		Mortality (%)[a]				
		Days After Treatment[b]				
		2	*4*	*6*	*8*	*10*
Paecilomyces farinosus (2538)	$1x10^5$	65.3±3.52 bc	73.3±4.80 bc	84.0±8.0 b	94.6±2.66 cd	97.3±1.33 bc
	$1x10^7$	90.6±7.42e	92.0 ± 6.11cd	98.6 ± 1.33de	100 ± 0.0e	100 ± 0.0c
Isaria fumosorosea (4501)	$1x10^5$	77.3 ± 11.3bcde	84.0 ± 8.0bcd	90.6 ± 4.80bcde	97.3 ± 1.33cde	98.6 ±1.33bc
	$1x10^7$	76.0 ± 0.0bcde	80.0 ± 2.30bcd	86.6 ± 5.33bc	97.3 ± 1.33cde	100 ± 0.0c
Beauveria bassiana (4984)	$1x10^5$	78.6 ± 13.3bcde	78.6 ± 13.3bcd	89.3 ± 4.80bcde	98.6 ±1.33de	100 ± 0.0c
	$1x10^7$	86.6 ± 6.66de	86.6 ± 6.66bcd	100 ± 0.0e	100 ± 0.0e	100 ± 0.0c
Lecanicillium muscarium (972)	$1x10^5$	62.6 ± 4.80b	70.6 ± 4.80b	90.6 ± 4.80bcde	98.6 ± 1.33de	98.6 ± 1.33bc
	$1x10^7$	84.0 ± 12.0cde	92.0 ± 4.0cd	97.3 ± 2.66cde	100 ± 0.0e	100 ± 0.0c
Isaria farinosa (3580)	$1x10^5$	72.0 ± 12.0bcde	72.0 ± 12.0b	85.3 ± 8.11b	98.6 ± 1.33de	100 ± 0.0c
	$1x10^7$	78.6 ± 2.30de	88.0 ± 9.33bcd	97.3 ± 1.33cde	100 ± 0.0e	100 ± 0.0c
Lecanicillium muscarium (5128)	$1x10^5$	69.3 ± 7.05bcd	74.6 ± 9.33bc	88.0 ± 6.11bcd	93.3 ± 3.52c	97.3 ± 2.66bc
	$1x10^7$	89.3 ± 5.33 e	96.0 ± 2.30d	98.6 ± 1.33de	100 ± 0.0e	100 ± 0.0c
Commercial Control (*L. muscarium* (Mycotal))	$1x10^5$	78.6 ±1.33bcde	84.0 ± 2.30bcd	89.3 ± 1.33bcde	89.3 ± 1.33b	96.0 ± 2.30bc
	$1x10^7$	82.6 ± 7.42 cde	82.6 ± 7.42bcd	88.0 ± 2.30bcd	89.3 ± 1.33b	94.6 ± 3.52b
Negative Control (Tween20+steril water)	-	0.0 ± 0.0a	1.33 ± 1.11a	1.33 ± 1.11a	2.66 ± 1.11a	2.66 ± 1.11a

[a]: Numbers in each column are Mean ± SE of three replicates, each set-up with 25 adults; [b]: Exposure duration (day)
[*]: Values followed by different letters in the same column differ significantly at P<0.05

2. 4. Statistical analysis

Percent mortalities of *C. maculatus* adults were subjected to ANOVA with SPSS 17.0 software package and means were separated by Duncan's multiple range test at P<0.05.

3. Results

Mortality rates of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) adults treated with six entomopathogenic fungi isolates are provided in Table 1. The results showed that all tested entomopathogenic fungi isolates had different mortality effects on *C. maculatus* adults as compared to control treatments. In all experiments, the mortality rates generally increased with increasing exposure durations. The strain used as commercial control (*Lecanicillium muscarium*, Mycotal) was failed to achieve 100% mortality and the greatest rate of mortality with this *L. muscarium* strain was 94.6%. According to the results, the earliest 100% mortality on *C. maculatus* adults were obtained at 6 days after treatment (DAT) with *Beauveria bassiana* (4984) sprayed at $1x10^7$ spore concentration, under 26±2 °C and 70±5% RH. On the other hand, mortality rates were considerable higher even in the first day counts (2 DAT), $1x10^7$ concentration of *Paecilomyces farinosus* (2538) provided over 90% average mortality, where *B. bassiana* isolate (4501) killed 78.6% of the adults at lower ($1x10^5$) dosage, which was on par with the same dosage mortality results of commercial control (*L. muscarium*). In the second count day (4 DAT), one of the *L. muscarium* isolates (5128) were able to kill about 96% of the adults at $1x10^7$ dosage while *Isaria fumosorosea* (4501) treatments at lower dose have the highest mortality (84.0%) among the others. All of the isolates, except for *I. fumosorosea*, displayed over 95% mortality at 6^{th} day after treatments. The second greatest mortality at that day was achieved in *L. muscarium* (5128) treated Petri dishes, 98.6% of adults were recorded dead at high-dose treatments, which was closely followed by *I. farinosa* (98.6%), again at $1x10^7$ spore concentration. In the 8^{th} day after treatments, 100% of the adults were dead in Petri dishes treated with the higher dose of entomopathogenic isolates, again, with the exception of *I. fumosorosea*. And finally, at 10^{th} day, over 97% mortality was recorded in all fungi, tested. As seen in Table 1, the first two days (2^{nd} and 3^{rd}) and second two days (6^{th} and 8^{th}) had different efficiency levels. It is also worth to mention that all isolates yielded significantly different results at lower and higher dosages, but *I. fumosorosea* treatment yielded similar outcomes throughout the experimental period, except for 4^{th} day.

4. Discussion

In this study, six entomopathogenic fungi isolates [(*Paecilomyces farinosus* (2538), *Isaria fumosorosea* (4501), *I. farinosa* (3580), *Beauveria bassiana* (4984), *Lecanicillium muscarium* (972) and *L. muscarium* (5128)] were found as pathogenic on *C. maculatus* adults. Mortality rates of all isolates on *C. maculatus* adults, at both doses (1×10^5 and 1×10^7), increased gradually with longer exposure durations. Mortality of *C. maculatus* adults varied between 97.3 and 100%. Specifically, the adult mortalities were 97.3% with *L. muscarium* (5128) and *P. farinosus* (2538) at $1x10^5$ dose on 10^{th} day of treatment and 100% with all tested entomopathogenic fungi isolates at $1x10^7$ dose (Table 1).

Many studies were carried out by different researchers about the mortality effects of various entomopathogenic fungi against *C. maculatus*. Currently, *B. bassiana* has been identified as one of the most successful entomopathogenic fungi. The use of *B. bassiana* to control *C. maculatus* was studied by various researchers worldwide. Cherry et al. (2005) reported that the evolution of cumulative mortality among *C. maculatus* adults following immersion in aqueous conidial suspensions of *B. bassiana* 0362 yielded greater efficacy for *B. bassiana* 0362. The same authors pointed out that this was the most noticeable at $1x10^8$ conidia mL^{-1} where 100% mortality was achieved with *B. bassiana* 0362 after 6 days of treatment. Draganova et al. (2007) found that the isolates 417, 412, 414 and 426 of *B. bassiana* caused mycoses on *C. maculatus* adults, and the highest lethal effect to *C. maculatus* adults was expressed as 100% mortality on the 6^{th}, 7^{th} and 8^{th} day, respectively. Shifa Vanmathi et al. (2011) recorded the mortality rates at 1×10^5 (40%, 80%, 85.70%, 91.52%, 97.90%) and 1×10^7 doses (13.33%, 33.33%, 71.42%, 71.42% and 97.78%) after 24, 48, 72, 92 and 120 h on *C. maculatus* adults, respectively. Kılıç et al. (2019) determined that nine different isolates of *B. bassiana* led to different mortality rates at 1×10^7 dose and after 12 h of treatment on *Helicoverpa armigera* (Hübner) (51.9-68.1%), *Spodoptera littoralis* (Hübner) (45.5-54.4%), *Tenebrio molitor* (L.) (66.7-81.5%) and *Blattella germanica* (L.) (3.33-6.70%). In present study, *B. bassiana* (4984) isolates caused the mortality rates at 1×10^5 (86.60%, 86.60%, 100%, 100%, 100%) and 1×10^7 doses (78.60%, 78.60%, 89.30%, 98.60%, 100%) after 2, 4, 6, 8 and 10 days of the treatments on *C. maculatus* adults, respectively (Table 1). *I. fumosorosea* has been known as a common entomopathogenic fungus all over the world for more than 30 years (Zimmermann 2008). Sevim et al. (2013) determined that *I. fumosorosea* KTU–42 caused different mortality rates on *C. ciliata* adults (63%) and nymphs (50%). In the present study, we found that *I. fumosorosea* (4501) isolate caused different mortality rates in the 1×10^5 (76 %, 80%, 86.6%, 97.3%, 100%) and 1×10^7 (77.3%, 84%, 90.6%, 97.3%, 98.6%) doses after 2, 4, 6, 8 and 10 days of the treatments on *C. maculatus* adults, respectively (Table 1). *I. farinosa* is one of the most the commercially produced entomopathogenic fungi, which have an estimated 700 species of entomopathogenic fungi in approximately 90 genera. Yang et al. (2009) stated that *I. farinosa* had mortality effect on the larvae, pupae and adults of *Pissodes punctatus* (Coleoptera: Curculionidae) up to 88%. In another study, Muştu et al. (2011) recorded that on the 9^{th} and 12^{nd} days of the incubation, *I. farinosa* caused different mortality rates (22.5 and 45%) at $1x10^6$ and $1x10^8$ conidial concentrations (mL^{-1}) (52.5 and 70%) on *A. rostrata* adults. Correlatively, Demirci et al. (2011) found that *I. farinosa* had different mortality rates at 1×10^8, 1×10^7, 1×10^6, 1×10^5 doses and 95% RH on the adults of *Planococcus citri* (Risso) (84.53%, 32.29%, 19.24%, 20.54%), respectively. Muştu et al. (2014) determined that *I. farinosa* isolate had significant mortality on the sun pest adults (*Eurygaster austriaca* (Schrk.)). They found that *I. farinosa* caused different mortality rates (0.00%, 5.00%, 10.00% and 33.75%, 63.75%, 86.25%) at 1×10^6 and 1×10^8 conidia concentration doses mL^{-1} after 6, 9 and 12 days of treatments on *E. austriaca* adults, respectively. In present study, *I. farinosa* (3580) isolate caused different mortality rates (72%, 72%, 85.3%, 98.6%, 100%; and 78.6%, 88%, 97.3%, 100%, 100%, respectively) at 1×10^5 and 1×10^7 doses on the 2^{nd}, 4^{th}, 6^{th} and 10^{th} days of the treatment on *C. maculatus* adults. These percentages were significantly higher than the control ($P<0.05$) (Table 1). *P. farinosus* has been known as an effective entomopathogenic fungus for more than 30 years. Today, because it is one of the most important biocontrol agents, its various strains are successfully used for biocontrol of different pestiferous insects, such as whiteflies (Zimmermann 2008). Vidal et al. (1997) demonstrated that 29 isolates of *P. farinosus* had highly significant mortality (from 68 to 94%) on the silverleaf whitefly, *Bemicia argentifolii* Bellows & Perring. Simova & Draganova (2003) found that *P. farinosus* had a mortality effect on two spotted spidermite, *Tetranychus urticae* K.. In another study, it was stated that *P. farinosus* isolates (290 and 290re) showed lethal effect on *Ips sexdentatus* Boer. and *I. acuminatus* Gyll. adults (45.00 and 66.67%, respectively) at 1×10^8 conidia/mL (Draganova et al. 2007). In this study, it was found that *P. farinosus* isolate (2538) caused different mortality rates on *C. maculatus* adults in the 1×10^5 and 1×10^7 doses after 2, 4, 6 and 10 days of the treatments, as 90.6%, 92.0%, 98.6%, 100%, 100%; and 65.3%, 73.3%, 84.0%, 94.6% and 97.3%, respectively (Table 1). *L. muscarium* is also among the important entomopathogenic fungi. It is known as one of the important natural enemies of *Scolypopa australis* (Walker) in kiwi orchards (Marshall et al. 2003). This fungus is a commercially produced entomopathogenic fungi and has been commercialized worldwide as the biopesticides Mycotal (a commercial formulation of *L. muscarium* produced) against whiteflies and thrips; and Verticillin against whiteflies, aphids and mites (Faria & Wraight 2007). In a previous study, it was stated that *L. muscarium* isolates had mortality effects on the adults and nymphs of *Bactericera cockerelli* (Sulc) (up to 100% for adults and 70% for nymphs) at the 1×10^7 dose after seven days of the treatments (Mauchline & Stannard 2013). In present study, *L. muscarium* isolate (972) caused greater mortality effect (84.0%, 92.0%, 97.3%, 100%, 100%) at 1×10^7 dose than at 1×10^5 dose (62.6%, 70.6%, 90.6%, 98.6%, 98.6%) within all exposure times of the treatments on *C. maculatus* adults. However, *L. muscarium* isolate (5128) caused 89.3%, 96.0%, 98.6%, 100% and 100% mortality rates at 1×10^7 dose and after 2, 4, 6, 8 and 10 days on *C. maculatus* adults in this study, respectively. But, the same fungus isolate had lower mortality effect (69.3%, 74.6%, 88.0%, 93.3% and 97.3%) at 1×10^5 dose and within the same exposure times. These percentages were significantly higher than the control ($P<0.05$) (Table 1). Komaki et al. (2017) tested six entomopathogenic fungi isolates ((*P. farinosus* (2538), *I. fumosorosea* (4501), *I. farinosa* (3580), *B. bassiana* (4984), *L. muscarium* (972) and *L. muscarium* (5128)) against *Tribolium confusum* du Val., 1863 adults under laboratory conditions. In their study, these entomopathogenic fungi isolates led to the mortalities between 37.3 (for *I. farinosa* (3580) at 1×10^5 dose) and 100% (for *P. farinosus* (2538) and *B. bassiana* (4984) at 1×10^7 dose) on *T. confusum* adults after 10 days of treatment. In present study, the mortality rates were recorded as between 62.6 (for *L. muscarium* (972) at 1×10^5 dose) and 100% (for all fungi isolates at different doses) on *C. maculatus* adults after 10 days of treatment. According to these results, six fungi isolates showed greater effect on *C. maculatus* adults than *T. confusum* adults (Table 1).

5. Conclusions

In the present study, six entomopathogenic fungi isolates [(*P. farinosus* (2538), *I. fumosorosea* (4501), *I. farinosa* (3580), *B. bassiana* (4984) and *L. muscarium* (2 isolates)] were tested against *C. maculatus* adults under laboratory conditions. Among the tested fungi isolates, *L. muscarium* (972) caused the greatest mortality rates (between 84 and 100%) on *C. maculatus* adults at 26±2 °C, 70±5% RH and with $1x10^7$ conidial concentrations (mL^{-1}) on the 2nd, 4th, 6th, 8th and 10th days. The lowest mortality was recorded at $1x10^5$ conidial concentration of *L. muscarium* (972) isolate as 62.60% on the 2nd day of the treatment. Present findings showed that the fungal isolates used in this study could be used as possible biocontrol agents against *C. maculatus* adults. Further studies should be carried out to evaluate the effectiveness of these isolates in the field. It was concluded that use of biological control agent of *L. muscarium* (972) isolate as a part of integrated pest management strategy may reduce the future dependence on chemical control.

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