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This contribution is dedicated to the memory of Prof. Dan Gerling, a scientist, a colleague and a friend

Potential of entomopathogenic fungus *Isaria javanica* for controlling the potato tuberworm *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae)

Xinyue Cheng[1*], Yaoyao Gao[1], Chunlei Yang[1], Xi Zhang[1], Feifei Qin[1], Jun Lu[1], Yulin Gao[2] & Rumei Xu[1*]

[1]*College of Life Sciences, Beijing Normal University, Beijing, 100875 China*
[2]*Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, 100193 China*
**Corresponding authors: chengxy@bnu.edu.cn, xurumei@bnu.edu.cn*

ABSTRACT

The potato tuberworm *Phthorimaea operculella* is an important cosmopolitan quarantine pest of the Solanaceae crops. Comparing the relative virulence of five entomopathogenic fungi against *P. operculella*, the results showed that *Isaria javanica* was more effective against eggs, larvae and pupae compared to the other tested isolates. Mean infection rates of *ca* 80% were obtained for individuals at different life stages treated with 1×10^8 conidia/mL. Based on the linear regression analyses of the mortality data, a median lethal concentration $<1\times10^6$ conidia/mL was calculated for eggs, larvae and pupae, suggesting that this isolate of *I. javanica* may be a useful biocontrol agent for *P. operculella*. Variation was observed in the growth rates and sporulation of *I. javanica* on different culture media and at different temperatures. When cultured on peptone maltose agar, the fungus grew quickly, but sporulation rates were higher on corn meal agar and potato dextrose agar, producing 8.16×10^3 and 7.39×10^3 conidia/cm^2, respectively. When cultured at different temperatures, the fungus grew faster at 27 °C, and produced more conidia when grown at temperatures under 30 °C. Our results indicate that *I. javanica* is a promising biocontrol agent for the potato tuberworm.

KEYWORDS: Biological control, entomopathogenic fungus, *Isaria javanica*, *Phthorimaea operculella*, potato tuber moth, potato tuberworm.

摘要

马铃薯块茎蛾 *Phthorimaea operculella* 是茄科作物上重要的世界性检疫害虫。本文通过比较五种不同昆虫病原真菌对马铃薯块茎蛾的相对毒力，发现与其它测试菌株相比，爪哇棒束孢 (*Isaria javanica*) 对马铃薯块茎蛾的卵、幼虫和蛹的作用效果更为明显，用浓度为1×10^8 孢子/mL 的孢子悬浮液处理各虫态，平均死亡率约80%。通过线性回归分析所得爪哇棒束孢对卵、幼虫和蛹的平均致死中浓度（LC_{50}）均小于1×10^6 孢子/mL，表明爪哇棒束孢对马铃薯块茎蛾有较好的生防效果。该生防真菌在不同培养基上和不同温度条件下的生长速率和产孢能力具有一定的差异。在蛋白胨麦芽糖琼脂（SMA）培养基上，其生长速率最快，但在玉米粉琼脂（CMA）培养基和马铃薯葡萄糖琼脂（PDA）培养基上，其产孢量最大，分别为 8.16×10^3孢子/cm^2 和7.39×10^3 孢子/cm^2。在27 ºC 培养条件下，其生长速率最快，但在30 ºC 培养条件下，其产孢量最大。以上研究结果表明，爪哇棒束孢是具有广阔应用前景的生防因子，可用于马铃薯块茎蛾的生物防治。

关键词：生物防治，昆虫病原真菌，爪哇棒束孢，*Isaria javanica*，马铃薯块茎蛾，*Phthorimaea operculella*.

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INTRODUCTION

The potato tuberworm *Phthorimaea operculella* (Zeller, 1873) (Lepidoptera: Gelechiidae), also known as the potato tuber moth or tobacco splitworm, is now a worldwide pest, which originated in tropical mountainous regions of South America (Rondon 2010). The larvae feed on plants of the Solanaceae, damaging potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*), both in the field and in storage. Outdoor, larvae feed by mining leaves, stems, petioles and tubers, causing considerable yield loss. In stored potatoes, larvae bore tunnels and feed inside the tubers, causing deterioration of the crop quality (Trivedi & Rajagopal 1992; Rondon 2010; Gill *et al.* 2014). In China, the potato tuberworm is an important quarantine pest. It was first reported on tobacco in Guangxi (Southern China) in the 1930s, and has now spread to more than ten provinces. It has become a devastating pest of potatoes in Southwest China, causing production loss of 20–30 % in the field and up to 100 % in stored potatoes (Li *et al.* 2005; Liu *et al*. 2015). Several approaches have been undertaken to develop integrated pest management systems for *P. operculella*. These include modified agricultural techniques, chemical control, biological control, sex pheromone trapping, *etc*. (Rondon 2010). Among them, biological control is an effective and environmentally friendly method. To date, some predators, parasitoids, pathogenic nematodes and entomopathogenic microbes including viruses, bacteria and fungi attacking *P. operculella* have been identified, but few have been utilized commercially (Li & Zhang 2005; Rondon 2010). Additional investigations are needed to assess their utility as crop protection tools.

Entomopathogenic fungi are important components in biocontrol programs. *Metarhizium anisopliae* and *Beauveriat bassiana* are well-known biocontrol agents that have been successfully commercialized. Laboratory studies showed that both fungi are active against *P. operculella*, particularly when applied to younger larvae (Hafez *et al.* 1997; Sewify *et al.* 2000; Li & Zhang 2005), but there are limited data on the feasibility of using these organisms for potato tuberworm control (Lacey & Kroschel 2009). The entomopathogenic fungus *Isaria javanica* (Friedrichs & Bally) Samson & Hywel-Jones (syn. *Spicaria javanica*, *Paecilomyces javanicus*) (Ascomycota: Pezizomycotina: Sordariomycetes: Hypocreales: Cordycipitaceae) (Luangsa-ard *et al.* 2005), is a potential biocontrol agent. This fungus is commonly isolated from corpses of lepidopteran insects (Samson 1974; Chen *et al.* 2007; Specht *et al.* 2009; Shimazu & Takatsuka 2010), beetles (Samson 1974; Cabanillas & Jones 2009) and whiteflies (Scorsetti *et al.* 2008), as well as from soil and insect hibernation sites (Hu *et al.* 2011; Hasan *et al.* 2012). Pathogenicity tests have shown that the fungus is efficient against caterpillars (Lepidoptera) (Hu *et al.* 2007; Shimazu & Takatsuka 2010), sap-sucking insects (Hemiptera), such as whiteflies (Scorsetti *et al.* 2008; Kim *et al.* 2014; Xie *et al.* 2016), psyllids (Gallou *et al.* 2016), aphids (Hasan *et al.* 2012), leafhoppers (Chen *et al.* 2014), and other insects—termites (Isoptera) (Lopes *et al.* 2011), thrips (Thysanoptera) (Hu 2014),

and fire ants (Hymenoptera) (Hu *et al.* 2011). An isolate of *I. javanica* [Garusajang] has been developed to control *Bemisia tabaci* (Gennadius) in Korea (Kim *et al.* 2014). However, its efficacy against the potato tuberworm is unknown.

In this paper, we present results of a preliminary screening assay comparing relative virulence of five entomopathogenic fungi, viz. *I. javanica*, *Paecilomyces lilacinus*, *Lecanicillium psalliotae*, *L. attenuatum* and *L. muscarium*, against the pest, and of a series of assays to evaluate *I. javanica* against different developmental stages of the potato tuberworm. Based on these results, we then evaluated growth parameters of the fungus to determine its potential as a microbial biocontrol agent for *P. operculella*.

MATERALS AND METHODS

Insect rearing

Adults of the potato tuberworm collected from a potato field in the Yunnan province (Southwest China) were put in a jar for eggs collection, and hatched larvae were reared on potato tubers (Fig. 1). A stable population was established and maintained on potato tubers in an insect rearing chamber at 27±0.5 °C (with a photoperiod of 12L:12D). For the bioassays, eggs were collected on filter paper during oviposition, and 4th instar larvae and pupae were collected from potatoes.

Fungal culture and preparation of conidia suspensions

Isaria javanica (strain Pj01) was isolated from the common cutworm *Spodoptera litura* Fabricius (Lepidoptera) (Hu *et al.* 2007). The other four fungi—*Paecilomyces lilacinus*, *Lecanicillium psalliotae*, *L. attenuatum* and *L. muscarium*—were originally recovered from tomato roots, knot-root nematodes, cysts of nematodes and aphids, respectively. All these fungal strains kept at -80 °C were first cultured on potato dextrose agar (PDA, Table 1) at 25±0.5 °C. Pure cultures were then prepared from single-spore isolates and their identity confirmed by ITS-PCR (Zare

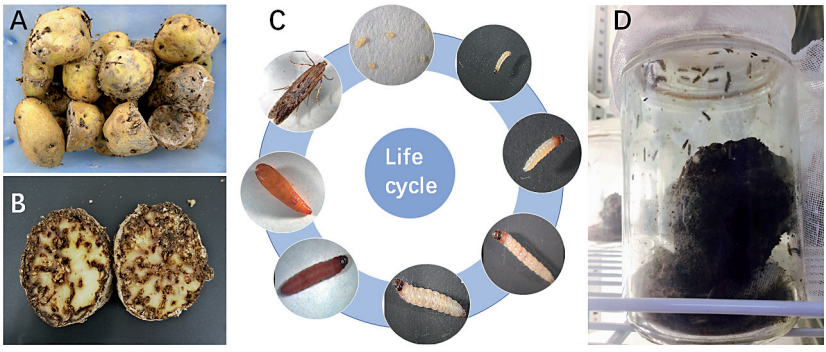

Fig. 1: Potato tuberworm *Phthorimaea operculella* rearing in the laboratory: (A) larvae feed on potatoes; (B) inside of a damage potato; (C) life cycle of *P. operculella*; (D) the insect cultured in a jar for egg collection.

Table 1. Components of culture media.

Medium	Component (for 1000 mL)
PDA	Potato 200 g, dextrose 20 g, agar 20 g
SMA	Peptone 10 g, maltose 20 g, agar 20 g
SDA	Peptone 10 g, dextrose 20 g, agar 20 g
CMA	Corn meal 40 g, agar 20 g
Czapek	$NaNO_3$ 3 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, sucrose 30 g, agar 20 g

& Gams 2008). Each isolate was then cultured in potato dextrose broth (PDB) that was shaken at 140 rpm at 27 °C for 4–5 days. After having filtered out mycelium, conidia were collected by centrifugation (4000 rpm) and washed with sterile double distilled water (DDW) 3 or 4 times. Suspensions were adjusted to definite concentration that were determined using a hemocytometer. The initial concentration was 1×10^8 conidia/mL.

Comparison of virulence among five entomopathogenic fungi

Each egg batch was treated with 0.1 mL of conidial suspension (1×10^8 conidia/mL), whereas 4th instar larvae and pupae of *P. operculella* were applied with 10 µL per individual (1×10^8 conidia/mL). Control batches of eggs/larvae/pupae were treated with DDW only. Then, eggs and pupae were transferred into 9-cm diameter Petri dishes, while larvae were put into separate wells of 24-well plates containing a piece of potato as food. A wet cotton ball was put in each dish and plate to maintain humidity. Dishes and plates were held in a growth chamber at 27 °C. For the egg assays, each treatment batch contained more than 40 eggs and five replicate batches were inoculated. For larvae and pupae, at least 30 individuals were treated with each fungus, and each treatment was replicated three times. Insects/eggs were examined four days later after inoculation for signs of infection, and then recording mortality/infection continued for several days until the insects died or molted to the next stage.

Defining the *I. javanica* virulence

Eggs, 4th instar larvae and pupae of *P. operculella* were collected as described above and treated with different concentrations of *I. javanica*; controls were treated with DDW only. Eggs were treated with seven concentrations (1×10^6, 5×10^6, 7.5×10^6, 1×10^7, 5×10^7, 7.5×10^7, 1×10^8 conidia/mL), while larvae and pupae were treated with five (1×10^6, 5×10^6, 1×10^7, 5×10^7, 1×10^8 conidia/mL). At least 30 individuals were treated with each concentration and the experiment was replicated three times. Corrected mortality was calculated as the following:

$$\text{Corrected mortality (\%)} = \frac{\text{treatment mortality} - \text{control mortality}}{100 - \text{control mortality}} \times 100$$

Growth and sporulation of *I. javanica*

To assess how nutritional component and culture conditions influence the fungal growth rate and conidial production, we tested growth rate and conidiation of *I. javanica* on the five commonly used media, i.e., potato dextrose agar (PDA), peptone maltose agar (SMA), peptone dextrose agar (SDA), corn meal agar (CMA) and Czapek-Dox agar (Table 1), and at five temperatures (i.e., 18, 21, 24, 27 and 30 °C) on PDA only. To quantify growth, a 6 mm diameter PDA agar plug was cut down from a Petri dish, where the fungus was cultured on, and placed in the center of each agar medium, and then plates were held at 25±0.5 °C. The radial diameter of each developing colony was measured in two directions every two days for 14 days. At that time, two pieces of mycelium (6 mm diameter) were cut from the centers of the developing fungal colony along the two axes, and each was placed into a separate 50-mL centrifuge tube containing 10 mL 0.1 % tween-80. Tubes were shaken at *ca* 400 rpm for 100 min to displace conidia from the growing medium. The concentration of conidia in the resulting suspension was determined using a hemocytometer. For each treatment, samples were taken from six culture plates and the experiment was repeated three times.

Statistical analysis

Independent samples T-test was performed to compare difference of virulence between *I. javanica* and each other fungus, and ANOVA analysis to compare differences of growth and sporulation of *I. javanica* under different culture conditions, using SPSS software (version 20.0) with a 95 % confidence interval. Simple linear regression analysis between conidial concentration (logarithmic value) and mortality was performed. Statistical significance was considered when *P*-value was less than 0.05.

RESULTS

Comparison of virulence

Of the five entomopathogenic fungi tested against *P. operculella* at 1×10^8 conidia/mL, *I. javanica* appeared to be the most virulent (Fig. 2). The mean infection rates of *I. javanica* on egg, larvae and pupae were 86.85, 79.44 and 88.89 %, respectively. Infection rates of the other four pathogenic fungi (*L. attenuatum*, *L. muscarium*, *L. psalliotae* and *P. lilacinus*) were significantly lower. The highest infection rate on eggs was 30 % (*L. psalliotae*), on larvae, 22 % (*L. muscarium*) and on pupae, 46 % (*L. muscarium*). *Isaria javanica* infection levels were significantly higher on eggs ($df=(4,24)$, $F=130.039$, $P<0.001$), larvae ($df=(4,13)$, $F=21.278$, $P<0.001$) and pupae ($df=(4,13)$, $F=257.940$, $P<0.001$).

Susceptibility of *P. operculella* to different concentrations of conidia

Eggs, larvae and pupae of the potato tuberworm were exposed to a range of *I. javanica* conidia concentrations. As concentration increased, mortality levels for each developmental stage also increased. When treated with suspension containing 1×10^6 conidia/mL, corrected mortality rates of *ca* 60 % (eggs and larvae) or

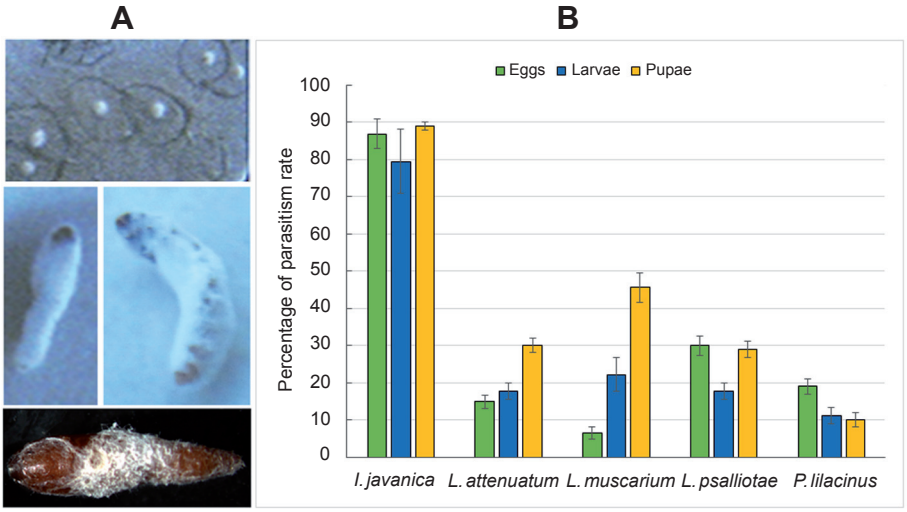

Fig. 2: Infection of the potato tuberworm by different entomopathogenic fungi: (A) *Isaria javanica* infection of eggs, larvae and pupa of *P. operculella*; (B) comparison of obtained infection levels following treatment of *P. operculella* eggs, 4th instar and pupae with 1×10^8 conidia/mL of five entomopathogenic fungi. The standard error of the mean is shown on each bar.

higher (pupae) were obtained. When treated with suspension containing 5×10^6 conidia/mL, mortality rates increased to >70% for all developmental stages. This increased to *ca* 80% when treated with suspension with 1×10^7 conidia/mL. Regression analyses show a clear linear relationship between conidial concentration and insect mortality; regression equations showed high correlation coefficient *r* values (Fig. 3, Table 2). Based on linear regression equations, it is estimated that the median lethal concentrations (LC_{50}) for eggs, larvae and pupae of *P. operculella* are all $<1\times10^6$ conidia/mL.

Influences of culture condition on the growth and sporulation of *I. javanica*

Differences in the radial growth rate of *I. javanica* were observed on the growing media tested, although temperature appeared to have a greater influence. The fungus grew relatively quickly on SMA compared to the other four media (PDA, CMA, SDA and Czapek-Dox) (Fig. 4A). No obvious differences in growth were detected among the media by day 5. The growth rate on PDA was slower than on the other media on days 8 and 11, and was clearly influenced by temperature. Growth was slowest at 18 °C, increased at higher incubation temperatures, peaking at 27 °C and declining at 30 °C (Fig. 4B). Analysis of variance (ANOVA) LSD test showed that the growth rate of the fungus at 18 °C was significantly slower than at other temperatures, whereas the growth rate at 27 °C was significantly faster (all $P<0.001$), while differences in growth rate among the other three temperatures (21, 24 and 30 °C) were not significantly different.

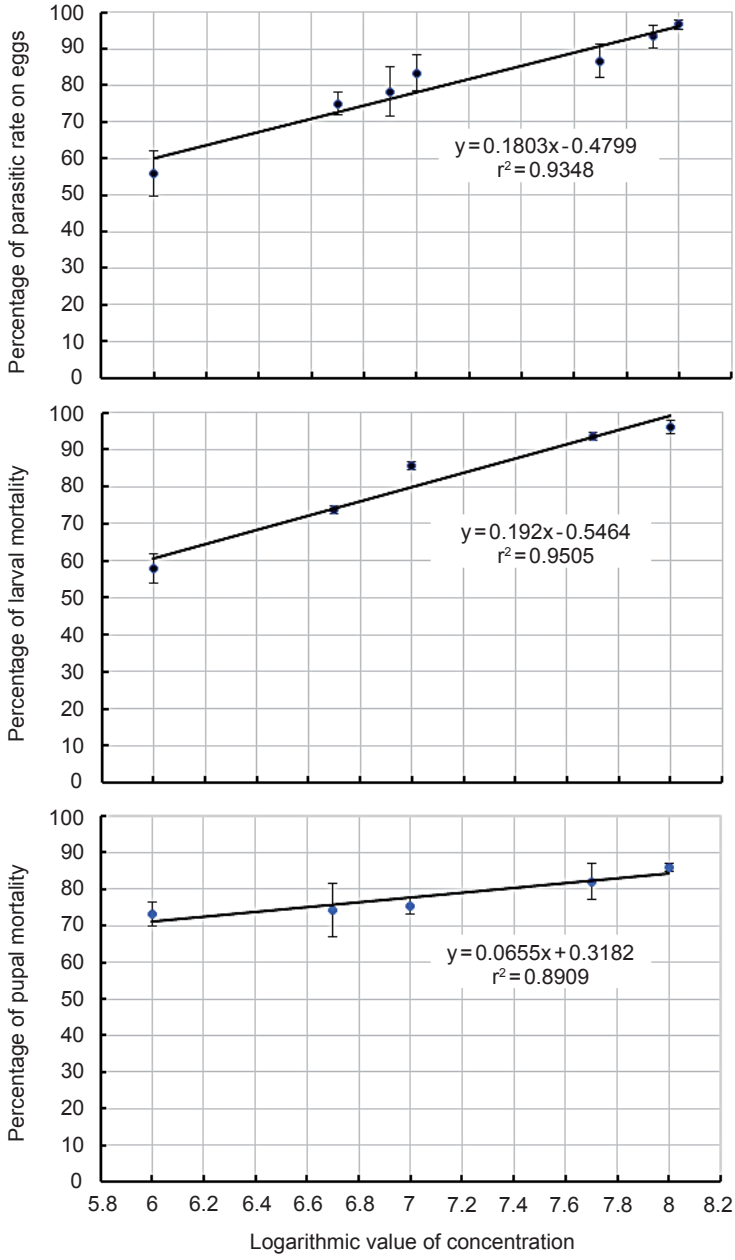

Fig. 3: Regression analysis between conidial concentration of *I. javanica* and mortality of eggs, larvae and pupae of the potato tuberworm. Each bar shows the standard error of the data point.

Table 2. Parameters of the regression analysis.

Stage	N	Adjusted R^2	Standard Error	Significance F	Intercept	Slope	t Stat	P-value	Lower 95%	Upper 95%
Egg	7	0.9218	0.0381	0.0004	-0.4799	0.1803	-3.1289	0.0260	-0.8742	-0.0856
Larva	5	0.9339	0.0404	0.0048	-0.5464	0.1920	-3.0330	0.0562	-1.1196	0.0268
Pupa	5	0.8545	0.0211	0.0158	0.3182	0.0655	3.3796	0.0431	0.0186	0.6178

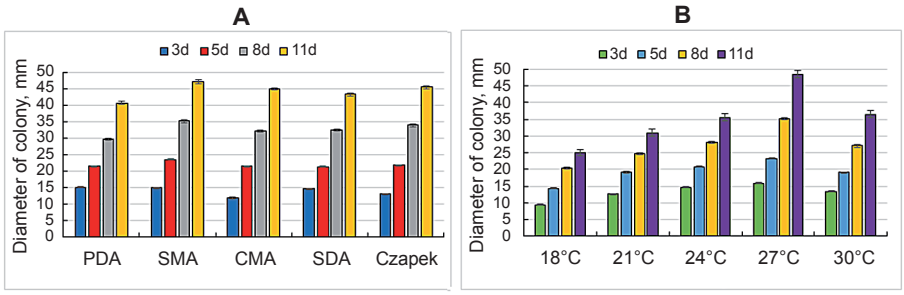

Fig. 4: Growth rate of *I. javanica* mycelium cultured on different media (A) and at different temperatures (B).

Production of conidia by *I. javanica* on the five media and at five different temperatures was also evaluated. Conidiation was notably different depending on the culture medium (at 25 °C) (Fig. 5A). Conidia production was highest on the PDA and CMA media, with a mean of 7389 and 8155 conidia/cm^2, respectively. On SDA, conidia production was significantly lower, with a mean value of 1333 spores/cm^2. Very few conidia were produced on the SMA medium, and no conidia developed on the Czapek medium. The differences in conidia productions are statistically significant (df=(4,69), F=7.0914, P<0.001). When grown on PDA at various temperatures, differences in conidiation were also observed (Fig. 5B). At lower temperatures (18 and 21 °C), few conidia were produced, with mean values of 1333 and 2500 spores/cm^2, respectively. As the temperature increased, the production of conidia raised exponentially, with the mean of 7167, 37738 and 332500 conidia/cm^2 at 24, 27 and 30 °C, respectively. The differences are statistically significant (df=(4,69), F=64.98, P<0.001). Therefore, conidiation is enhanced at higher incubation temperatures.

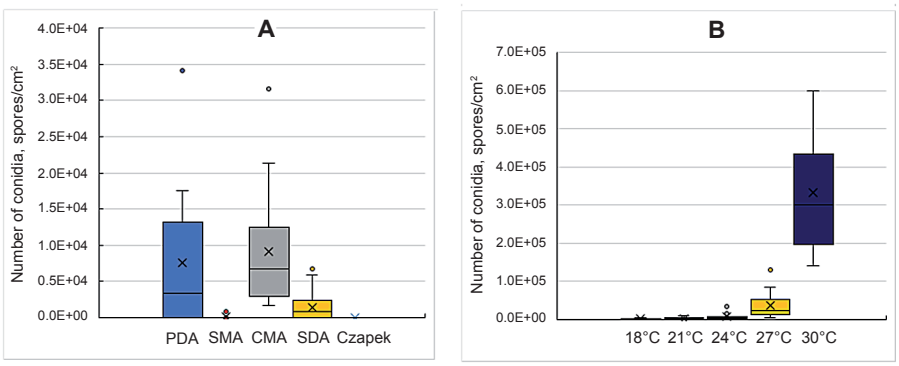

Fig. 5: Sporulation of *I. javanica* on different media (A) and at different temperatures (B).

DISCUSSION

In previous studies, *I. javanica* has shown great potential as biocontrol agent for several insect pests. It was reported that *I. javanica* URM4993 caused 100% mortality of the Asiatic termite *Coptotermes gestroi* (Wasmann) after six days (LC_{50} of 4.86×10^5 conidia/mL and LT_{50} of 2.26 days) (Lopes *et al.* 2011). It also caused 88.15% mortality of green peach aphid *Myzus persicae* (Sulzer) with LT_{50} of four days; 85% mortality in five days against southern yellow thrips *Thrips palmi* Karny; and 79.62% mortality in eight days against the tea moth *Andraca theae* (Matsumura) (Hu 2014). Another study demonstrated that *I. javanica* was pathogenic to two aphid species, *Hyalopterus pruni* (Geoffroy) and *Aphis pomi* de Geer, causing 66.67 and 75.59% mortality, respectively, after six days following inoculation with a suspension containing 1×10^8 conidia/mL (Hasan *et al.* 2012). The conidial suspensions (10^7 conidia/mL) of *I. javanica*, produced on barley alone or on barley supplemented with 5% of either silkworm powder or ground *Spodoptera exigua* larvae and sprayed onto eggplant leaves infested with 2nd instar nymphs of sweet potato whitefly *Bemisia tabaci* biotype Q, caused >90% mortality (Xie *et al.* 2016). *Isaria javanica* was moderately virulent against the gypsy moth *Lymantria dispar* (Linnaeus) (mortality 58–100%) when dipping of larvae into the conidial suspension (10^8 conidia/mL) (Shimazu & Takatsuka 2010), and also pathogenic to the fire ant *Solenopsis invicta* Buren, causing mortality ranging from 81–92% depending on the isolate tested (Hu *et al.* 2011). Our present results show that *I. javanica* is more effective than the other four fungal isolates tested against eggs, larvae and pupae of *P. operculella*, with mean infection rates of *ca* 80% when treated with 1×10^8 conidia/mL (Fig. 2). Based on regression analyses, all median lethal concentrations (LC_{50}) for eggs, larvae and pupae were $<1\times10^6$ conidia/mL. These values are considerably lower than those obtained with *B. bassiana* in a previous study, where LC_{50} values of 4.7×10^8 conidia/mL were calculated for 1st to 4th instars (Hafez *et al.* 1997).

To develop a fungal bioinsecticide, the agent must have a strong sporulation ability and be able to produce large numbers of conidia on artificial substrates. In previous studies, *I. javanica* strain Pf04 produced 10^8 conidia/g on barley substrate at 25 °C; the maximum conidial production obtained was 3.5×10^9 conidia/g on dry substrate after 15 days of cultivation using optimal conditions (Xie *et al.* 2016). Another study showed that *I. javanica* produced 3.43×10^{10} conidia/g when cultivated on barley and 3.05×10^{10} conidia/g when grown on brown rice. By supplementing the growing substrate with additives, the yield and pathogenicity of *I. javanica* conidia could be increased (Kim *et al*. 2014; Xie *et al.* 2016). In our study, *I. javanica* strain Pj01 produced 332,500 spores/cm^2 when cultured on PDA at 30 °C. These results suggest that *I. javanica* has characteristics that favor further investment in its development as a biological insecticide to control the potato tuberworm.

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