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Efficacy of *Clonostachys rosea*, as a promising entomopathogenic fungus, against coleopteran stored product insect pests under laboratory conditions

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Abstract

Background: Efficacy of new isolates of the entomopathogenic fungus (EPF), *Clonostachys rosea*, against adult stage of the most serious coleopteran stored product insect pests in Iraq, *Trogoderma granarium* Everts (Coleoptera: Dermestidae), *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) and *Callosobruchus maculatus* Fabricius (Coleoptera: Chrysomelidae), was evaluated under laboratory conditions. Two isolates of *C. rosea*, associated with the green peach aphid (*Myzus persicae* Sulz.), were isolated and investigated. Efficacy of *C. rosea* isolates was evaluated by two concentrations (1×10^8 and 1×10^6 conidia ml^{-1}).

Results: Corrected mortality rates caused by both *C. rosea* isolates, 6 days post-treatment, with 1×10^8 conidia ml^{-1}, ranged from 70.7 to 75.7%. Fungal infection caused 37–53% reduction in total fecundity of the adult females of the three tested insect species, 6 days post-treatment.

Conclusion: Obtained results demonstrated that *C. rosea* isolates had potentials as a biological control agent against coleopteran stored product insect pests. However, further studies under commercial storage conditions are required.

Keywords: Entomopathogenic fungus, *Clonostachys rosea*, Stored product insect pests, *Trogoderma granarium*, *Tribolium castaneum*, *Callosobruchus maculatus*, Potential, Iraq

Background

The khapra beetle *Trogoderma granarium* Everts (Coleoptera: Dermestidae), the red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) and the cowpea weevil *Callosobruchus maculatus* Fabricius (Coleoptera: Chrysomelidae) are among the most important and widespread insect pests of stored grains and products worldwide (Lal et al. 2017). These stored insect pests cause economic damages including seed weight loss, decrease of market value, decrease in germination viability of seeds and decrease in nutritional value, particularly proteins (García-Lara and Saldivar 2016).

Chemical insecticides such as cypermethrin, deltamethrin and pirimiphos-methyl are still the main control method to manage these stored insect pests. However, extensive use of such chemical insecticides has been associated with many problems (Kavallieratos et al. 2017) including development of insecticide resistance, negative effects on human and animal health and other non-target organisms and environmental contamination (Koureas et al. 2012). To reduce the adverse effects of insecticides, scientists are attempting to develop alternative methods for management of these insect pests in storage environments.

Microbial control agents have been widely used as alternatives for controlling stored insect pests (Mohammed et al. 2019). Several entomopathogenic fungi (EPF),

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such as *Metarhizium anisopliae* Metchnikoff (Hypocreales: Clavicipitaceae), *Beauveria bassiana* Bals.-Criv. (Hypocreales: Cordycipitaceae), *Lecanicillium* spp. Gams and Zare (Hypocreales: Cordycipitaceae) and *Isaria fumosorosea* Wize (Hypocreales: Clavicipitaceae) have been well reported as a promising biocontrol agent of stored insect pests including *T. granarium* (Mohammed et al. 2019) *T. castaneum* (Rehman et al. 2020) and *C. maculatus* (Ozdemir et al. 2020).

The entomopathogenic fungus (EPF), *Clonostachys rosea* Schroers (Hypocreales: Bionectriaceae) (formerly *Gliocladium roseum*), has a broad-spectrum mycoparasite effect on reducing the growth and associated diseases of several plant-pathogenic fungi, including *Alternaria* spp. Nees (Pleosporales: Pleosporaceae), *Bipolaris sorokiniana* Drechsler ex Dastur (Pleosporales: Pleosporaceae), *Botrytis cinerea* Pers. (Helotiales: Sclerotiniaceae) and *Fusarium culmorum* Sacc. (Hypocreales: Nectriaceae) (Xue 2003; Jensen et al. 2004; Nobre et al. 2005). Potential effects of *C. rosea* as a microbial control agent for the control of insect species including leafhopper, *Sonesimia grossa* and *Oncometopia tucumana* (Hemiptera: Cicadellidae) (Toledo et al. 2006), and sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Anwar et al. 2018), have been also reported. However, there are no available published articles on the efficacy of *C. rosea* isolates as EPF against stored product insect pests. Thus, such information may help in developing management strategies of utilizing microbial control as part of an integrated pest management strategy against *T. granarium*, *T. castaneum* and *C. maculatus*.

The objective of this study was to evaluate the biocontrol potential of two *C. rosea* isolates against the most important stored product insect pests (*T. granarium*, *T. castaneum* and *C. maculatus*) under laboratory conditions. The effect of fungal infection on the fecundity of the studied insect species was also examined.

Methods

Insect culture

Colonies of *T. granarium*, *T. castaneum* and *C. maculatus* were collected initially from the Entomology Laboratory, Faculty of Agriculture, Iraq. Insects of each species (50 male and female pairs) were reared on their main stored product in 300-ml plastic jars, secured with a muslin cloth and rubber bands and maintained at 30±2°C and 65±3% RH at continuous darkness for 2 generations. Grains were sterilized by placing them in a freezer (Samsung Ltd, Thailand) at −20°C for at least 2 days before each experiment. *T. granarium* was reared on whole sterilized wheat grains, *C. maculatus* was reared on sterilized cowpeas (*Vigna unguiculata* L. var. Parastoo) and *T. castaneum* was reared on sterilized wheat flour. Adults were removed after 5 days, and wheat grains, cowpea grains and wheat flour with the insect eggs were maintained under the laboratory conditions described above. Same-age cohorts of adult *T. granarium*, *T. castaneum*, or *C. maculatus* were obtained after 40–45 days and used in the treatments.

Source and preparation of *C. rosea* isolates

Two isolates of *C. rosea* [AA80 (MT366561) and AA82 (MT366214)] were obtained from dead *M. persicae* adults, collected from a greenhouse at the Faculty of Agriculture. Isolates were identified as *C. rosea*, following general and specific identification keys of Schroers et al. (1999). DNA amplification, sequencing and phylogenetic analysis technique were used to confirm morphological identification. Both isolates were cultivated on Sabouraud dextrose peptone yeast extract agar (SDAY) at 25°C. Aerial conidia were harvested from 14-day-old cultures by adding 15 ml of 0.01% Tween 80 to culture agar Petri dishes and gently scraping the surface of the cultures with a sterile inoculating loop to dislodge the conidia from the surface of the agar plates. The conidial suspension was pipetted from the Petri dish and filtered through 3 layers of cheesecloth. The number of conidia in the suspension was estimated, using a haemocytometer (Neubauer improved, Superior Marienfeld, Germany). The resulted suspension was diluted to the desired concentrations with 0.01% Tween 80 as required. The viability of the conidia was determined by spraying (0.1 ml of 1×10^6 conidia ml^{-1}) on a sterile Petri dish with 1.5% SDAY. The dishes were sealed by a parafilm and incubated at 20°C, 90±2% RH, and a photoperiod of 16:8 (L:D) h. After 24 h, the number of germinated spores per 100 spores of each Petri dish was assessed under the microscope (×400 magnification). Germination was considered positive when the length of germ tube was at least half of the spore length. The viability exceeded 96% for both isolates.

Virulence of *C. rosea* isolates

Ten Petri dish replicates, each with 5 pairs (male and female adults), were used for each EPF isolate and control (0.02% sterile aqueous Tween 80 only) for either *T. granarium*, *T. castaneum*, or *C. maculatus*. Whatman No. 1 filter paper was placed in a Petri dish. Afterwards, pairs of each of *T. granarium*, *T. castaneum*, or *C. maculatus* were released into the dishes and sprayed with 2 ml of conidial concentrations of each *C. rosea* isolate (1×10^6 or 1×10^8 conidia ml^{-1}), using 1l handheld sprayer and air-dried for 30 min at room temperature. Then, 5 g of sterilized wheat grain, cowpea grain or wheat flour were added to each Petri dish. All Petri dishes were sealed by a parafilm and incubated at 25±2°C, 70±2% RH and a photoperiod of 12:12 (L:D) h.

Table 1 The corrected mortalities of *Callosobruchus maculatus*, *Trogoderma granarium* and *Tribolium castaneum* adults treated with conidia of two *Clonostachys rosea* isolates. The conidial concentration of each entomopathogenic fungal isolate used for treatment was 1×10^8 conidia ml^{-1}

Insect species	EPF isolate	Corrected mortality (%)					
		1 day	2 days	3 days	4 days	5 days	6 days
Callosobruchusmaculatus	*Clonostachys rosea* isolate AA80	0.0 aA	3.0 aA	20.0 aB	38.2 aC	57.3 aD	75.7 aE
	Clonostachysrosea isolate AA82	0.0 aA	2.0 aA	21.4 aB	39.1 aC	58.0 aD	75.1 aE
Trogoderma granarium	*Clonostachysrosea* isolate AA80	0.0 aA	0.0 aA	19.0 aB	37.0 aC	57.2 aD	73.5 aE
	Clonostachys rosea isolate AA82	0.0 aA	1.0 aA	20.0 aB	36.1 abC	55.0 abD	71.7 abE
Tribolium castaneum	*Clonostachys rosea* isolate AA80	0.0 aA	0.0 aA	17.6 aB	34.8 bC	54.9 bD	70.7 abE
	Clonostachys rosea isolate AA82	0.0 aA	0.0 aA	17.4 aB	35.3 bC	53.2 bD	71.4 bE

Values within a column followed by the same lowercase letters indicate no significant differences among insect species of both EPF isolates; values within a row followed by different uppercase letters indicate significant differences among days after treatment within each insect species and EPF isolate combination at $P = 0.05$ using LSD test

Mortality rate was recorded daily for 6 days only, because the number of dead *T. granarium* and *C. maculatus* in the control significantly increased after 6 days of treatment, which was likely to influence the death rate of the fungus-treated insects. Dead insects were surface sterilized by rinsing twice with 70% ethanol for 30 sec, then rinsed with sterilized distilled water, before being placed on water agar (3 g of agar/l of water) in 9-cm Petri dishes for 5 days to confirm infection by EPF (Mohammed and Hatcher 2016). Cadavers were regarded as died from infection with these fungi, if conidia were recovered from the cadavers. The median lethal time (LT_{50}) values for each fungal isolate were calculated. The entire experiment was repeated twice.

Effect of *C. rosea* treatment on the fecundity of coleopteran stored pests

The effect of infection with *C. rosea* isolates on the fecundity was determined for *T. granarium*, *T. castaneum* and *C. maculatus* adult females, male-female (1–2 days old) pairs of each insect species (each pair was a replicate). The experiment followed the method described in the 'Virulence of C. rosea isolates' section, where each pair was sprayed by 2 ml of each fungal isolate at a concentration of 1×10^6 conidia ml^{-1}. Insects in the control treatment were treated by 0.02% sterile aqueous Tween 80 only. Each treatment was replicated 20 times. The number of eggs produced by each female was recorded daily until the female's death using a magnifying lens.

Statistical analysis

Cumulative mortality was corrected for natural death in the control using Abbott's formula (Abbott 1925). Normal distribution of data was assessed, using the Shapiro-Wilk test. Corrected mortalities were login transformed when necessary to meet the assumption of normality. Data used to determine the LT_{50} values of *C. rosea* isolates were calculated, using the probit analysis method. Two-factor repeated measurement was used to determine the effect of fungal isolate and insect species on the efficacy of *C. rosea* isolates. The effect of fungal infection on the fecundity of either *T. granarium*, *T. castaneum* or *C. maculatus* adult females was analysed separately using one-way repeated measurement ANOVA. Mean comparisons were performed using LSD test at 5% level of significance ($P < .05$).

Table 2 Median lethal time (LT_{50}) of two isolates of *Clonostachys rosea* against adult stage of *Callosobruchus maculatus*, *Trogoderma granarium* and *Tribolium castaneum*. The conidial concentration of each entomopathogenic fungal isolate used for treatment was 1×10^8 conidia ml^{-1}

Insect species	EPF isolate	Slope ± SE [log 10 (dose)]	LT_{50}	95% CL	χ^2
Callosobruchus maculatus	*Clonostachys rosea* isolate AA80	3.28 ± 0.69	4.83	4.30–5.63	1.54
	Clonostachys rosea isolate AA82	3.77 ± 0.45	4.79	4.04–5.80	2.73
Trogoderma granarium	*Clonostachys rosea* isolate AA80	4.12 ± 0.82	4.81	4.23–6.11	0.96
	Clonostachys rosea isolate AA82	2.98 ± 0.57	4.86	4.35–5.95	1.79
Tribolium castaneum	*Clonostachys rosea* isolate AA80	3.49 ± 0.72	4.93	4.47–6.24	2.48
	Clonostachys rosea isolate AA82	4.32 ± 0.84	4.95	4.15–6.33	1.24

Table 3 The corrected mortalities of *Callosobruchus maculatus*, *Trogoderma granarium* and *Tribolium castaneum* adults treated with conidia of two *Clonostachys rosea* isolates. The conidial concentration of each entomopathogenic fungal isolate used for treatment was 1×10^6 conidia ml^{-1}

Insect species	EPF isolate	Corrected mortality (%)					
		1 day	2 days	3 days	4 days	5 days	6 days
Callosobruchus maculatus	*Clonostachys rosea* isolate AA80	0.0 aA	2.0 aA	9.0 aB	27.2 aC	41.3 aD	50.7 aE
	Clonostachys rosea isolate AA82	0.0 aA	0.0 aA	9.4 aB	26.1 aC	41.0 aD	50.1 aE
Trogoderma granarium	*Clonostachys rosea* isolate AA80	0.0 aA	0.0 aA	8.3 aB	24.7 aC	38.2 aD	50.5 aE
	Clonostachys rosea isolate AA82	0.0 aA	1.0 aA	8.6 aB	25.6 aC	39.0 aD	49.7 aE
Tribolium castaneum	*Clonostachys rosea* isolate AA80	0.0 aA	0.0 aA	7.6 aB	24.8 aC	38.9 aD	47.7 aE
	Clonostachys rosea isolate AA82	0.0 aA	0.0 aA	8.4 aB	25.3 aC	39.2 aD	48.4 aE

Values within a column followed by the same lowercase letters indicate no significant differences among insect species of both EPF isolates; values within a row followed by different uppercase letters indicate significant differences among days after treatment within each insect species and EPF isolate combination at $P = 0.05$ using LSD test

Results

Virulence of *C. rosea* isolates

The pathogenicity of two isolates of *C. rosea* [AA80 (MT366561) and AA82 (MT366214)] was investigated against adults of *T. granarium*, *T. castaneum* and *C. maculatus*, using 2 conidial concentrations (1×10^8 and 1×10^6 conidia ml^{-1}). The results showed that conidial concentration of both *C. rosea* isolates significantly affected the corrected mortality of all the 3 tested species of insect adults after 6 days of application ($F_{(1, 719)} = 176.92$; $P < 0.001$). The effect of time after fungal application on the rate of corrected mortality was also significant ($F_{(5, 719)} = 265.45$; $P < 0.001$), but there were non-significant differences between both fungal isolates ($F_{(1, 719)} = 0.26$; $P = 0.61$) and insect species ($F_{(2, 719)} = 1.27$; $P = 0.17$). Corrected mortality of the 3 tested species of insect adults treated with the conidial concentration of 1×10^8 conidia ml^{-1} began 3 days post incubation, had a high percent mortality for both *C. rosea* isolates after 6 days and ranged between 70.7 and 75.7% (Table 1). Both *C. rosea* isolates showed the LT_{50} values ranged from 4.79 to 4.95 days (Table 2). Corrected mortality of *T. granarium*, *T. castaneum* and *C. maculatus* adults treated with either *C. rosea* isolate AA80 or *C. rosea* isolate AA82 at a conidial concentration of 1×10^6 conidia ml^{-1} after 6 days ranged from 47.7 to 50.7% (Table 3). Mortality in control treatments ranged between 8 and 10%.

Effect of *C. rosea* treatment on the fecundity of coleopteran stored pests

The fecundity of adult females of each of the 3 species tested, *T. granarium*, *T. castaneum* and *C. maculatus*, was affected by the treatment with *C. rosea* isolates (*T. granarium*: $F_{(2, 44)} = 199.51$; $P < 0.001$; *T. castaneum*: $F_{(2, 44)} = 173.22$; $P < 0.001$; *C. maculatus*: $F_{(2, 44)} = 122.31$; $P < 0.001$). The average number of eggs produced per *T. granarium* adult female in the control was 144.7 ± 8.6 eggs, which was higher than those exposed to both *C. rosea* isolates: AA80 (80.6 ± 6.5 eggs) and isolate AA82 (88.9 ± 9.1 eggs). The average number of eggs produced per *T. castaneum* adult female was 44.6 ± 4.5 eggs in control, which was higher than those exposed to both *C. rosea* isolates (Table 4). The average number of

Table 4 Effect of fungal infection on the mean numbers of eggs produced per adult female of either *Callosobruchus maculatus*, *Trogoderma granarium* or *Tribolium castaneum* compared to the control

Insect species	Treatment	Total fecundity mean No. of eggs per female (±SE)
Callosobruchus maculatus	*Clonostachys rosea* isolate AA80	60.8 ± 5.2 b
	Clonostachys rosea isolate AA82	58.2 ± 4.4 b
	Control	92.3 ± 7.6 a
Trogoderma granarium	*Clonostachys rosea* isolate AA80	80.6 ± 6.5 b
	Clonostachys rosea isolate AA82	88.9 ± 9.1 b
	Control	144.7 ± 8.6 a
Tribolium castaneum	*Clonostachys rosea* isolate AA80	24.8 ± 2.9 b
	Clonostachys rosea isolate AA82	21.6 ± 3.1 b
	Control	44.6 ± 4.5 a

Means within a column followed by different lowercase letters indicate significant differences among treatments at each insect species at $P = 0.05$ using LSD test

eggs produced per *C. maculatus* adult female in control was 92.30 ± 7.6 eggs, which was higher than those exposed to both *C. rosea* isolates (Table 4).

Discussion

Several studies have investigated the efficacy of different EPF isolates against stored grain pests (Khashaveh et al. 2011; Mohammed et al. 2019). The present study evaluated the efficacy of 2 different isolates of *C. rosea* against adult stages of *T. granarium*, *T. castaneum* and *C. maculatus*. The results showed that high concentration of both EPF isolates against all the 3 in the laboratory resulted in high levels of mortality, 6 days post the treatment. In addition, results reported that both *C. rosea* isolates had the LT_{50} values ranged from 4.79 to 4.95 days. The results concluded that *C. rosea* isolates displayed a potential as an important biological control of stored grain pests.

It is important for EPF to determine the optimal conidial concentration and lethal time to kill 50% (LT_{50}) of treated insect hosts also, to reduce the overall cost of insect pest control and achieving a high level of control. The results of the present study suggested (1×10^8 conidia ml^{-1}) for both *C. rosea* isolates as the recommended concentration to control the coleopteran stored product insect pests under laboratory conditions. However, more experiments are needed to evaluate this application in commercial storage conditions. The results obtained are consistent with those of Toledo et al. (2006) who recorded (82.5%) mortality rate of *O. tucumana* after 2 weeks of inoculation with *C. rosea* at a conidial concentration (1×10^8 conidia ml^{-1}). Uses of different insect hosts, environmental conditions and/or application methods have showed different results.

The results obtained in the present study led to accept the hypotheses that individuals of insects treated with EPE produce eggs at a different rate compared to untreated insects. The reduction in total fertility in infected insects is consistent with the results obtained by Mohammed et al. (2019) with other EPFs, who found that infection with *B. bassiana* isolate Bb25 and *M. anisopliae* isolate Ma42 showed (50%) reduction in total fertility of *T. granarium* females, than in the untreated ones. In addition, Fargues et al. (1991) reported that infection with *B. bassiana* reduced the total fecundity of Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), about 56% than in the untreated females. The indirect effect of EPF infection on the daily and total fecundity of stored product insect pests may be related to histological and cytological damage of the ovaries of treated *T. granarium*, *T. castaneum* and *C. maculatus* (Mohammed et al. 2019). Second, EPF can produce secondary metabolites, which may interfere egg production (Furlong et al. 1997). The decrease in total fecundity of infected coleopteran stored product pests of the present study could result from killing of adult females so that the duration of their reproductive period is effectively shortened. Furthermore, infection with certain EPF can cause muscle paralysis (tetany), followed by muscle weakness in infected insect hosts before death, which may reduce the number of eggs produced by infected females (Samuels et al. 1988).

Conclusions

The entomopathogenic fungus, *C. rosea* isolates, showed potential against the 3 stored product insect pests: *T. granarium*, *T. castaneum* and *C. maculatus* under laboratory conditions. As well, reductions in total fertility of the infected target insect pests were also recorded. However, further studies are required to confirm the efficacy of *C. rosea* isolates under commercial storage conditions. In addition, the potential effects of *C. rosea* in combination with some other control methods are also required to be evaluated.

Abbreviations

EPF: Entomopathogenic fungi; RH: Relative humidity; SDA: Sabouraud dextrose agar

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Authors' contributions

AAM, ASA and ANA designed and carried out all experiments, recorded the data and interpreted the results. AAM analysed the data and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Competing interests

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