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Clonostachys rosea, a new and promising entomopathogenic fungus infecting pupa of jujube fruit fly, *Carpomya vesuviana*

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Abstract: Jujube fruit fly, *Carpomyia vesuviana*, is the most important pest of jujube in Iran. During February and March 2013, a survey on fungi associated with pupae of *C. vesuviana* was conducted in Birjand, South Khorasan province of Iran. To isolate fungi, the samples were washed under running tap water, surface-sterilized in 0.5% sodium hypochlorite, then rinsed and placed onto potato dextrose agar. Out of eight isolates of *Clonostachys rosea* obtained in this study, only one showed considerable pathogenicity against the pupa of *C. vesuviana*. This isolate was additionally examined using the morphological and molecular studies, based on ITS-rDNA. Both morphological and molecular data confirmed the identity of this fungus as *Clonostachys rosea*. Based on pathogenicity assays, significant mortality of inoculated pupa (46%) was recorded with a conidial suspension of 10^{10} (spore/ ml). Median Lethal Time (LT_{50}) and LC50 values of this isolate against pupa of *C. vesuviana* were calculated 4.6 days and 5.1×10^4 spore/ml, respectively. A significant positive correlation between different 3 *C. rosea* concentrations and mortality of *C. vesuviana* was observed (r^2= 0.9986, p= 0.003). *Clonostachys rosea* is here reported as a new and promising entomopathogenic fungus on *C. vesuviana*, suggesting that it is likely to play roles in protecting jujube trees against this pest.

Key words: Morphology, phylogeny, insect pathogen, biocontrol agent

INTRODUCTION

Jujube (*Zizyphus jujuba* Mill.), belonging to the family Rhamnaceae, is distributed in the warm temperate and sub-tropical regions of the world (Mirzaee et al. 2013). The jujube fruit fly, *Carpomyia vesuviana* Costa (Diptera: Tephritidae) is the most important pest of jujube in Iran (Moodi 2002), causing considerable damage to this crop at the fruiting stage. A variety of control methods including chemical pesticides, cultural practices, resistant cultivars, and attractant agents have been used for its control in Iran (Tavakoli Korghond et al. 2012). However, an attractive alternative method to control pests of agricultural crops is biological control. Microbial biocontrol agents (MBCAs) are as the natural enemies of the pest population that have no hazard effects on human health and the environment. Application of non-chemical entomopathogenic agents for control of some pests have been conducted and recommended. For example, Andreev et al. (2008) suggested *Beauveria bassiana* (Bals.-Criv.) Vuill. as the best agent for control of black cherry aphid, *Myzus cerasi* (Fabricius). Besides, Sabbour et al. (2012) have evaluated the efficiency of *Beauveria bassiana*, *Metarhizium anisopliae* (Metschn.) Sorokin and *Isaria fumosorosea* Wize against three important stored product pests, *Plodia interpunctella* (Hubner),

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Ephestia cautella (Walker) and *E. kuehniella* Zeller. Due to their environmental safety and specificity, bio-pesticides are gaining popularity in the management of pests of horticultural crops. *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (teleomorph: *Bionectria ochroleuca* (Schwein.) Schroers & Samuels) has been reported as a potential parasite of insects (Toledo et al. 2006) and nematodes such as *Heterodera* spp. and *Globodera* spp. (Silva et al. 2015). This fungus also has been reported as pathogenic on sclerotia of *Sclerotinia sclerotiorum* (Lib.) de Bary, *Phymatotrichum omnivorum* Duggar, *Rhizoctonia solani* J.G. Kühn, *Botrytis* spp. and *Verticillium* spp. (Sutton et al. 1997). This widely distributed fungal species, often naturally found in agricultural soils, is a promising effective biological control agent (Keyser et al. 2016; Sun et al. 2017). This study describes a fungal antagonistic agent of the jujube fruit fly as the first step of the biological control program of this pest.

MATERIALS AND METHODS

During surveys on jujube orchards in Birjand, South Khorasan province of Iran in February and March 2013, the pupae of jujube fruit fly showing symptoms of infection and emerging fungal structures were observed in the soil under trees. These pupae were collected in sterile Petri dishes and transferred to the laboratory. To isolate fungi, the infected pupae (fig1-b) were washed under running tap water, surface-sterilized in 0.5% (v/v) sodium hypochlorite, rinsed several times in sterile distilled water, placed onto potato dextrose agar (PDA, Merck), and incubated at 25°C in the dark. Out of eight isolates of *Clonostachys rosea* obtained in this study, only one showed considerable pathogenicity against the pupa of *C. vesuviana*. This isolate was additionally examined using morphological and molecular studies. Measurement of fungal structures (conidiophores, phialides and conidia) was based on single spore cultures on both PDA and Oat Meal Agar media (OMA, East Molesey). The pathogenic strain of *C. rosea* on pupae of jujube fruit fly is preserved at the Iranian Fungal Culture Collection (IRAN 3253C), Iranian Research Institute of Plant Protection (Tehran, Iran).

For pathogenicity assays and LC50 and LT_{50} calculation of the representative strain of *C. rosea*, thirty individuals of jujube fly pupae were exposed to different conidial suspension of 10^3, 10^5, 10^7, 10^{10} spore/ml, prepared from 10-day-old cultures grown on PDA at 25°C, for 10 sec and placed on sterile Petri dishes containing filter paper dampened with sterile distilled water. Each of 10 pupa was used for each concentration at three replicates. The control was treated with sterile distilled water. Treated and control pupae were maintained at 25°C. Percentage of pupal colonization was recorded after 14 days. Probit program of SAS (version 9.2) software was used to determine the concentration of the suspension of the fungus that killed 50% (LC50) of the examined pupa.

Genomic DNA of the representative strain of *C. rosea* was extracted using a modified Chelex 100 chelating resin method with an initial step of grinding the mycelia in liquid nitrogen (Walsh et al. 1991). The very small mycelium was transferred to a sterile micro-tube and crushed with liquid nitrogen. The crushed mycelium was transferred into a tube containing 200 µl of 5% w/v Chelex 100 resin suspension. The tubes were vortexed and incubated at 56°C overnight, then at boiling water bath for 7 min. The extracts were thoroughly vortexed, then incubated again in a boiling water bath for 8 min and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to a new sterile tube and diluted with TAE buffer (1:10). PCR amplification of the internal transcribed spacer (ITS) of the causal fungus was carried out using the primers of ITS4 and ITS5 (White et al., 1990). PCR was performed in a 25 µl volume reaction mixture with 1 µl Mgcl2, 2.5 µl PCR buffer (10X), 0.5µl dNTP mix, 1 µl of each primer, 0.4 µl Enzyme taq polymerase and 13.6 ml sterile water and 5µl DNA template. The amplification was performed on Eppendorf Thermo-cycler according to the following profile: an initial denaturation step at 94°C for 3 min, followed by 30 amplification cycles at 94°C for 45 sec, 55°C for 45 sec, 72°C for 2 min and a final extension step at 72°C for 7 min. The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after the novel juice (Gene DireX) staining. PCR products sequenced by Macrogen Company (South Korea) and deposited in GenBank (NCBI) database (www.ncbi.nlm.nih. gov/genbank/) with accession number MK005284. The sequences were assembled and edited by MEGA software version 6 (Schroers. 2001). BLAST searches were done using the NCBI server (http:// www.ncbi.nlm.nih.gov/blast). Sequence alignments were generated by CLUSTALW implemented in MEGA software version 6 (Tamura et al. 2013). The phylogenetic tree was constructed using the neighbor-joining (NJ) method using the Tamura–Nei model (Tamura and Nei 1993) in MEGA 6.0. The robustness of the resulting tree was tested by bootstrapping with 1000 re-samplings of the data (Saitou and Nei 1987). *Bionectria levigata* (AF210680) was used as outgroup (table 1).

RESULTS

Eight strains of *Clonostachys rosea* were isolated from which the only one presented considerable pathogenicity on pupa of *Carpomya vesuviana*. This isolate (IRAN 3253C) was additionally examined using morphological and molecular studies. The microscopic and macroscopic observations were made from potato-dextrose agar (PDA) and oatmeal agar (OA) cultures as described by Schroers et al. (2001). Colonies were white to light yellow, producing orange pigments on cultures. The colony diam. reached 38–40 mm after 7d at 28 °C. The fungal isolate was

characterized by two kinds of conidiophores. Primary conidiophores were verticillium-like, bearing phialides measuring 8–12.5 × 1.2–1.6 µm (n=100) (Fig 1-d), and secondary conidiophores were penicillate (Fig. 1-c).

Conidia from both types of conidiophores were hyaline, smooth-walled, slightly curved, 4–7 × 2–3.1 µm (fig. 1-e). Based on the above-mentioned characteristics, the fungus was identified as *Clonostachys rosea*.

Pupae inoculated with *C. rosea* were infected (Fig 1-a). The fungus re-isolated from inoculated pupa was identical to the original isolates, confirming Koch's postulates. The LC50 value was calculated by 5.1×10^4 spore/ml. Statistical analysis showed a significant difference between the different spore concentrations of the *C. rosea* and mortality of *C. vesuviana* ($p<0.001$). Mortality of inoculated pupa with 10^{10} (spore/ml) was recorded 46% compared with control (Table 2). A significant positive correlation between concentrations and mortality was observed ($r2 = 0.9986$, $p = 0.003$) (Fig. 3). Median lethal time (LT_{50}) values of this isolate against pupa of *C. vesuviana* was calculated 4.6 days and ranged from 3.1 to 6.1 days (Table 3).

The phylogenetic analyses performed using ITS region sequences including our isolate and the others obtained from GenBank. The phylogenetic tree showed that the examined isolate belonging to a moderately supported clade in NJ analysis (support value= 72), is nested with some strains of *C. rosea* (Fig. 2).

Table.1. The taxa were used in the phylogenetic analysis.

Taxon	Strain	Substrate	Country	GenBank accession no. (ITS)	References
Clonostachys rosea	IRAN 3253C	*Carpomyia vesuviana*	Iran	**MK005284**	This study
Bionectria ochroleuca	BBA 68698	-	-	AF106532	Hagedorn and Scholler 1999
Clonostachys sporodochialis	CLL-GUY-12-046	-	French Guiana	KJ802125	Lechat et al. 2014
Bionectria ochroleuca	CBS 710.86	Soil	Netherlands	AF358235	Schroers 2001
Clonostachys rosea	-	*Bemisia tabaci*	-	LT604480	Anwar 2016
Bionectria levigata	CBS 948.97	*Buxus sempervirens*	France	AF210680	Schroers 2001

The sequence written in bold is generated in this study. CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; IRAN: Iranian Fungal Culture Collection, Iranian Research Institute of Plant Protection, Tehran, Iran; LPSC: La Plata Spegazzini Collection, Argentina.

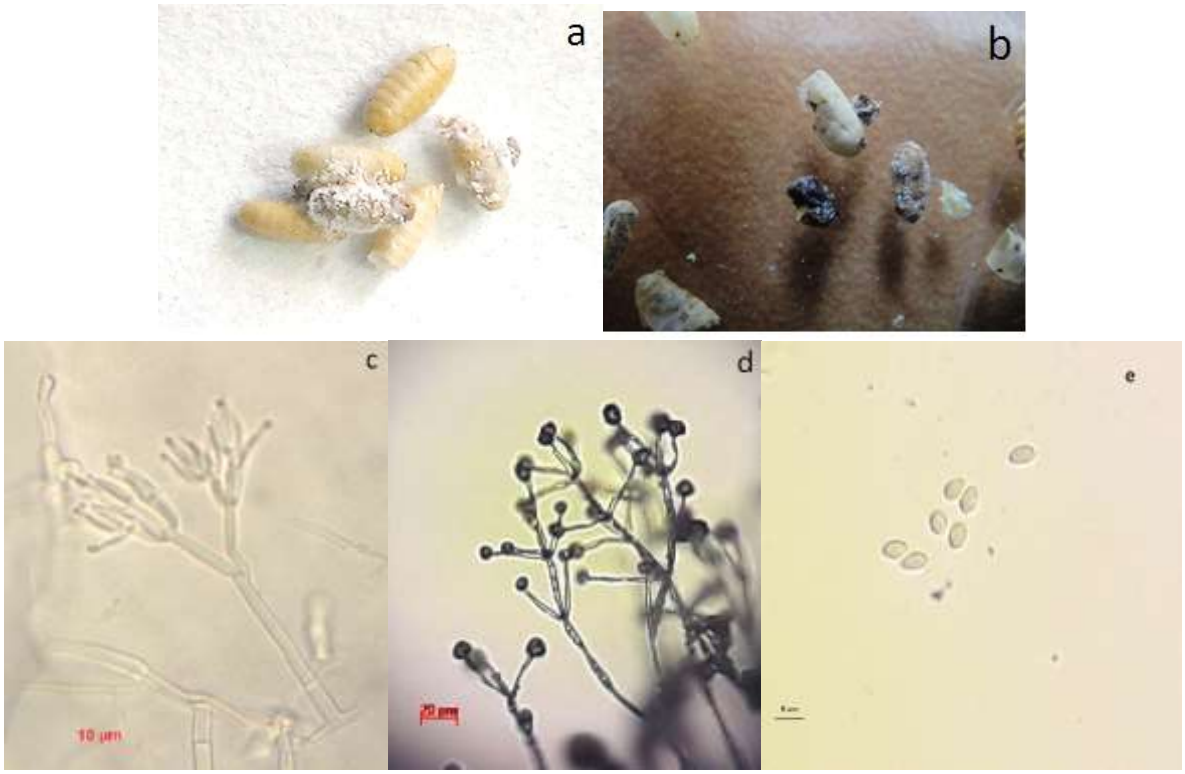

Fig. 1. a. Infected pupae of *Carpomya vesuviana* by *Clonostachys rosea* in pathogenicity tests, **b.** naturally infected pupae of *C. vesuviana* showing fungal structure of *C. rosea*, **c.** secondary penicillate conidiophores, **d.** primary *Verticillium*-like conidiophores of *C. rosea*, **e.** conidia.

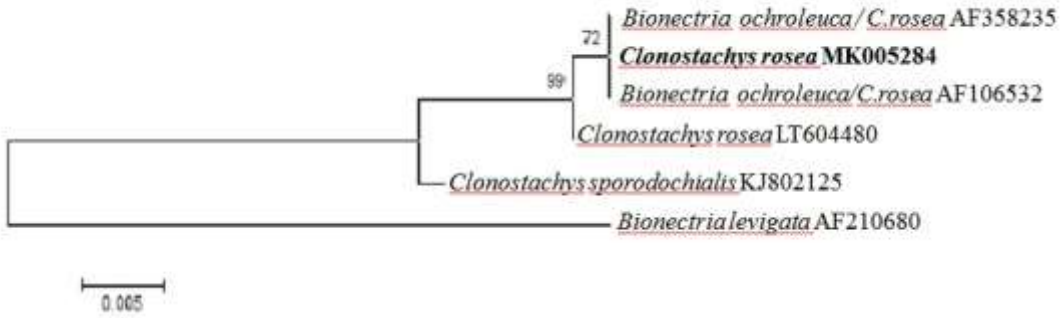

Fig. 2. Neighbor-joining tree based on ITS-rDNA sequences of *Clonostachys rosea*. Bootstrap values >50% (1000 replicates) shown at the nodes. *Bionectria levigata* serves as outgroup.

Table 2. Effect of conidial concentrations of *Clonostachys rosea* (IRAN 3253C) against *Carpomya vesuviana*.

Concentrations (per ml)	No. of pupa exposed	Number of infected pupae after spore spraying (day)							Mortality (%) *
		1	2	3	4	5	6	7	
0	30	30	30	30	30	30	30	30	0^{a}
10^{3}	30	28	27	27	27	27	27	27	10^{ab}
10^{5}	30	27	26	25	25	25	25	25	16.6^{ab}
10^{7}	30	30	30	28	24	22	22	22	26.6^{ab}
10^{10}	30	29	27	22	22	19	17	16	46^{b*}

* Mean followed by the same letter not significantly different

DISCUSSION

The taxonomic characteristics of the fungus described in this study were consistent with those reported by Schroers (2001) and Toledo et al. (2006). The destructive, necrotrophic, and unspecific mycoparasitism of *C. rosea* was first described by Barnett and Lilly (1962). *Clonostachys rosea* has been isolated from various plant substrates by Schroers (2001).

This research proposed that species of *Bionectria*/*Clonostachys* do not have serious plant-pathogenic abilities. As a mycoparasite, *C. rosea* was successfully tested as a biological control agent of gray mold, caused by *Botrytis cinerea* Pers. (Cota et al. 2008). A basic formulation of *C. rosea* strain ACM941 has been applied against fusarium head blight of wheat and root rot of soybeans in the field but there are few reports about entomopathogenic efficacy of this fungus. Toledo et al. (2006) reported *C. rosea* as an entomopathogenic fungus of *Oncometopia tucumana* (Schröder) and *Sonesimia grossa* (Signoret) in Argentina. Pathogenicity of *C. rosea* against coffee berry borer, *Hypothenemus hampei* (Ferrari), has been reported by Vega et al. (2008). Successful control of *Delia radicum* (Linnaeus), an important pest of Brassicaceous crops, using *Clonostachys solani* f. *nigrovirens* (J.F.H. Beyma) Schroers has been reported by Razinger et al. (2014).

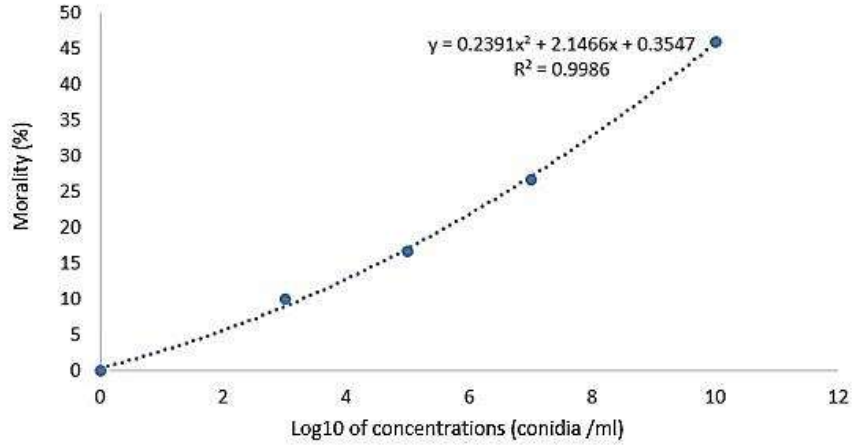

Fig. 3. Effect of different conidial concentrations of *Clonostachys rosea* on the mortality of pupa *Carpomya vesuviana* after 7 days.

Table 3. LT_{50} values of conidial suspension of *Clonostachys rosea* (1×10^{10} conidia/ml) on pupa of *Carpomya vesuviana**

Fungal Isolate	LT_{50} (days)	95% confidence limits	
		Lower limit	Upper limit
IRAN 3253C	4.6	3.1	6.1

The results obtained by this study showed that there is a high parasitic rate with increasing conidia concentration of *C. rosea* on pupa of *C. vesuviana.* Anwar et al. (2018) also showed a reduction in mortality *B. tabaci* nymphs by *C. rosea* at the lower spore concentrations. Our results showed that the examined *C. rosea* isolate leading to 46% pupae jujube fruit fly mortality 7 days after inoculation. Toledo et al. (2006) also observed 82.5% mortality of *Oncometopia tucumana* after 14 days of inoculation using *C. rosea* isolates. In similar research, Mustafa et al (2014) reported 55.1% mortality of Alfalfa weevil 6 days post-inoculation by *C. rosea*.

Several biocontrol mechanisms have been reported for *C. rosea* as a mycoparasite or Entomopathogenic agent. Zhang et al. (2008) reported that the pathogenesis started from the adherence of conidia to nematode cuticle for germination, followed by the penetration of germ tubes into the nematode body and subsequent death and degradation of the nematodes. Direct parasitism, production of fungal cell wall degrading enzymes, antibiosis, induction of plant defense reactions and toxic metabolites production by this fungus have been reported as biocontrol mechanisms of *C. rosea* (Nygren et al. 2018).

This fungus has been considered as an entomopathogenic fungus and therefore may contribute to biological control of insects (Keyser et al. 2016). To our knowledge**,** this is the first record of *C. rosea* on jujube fly throughout the world. As the pest overwinters as a pupa in the soil (Shan Yong et al. 2009), it has the potential to be considered as a biocontrol agent of the ber fruit fly pupae in soil. Therefore, it seems that this fungus is likely to play roles in protecting jujube trees against pests and also promoting plant growth simultaneously. However, further research on its efficiency as a biocontrol agent or as an endophyte is required.

CONCLUSION

With an emphasis on organic production of the jujube, biological control of jujube fruit fly (the most important pest of jujube in Iran) provides opportunities for reducing negative impacts of chemical pesticides on the environment, biodiversity and human health.

In this study, *C. rosea* is reported as a potential biological control agent of jujube fruit fly, *C. vesuviana.* However, further studies are recommended to evaluate its potential in field conditions, and determining its presumably endophytic role.

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قارچ *Clonostachys rosea* به عنوان بیمارگر جدید و امید بخش آفت مگس میوه عناب (*Carpomya vesuviana*)

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چکیده: مگس میوه عناب (*Carpomya vesuviana*) یکی از مهمترین آفات عناب در کشور است. طی اسفند و فروردین سال ۱۳۹۲ تحقیقی در ارتباط با قارچ‌های همراه با شفیره‌های این آفت در خراسان جنوبی شهرستان بیرجند انجام گرفت. به منظور جداسازی جدایه‌های قارچی، نمونه‌ها با هیپوکلریت سدیم رقیق شده با ۰/۵ درصد ماده موثر ضدعفونی و بعد از شستشو با آب مقطر استریل، روی محیط سیب زمینی دکستروز آگار کشت شدند. در این پژوهش، در مجموع هشت جدایه قارچی دارای ویژگی‌های عمومی *Clonostachys rosea* جداسازی شد که فقط بیماری‌زایی یکی از این جدایه‌ها روی شفیره‌های مگس میوه عناب اثبات شد. این جدایه تحت مطالعات ریخت‌شناسی و مولکولی (بر اساس توالی‌های ITS r-DNA) قرار گرفت و شناسایی آن تحت گونه *Clonostachys rosea* مورد تایید قرار گرفت. در آزمون‌های بیماری زایی، پارازیته شدن معنی دار شفیره‌های تلقیح شده مشاهده و این قارچ دوباره جداسازی شد. همبستگی مثبت و معنی دار بین غلظت‌های مختلف اسپورهای قارچ و میزان کشندگی شفیره‌های مگس عناب مشاهده شد ($r^2= 0.9986$، $p= 0.003$). بیشترین درصد کشندگی (۴۶ درصد) در غلظت $۱۰^{۱۰}$ اسپور/ میلی‌لیتر به دست آمد. مقدار LT_{50} و LC50 قارچ روی شفیره‌ها به ترتیب ۴/۶ روز و $۵/۱ \times ۱۰^{۴}$ اسپور/ میلی‌لیتر محاسبه شد. بر اساس یافته‌های به دست آمده در این پژوهش، برای اولین بار، گونه *C. rosea* به عنوان قارچ پارازیت کننده شفیره مگس میوه عناب گزارش می‌شود. به نظر می‌رسد این قارچ بتواند نقش مهمی در محافظت درختان عناب در برابر این آفت مهم ایفا نماید.

کلمات کلیدی: ریخت شناسی، فیلوژنی، بیمارگر حشره، عامل کنترل بیولوژیک

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