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CAN ENTOMOPATHOGENIC FUNGI BE EFFECTIVE IN CONTROLLING *PHYLLOCNISTIS CITRELLA* AND SUBSEQUENTLY CITRUS CANKER DISEASE?

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ABSTRACT

Citrus leafminer (CLM) is a drastic pest of citrus trees threatening citrus plants with canker disease caused by bacterial pathogen (*Xanthomonas axonopodis* pv. *citri*). In current studies, we evaluated the insecticidal effect of entomopathogenic fungi in four treatments viz. fungus treated citrus leaf miner released on healthy seedlings, fungus treated citrus leaf miners on infected seedlings, untreated citrus leaf miners on healthy seedlings and untreated citrus leaf miners on infected seedlings. The results clearly indicate that fungus treated CLM on infected leaves resulted in maximum mortality (100%) with minimum number of galleries (5.67), average no. adult emergence (2.33) and maximum mycosis (93%). On the other hand, the highest disease incidence (84.62%), with maximum number of galleries (31.83), and the highest number of emerged adults (21.33) was noted when untreated CLM released on infected seedlings. It was concluded that entomopathogenic fungus has a great potential to control CLM infestation and subsequently to manage the citrus canker disease in citrus plantation.

Keywords: *Phyllocnistis citrella*, entomopathogenic fungi, citrus canker

INTRODUCTION

The citrus leafminer, *Phyllocnistis citrella* Stainton (Lepidptera: Gracillariidae) commonly known as Citrus leafminer (CLM) is a major insect pest which harmfully affects fruit development (Urbaneja *et al*., 2003). It is the most prominent problem in new planting trees and nurseries (Diez *et al*., 2006). The plants damaged by CLM are highly susceptible for the secondary infestation by pathogenic bacteria causing citrus canker disease (Hoy *et al*., 2007). Adults of CLM are minute creatures with silvery-white scales with numerous black spots and marking on forewings (Kawahara *et al*., 2009), while larvae are tiny, greenish-yellow translucent and usually found in mines made on the leaves (De Prins and De Prins, 2005).

Lemon, grapefruit and lime are found to be more susceptible to CLM damage, however, it also attacks on different plants belonging to family Rutaceae (De Prins and De Prins, 2005). This insect damages the young foliage and has serious effects on growth and development of young groves, seedlings and mature trees ultimately resulted in yield reduction (Belasque *et al*, 2005). Newly hatched larvae chew the young and fresh leaves, start feeding inside the leaf parts, leaf chlorosis, leaf deformation and leaf dropping occur due to leaf mining which eventually resulted in reduced photosynthesis (Ujiye, 2000; Gottwald *et al*., 2001). Several studies have demonstrated a direct correlation of CLM damage with certain economic losses (Pena *et al*., 2000).

Different approaches including cultural, biological and chemical control have been used to manage this pest (Shivankar *et al*., 2002). Mostly growers rely on the use of insecticides, although these are often expensive (Heppner, 1993), harmful to beneficial insects including predators and parasitoids and also not much effective to attain maximum larval mortality because of several overlapping generations of this pest (Raga *et al*., 2001; Besheli, 2007). All these problems have collectively influenced the agriculture production sector and forced the researchers to focus on development and evaluation of alternative biological based control tactics (Khachatourians, 2009).

Among biocontrol strategies, entomopathogenic fungus *Beavueria bassiana* (Bals.-Criv.) Vuill. (Cordycipitaceae: Ascomycota) possesses a significant potential to reduce the population of CLM, therefore, the present study was designed to evaluate the effect of *B. bassiana* on citrus leafminer.

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MATERIALS AND METHODS

The study was done in the Laboratory of Plant Pathology, University College of Agriculture, University of Sargodha, Punjab, Pakistan.

Isolation of bacterial culture

The sample leaves infected with citrus canker disease was collected from the field area of College of Agriculture, University of Sargodha (32◦5′1″N 72◦40′16″E). The infected portions of leaves were cut into small pieces of diameter 2-3 mm approximately. Then, these pieces were surface sterilized with 1% solution of sodium hypochlorite for 3-4 minutes followed by two to three rinses with distilled water. Disinfected cuts were dried after placing them on the sterilized filter paper. With the help of sterilized forceps these pieces were placed in the petri plates containing nutrient agar solidified media. Petri plates were sealed with paraffin tape and placed in incubator under controlled conditions and the colony was observed after one week.

Bacterial suspension

The count method was used for counting bacterial culture in the plate. One ml of the fresh bacterial culture was taken to mix it with the 9 ml of distilled water and 6-8 serial dilutions were used to count the number.

Fungal culture

The fungal culture from laboratory already isolated from soil was sub-cultured on the potato dextrose agar (PDA) media to get a fresh grown colony of *B. bassiana*. After 7 days of inoculation, 10 ml of distilled water was added to the plate containing fungal culture and was shaken to detach the conidia from the fungal colonies. Then 100 µl of the fungal suspension was taken and spores were detached. The number of spores was counted using haemocytometer and 1 x 10^4 spores/ml concentrations was achieved.

Raising seedlings of sweet orange

Sweet orange seedlings were transplanted into the potted cups containing soil and well rotten FYM with ratio of 2:1. These potted cups were kept in the open condition till the flushing of seedlings. The transplanted seedlings were given using all recommended cultural practices and irrigations till the inoculation with the bacterial and fungal cultures.

Rearing of citrus leafminer

Seedlings were transplanted in the small potted cups; all the seedlings were kept pruned to get flushes. These flushes are the good food source for citrus leafminer under natural conditions. Larval stage of leafminer was taken and released on the citrus foliage to feed and pupate. After 2-3 weeks' the newly emerged adult leafminers were allowed to mate for egg laying and the homogenous population of CLM was established for further assays.

Bioassay

A set of 80 pots were covered by the net cages. Among these seedlings, 40 were taken as healthy, and 40 got infected by the application of bacterial solution. Test seedlings were wounded by syringes to facilitate the entry of the *Xanthomonas axonopodis*. In order to maintain the moisture level in the cages one side of the cage was covered with polythene and wet cotton was placed in the cage receiving continuous spray of water. Adults of citrus leafminers were exposed to fungal conidia by an improved but modified contamination method used by Dimbi *et al*. (2003) for tephritid flies. For this purpose, dry fungal conidia (0.1 g) of *B. bassiana* were uniformly spread on cotton (velvet) cloth wrapped inside a cylindrical shaped plastic tube. Then 1-2 days old adult leafminers were shifted into these plastic tubes and allowed to walk on cloth for one minute. The fungus treated adults were collected with aspirator and released into test healthy and infected seedlings. In total, four treatments were made, and each treatment was repeated for four times, each consisting of 20 seedlings and 50 adults per seedling. The four treatments were: fungus treated CLM released into healthy plants (T1), fungus treated CLM introduced into infected plants (T2), untreated CLM released into healthy plants (T3) and untreated CLM released into infected plants (T4). Then each pot was coverd individually with the help of net cages again and treatments were set for data recording. The data for adult morality was recorded on daily basis up to 8 days. Additionally, the number of galleries formed by the insect on test seedling and percent disease incidence was also recorded. In order to collect the developed pupae, leaves with mature galleries were removed from the seedlings and spread on sterile sand kept inside plastic trays. The complete randomized block design was used for the treatments.

Mycosis

At the end of assay the cadavers were collected, surface sterilized with 1% solution of sodium hypochlorite with subsequent three rinses of distilled water and shifted into Petri plates lined with sterile filter paper and inoculated at 25±2C and 75±5% R.H. in complete darkness to allow the fungal growth to determine the mycosis percentage on cadavers.

Statistical analysis

Data was statistically analyzed by using analysis of variance (ANOVA) in Minitab 13.2. Mortality data were subject to one-way analysis of variance and means were compared by using Tukey's test at 5% significant level (Sokal and Rohlf, 1995).

RESULTS

Effect of fungal treated and untreated leafminer on disease occurrence

It was shown that maximum galleries were produced (31.83%) in T4 (untreated CLM on infected plants) followed by 23.16% in T3 (untreated CLM on healthy plants), 8.83% in T1 (fungal treated CLM released on healthy plants) and

5.67% in T2 (fungal treated CLM on infected plants). Similarly, the disease incidence was found maximum against T4 (84.62%) while minimum disease incidence recorded against T1 (0.00%). In the same sequence, adult emergence was maximum against T4 (21.33) with the following order of 15.16 in T3, 2.33 in T2 and the lowest adult emergence (0.00) against T1 (Table 1).

Mortality and mycosis

Among all the treatments, maximum mortality of CLM (100%) observed in T2 (fungal treated CLM released on infected plants) compared with T1 (fungal infected CLM on healthy plant) (98.17%), T3 (untreated CLM released on healthy plant) (5.17%) and T4 (untreated CLM released on

Table1.
Average mean number of galleries, % disease incidence and average adult emergence as a result of four different treatments; T1 (fungal infected CLM on healthy plant), T2 (Fungal treated CLM released on infected plant), T3 (untreated CLM released on healthy plant) and T4 (untreated CLM released on infected plant). Means sharing same letter in each column, are not significantly different from each other.

Treatment	Average no. of galleries	Disease incidence (%)	Average no. of adult emergence
T1	8.83±0.60 c	0.00±0.00 c	0.00±0.00 d
T2	5.67±0.74 c	11.10±1.32 b	2.33±0.42 c
T3	23.16±0.76 b	2.46±0.78 c	15.16±0.60 b
T4	31.83±0.96 a	84.62±2.20 a	21.33±0.79 a

Table 2.
Mean (% mortality ±SE), and mycosis (%) against four different treatments; T1 (fungal infected CLM on healthy plant), T2 (Fungal treated CLM released on infected plant), T3 (untreated CLM released on healthy plant) and T4 (untreated CLM released on infected plant). Means sharing same letter in each column, are not significantly different from each other.

Treatment	Mortality (%)	Mycosis
T1	98.17±1.1 a	86.67±0.76 b
T2	100.00±0.00 a	93.50±0.77 a
T3	5.17±0.47 b	-
T4	1.67±0.33 c	-

healthy plant) (1.67%). Similar to the trend in mortality data, percentage of mycosed adults was the maximum against T2 treatment and minimum mycosis was noted when treatment T1 was applied against CLM (Table 2).

DISCUSSION

Any kind of injury to plant make the plant very susceptible for the introduction of pathogenic infection, as it has been demonstrated in study reported by Pohronezny *et al*. (1992). In our study, we introduced the inoculum with the help of syringes to ensure the establishment of the disease in test plants. Vernière *et al*. (2009) noted a significant difference between the disease incidence in mechanically wounded and non-wounded plants. The current study strengthens the earlier reports that the control of citrus canker is best possible by controlling the citrus leafminer in citrus orchards. Presently, when fungus treated insects were released on healthy plants, no disease symptoms were noted. The possible explanation for this fact would be that citrus leafminer infected with entomopathogenic fungi did not lead to disease incidence because released insects were died due to fungal infection prior to the initiation of the disease incidence. Compared to this, somehow disease symptoms (11%) were observed in case when fungus treated leafminers were released on artificially diseased/infected plants. But the level of disease occurrence was significantly lower than the infected plants which received fungus free leafminers where approximately 84% of the plants showed the disease symptoms. This showed that fungus treatment exhibited its effect on leafminer and played a role in reduction of disease occurrence by killing the insects. The potential impact of entomopathogenic fungi for the management of insect pests have been already shown in a study by Inglis *et al*. (2001). Prevalence of fungi and species spectrum in leafminer and their effect on host populations has been slightly studied (Samek *et al*., 2006; Sierpinska and Kubiak, 2011; Metla *et al*., 2013). Although, EPF has already been developed successfully as myco-insecticide involving different steps comprising isolation of fungi, selection of strain (based on virulence and formulation) and storage properties. Our results revealed that entomopathogenic fungal isolate was pathogenic and virulent to citrus leafminer adults. The mortality of insect and pathogenic activity of fungus reported in our study was similar to observations that stated for other arthropod insect pests (Quesada-Moraga *et al*., 2006, Bugeme *et al*., 2009) and immature stages of leafminer (Borisov and Ushchekov, 1997). Another study showed the similar results to our observations that different isolates of *Beauveria bassiana* were highly infectious to leafminer (Migiro *et al*., 2010). Several other studies also supported our findings that fungi (*B. bassiana*) were pathogenic to larvae of leafminer in laboratory and greenhouse conditions and highly effective against egg and larval stages of leafminer (Sabbour and Abd-El-Raheem, 2013; Sabbour, 2014).

These results suggested that decreasing citrus leafminer inoculation sources through appropriate biocontrol programs may help to minimize the incidence and severity of the

disease. CLM has a history of developing resistance to insecticides making it difficult to achieve its sustainable control. Therefore, in addition to the complete knowledge about the biology and ecology of parasitoid species, more substitutes to existing control methods are needed to be explored. For instance, after establishing the effectiveness of entomopathogenic fungi, it could be used as a component of integrated pest management program of the *P. citrella* and citrus canker disease in citrus orchards.

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