Pakistan Entomologist

Journal homepage: www.pakentomol.com

EXPLORING FUNGAL FLORA ASSOCIATED WITH INSECTS OF COTTON AGROECOLOGICAL ZONES OF PUNJAB, PAKISTAN

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ARTICLE INFORMATION

Received: January 10, 2015
Received in revised form: June 10, 2015
Accepted: June 18, 2015

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ABSTRACT

In this study, occurrence frequency as well as insect associated fungal flora from the soil of cotton zone was investigated. Insect associated fungi were classified as entomopathogens, secondary colonizers and opportunistic pathogens isolated from soils through Gallaria mellonella bait method. Twenty soil samples from Cotton zone (Rahim Yar Khan and Sahiwal) were analyzed for occurrence frequency of insect related fungi. Occurrence frequency of Entomopathogenic fungi among all types of observed fungal species was the highest in District Rahim Yar Khan that was found to be 7.35 % in comparison with that of 6.21 % in District Sahiwal. Occurrence frequency of opportunistic fungi among all types of observed fungal species was the highest in District Rahim Yar Khan that was found to be 55.86 % in comparison with 54.28 % in District Sahiwal. Frequency of occurrence of secondary colonizers was the highest in District Sahiwal that was found to be 39.55% among all types of observed fungal species in comparison with that of 36.75% in District Rahim Yar Khan. The absolute value of Beta Diversity (β) of entomopathogenic fungi was 2 in cotton zone while in opportunistic fungi and secondary colonizers; it was zero in cotton agroecological zone.

Keywords: Beta diversity, Cotton agroecological zone, Entomopathogens, Frequency of occurrence,

INTRODUCTION

Pesticides have been utilized by human being since 20 B.C. (Ritter, 2009). Beside benefits, there are also tremendous drawbacks of the use of pesticides, such as potential toxicity to humans and other animals. Agricultural water is contaminated due to extensive use of pesticides in Pakistan and even it is contaminating groundwater rapidly (WWF, 2007). In Pakistan, extensive usage of pesticides has reduced the pest population, but like other countries, it has created environmental issues in the region. Extensive use of pesticides has been contaminated the ground water in some areas of Punjab and Sindh. Biological monitoring studies have proved that farmers suffered from severe and chronic health problems due to pesticides. Moreover, the excessive use of pesticides in cotton growing areas is at risk for pickers, field workers and undesirable residue concentration in cotton seed oil and cakes (Ilyas *et al*., 2007).

Biological control is a basic approach for suppression of pest within an effective integrated pest management (IPM) program. Natural enemies can be used against a pest population to minimize its density to a level lower than economic threshold. Biological control as a managing tool dates back over 1,000 years when ancient citrus growing Chinese were using ants to control caterpillar larvae that infest their trees. Biological control is a safe method to control pest because it is not toxic or injurious to humans (Bellows and Fisher, 1999). It is indispensable to describe the two wide approaches to biological control: classical and indundative. Classical biological approach means the usage of natural enemy of the target pest from its native range into exotic one. This approach resulting in a self- sustainable and balanced system and pest population is limited at a less damaging level, as its fitness is tough (Evans and Ellison, 1990). In case of fungal pathogens, Inundative control involves the formulation, mass production and application of a product that can be marketed and employed in the same way as a conventional chemical pesticide.

Cite this article as: Anwar, W., S.N. Khan, M. Aslam, M.S. Haider, A.A. Shahid and Muhammad Ali, 2015. Exploring fungal flora associated with insects of cotton agroecological zones of Punjab, Pakistan. Pak. Entomol., 37(1):27-31.

Fungi that play their role to the regulation of host community, arachnids and insects use entomopathogenic fungi as their natural enemies. Fungi have been found to cause mortality in pest population and numerous fungal species were evaluated as biocontrol agents in agriculture. In biological control, conventional strategy with the application of entomopathogenic fungi has been increasing to cropping system (Eilenberg *et al*., 2001).

In agroecosystems, insect associated fungi are very common. Arthropod hosts are found to be attacked by many members of order Hypocreales and Entomophthorales from Phylum Ascomycota in temperate regions. In agroecosystems, hypocrealean species *Metarhizium anisopliae* and *Beauveria bassiana* have wide host ranges. Recent advances in research have evaluated different prospects of fungal ecology that are related for conservation biological control. It is studied that *B. bassiana* are specifically linked with above ground insect hosts and *M. anisopliae* is particularly linked with hosts on or below the soil surface (Meyling and Eilenberg, 2007). Moreover, these two fungal reservoirs are found in soil environment and easily isolated from the same soil sample (Keller and Zimmermam, 1989; Meyling and Eilenberg, 2006).

It is essential to use a conservation biological control approach for the knowledge of population of natural enemies along with the effect of agronomical practices on these organisms. Entomopathogens roles in regulation of pest populations has mostly ignored during the surveys of delivery and diversity of ecosystem services to agricultural production (Altieri, 1999; Gurr *et al*., 2003; Tscharntke *et al*., 2005). Although, insect associated pathogen are amongst the natural enemies of arthropod pest in agrosystems. A reliable perceptive of the ecology of native community of the valuable organisms is a precondition for the assessment of their role in control of insect pests.

In this work, we described the present understanding of these ecological aspects of cotton agroecosystem in Punjab, Pakistan. The purpose of this research work was to lay foundation for future prospects on native population of insect associated fungi as a biological control agent in different regions of Punjab using conservation biological control strategy.

MATERIALS AND METHODS

Soil Samples Collection

Soil samples were collected from 10 sites covering whole area and different soil types in each selected district by digging out soil in agro-ecological system at different spots around plant rhizosphere covering an area of 5 meter square at the depth of 12 cm with the help of either soil core sampler (ϕ= 20mm) or through auger (for sandy soil). Five soil samples from each field were collected, pooled and mixed together into a single upto 01 kilogram. The altitude and geographical location of each zone was measured by Global Positioning System.

Rearing of *Galleria mellonella*

In plastic boxes rearing was performed and incubated in the dark climate controlled room at 20^{0}C temperature. Honey and water solutions were provided to adults moths. Folded paper strips were used for oviposition under the lid of box containing adults and females laid their eggs in crevices of folded paper. Eggs were removed attached with the paper and placed in a new box containing a ball of food for early instars. The neonate larvae moved toward food and started feeding after hatching. Larvae of 1 cm in length were provided with food for late instars. For baiting soil samples, larvae of 2.5-3 cm in length were suitable.

Insect bait bioassay

Insect bait method for selective isolation of entomopathogenic fungi was suggested by Zimmermann (1986). Several researches have been done using insect bait technique; especially *G. mollonella*. Sieve of 2-mm pore size was used to remove molding gravel and plant tissues from each soil sample. Soil samples were mixed completely and put in polythene bags. Soil samples were moistened with distilled water and remoistened to a suitable level to avoid infections by entomopathogenic nematodes. For each soil sample 3-5 larvae were used and baiting was replicated five times. Soil samples were incubated at 21±1ºC in dark area and containers were daily inverted during the first week of incubation. Soil samples were inspected after 5 days and inspection was repeated after 3-4 days for a period of three weeks.

Media preparation

For the isolation of insect associated fungi, Potato Dextrose Agar (PDA) media was used. The ingredients of the respective media were taken in 1000 ml conical flasks, and thoroughly mixed by vigorous shaking. The suspensions obtained were stream sterilized in autoclave for 15 minutes at 121°C at 15 PSI. Just before pouring antibacterial tetracycline and streptomycin (1/2 of capsule per 250 ml media) was added to each flask and mixed by shaking. The media were then poured equally (about 20 ml in each Petri dish) into already sterilized Petri dishes and were allowed to settle before inoculation of Galleria Larvae.

Inoculation of *Galleria mellonella* larvae

The dead bait larvae were surface sterilized with 1% Na-Hypochlorite for few seconds and inoculated to PDA Petri plates at 25°C in incubator for 7 days.

Isolation and identification of fungi

Entomopathogenic fungi were isolated from Galleria larvae and identified by their morphological characteristics like colony color and shape of culture on potato dextrose agar (PDA), conidiophore shape, and conidia shape and color with different taxonomic keys (Domsch *et al*., 1980; Nelson *et al*., 1983; Samson *et al*., 1988).

Pathogenicity test

Galleria mellonella larvae were bioassyed with the isolates of unknown pathogenicity. PDA media were prepared and tested fungus was inoculated on plates for 2 weeks, spores were rinsed with distilled water into 1.5 ml vials. *Galleria mellonella* larvae of final instar were dipped into spore suspension with the help of forceps for 3-5 s and transferred to Petri dishes with moistened filter paper. Parafilm was wrapped around the Petri dishes to maintain humidity and incubated in dark area at 20-25^{0}C temperature. Infected larvae were daily inspected till death and fungal structure of deceased larvae were recognized to confirm the fungus that was inoculated. Five larvae were treated for each fungus and experiment was repeated 3 times to assess the fungus pathogenicity.

Sun and Liu (2008) divided the isolated fungi into three categories on the basis of pathogenicity.

Entomopathogens

The isolated fungi that showed 100% mortality rate on larvae of Galleria were categorized as Entomopathogenic Fungi or Entomopathogens.

Opportunistic fungi

The isolated fungi showing mortality rate from 1-90 % on the larvae of Galleria were categorized as opportunistic fungi.

Secondary colonizers

The fungi isolated during Galleria baiting method that did not show any mortality on the larvae of Galleria were categorized as secondary colonizers.

Analysis of data

The occurrence frequency of insect associated fungi was measured by following simple formula:

$$\text{Frequency of Occurrence (\%)} = \frac{\text{No. of Individual Species}}{\text{No. of total Species}} \times 100$$

The species diversity was measured using beta diversity (β) which is the total number of species that are unique between communities (Meffe *et al.,* 2002). Beta diversity was determined by the following equation:

$$\beta = [S_1 - C] + [S_2 - C]$$

where 'S_1' is total number of species recorded in first community, 'S_2' is total number of species recorded in second community while 'C' represents the number of species common to both communities.

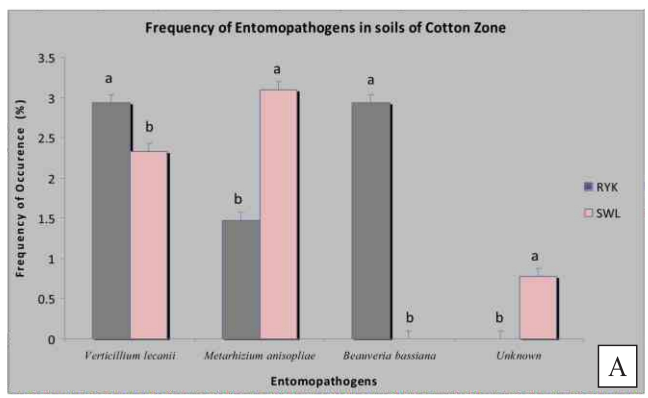
All data were analyzed by analysis of variance followed by Duncan's Multiple Range Test (Steel and Torrie, 1980) using computer software COSTAT.

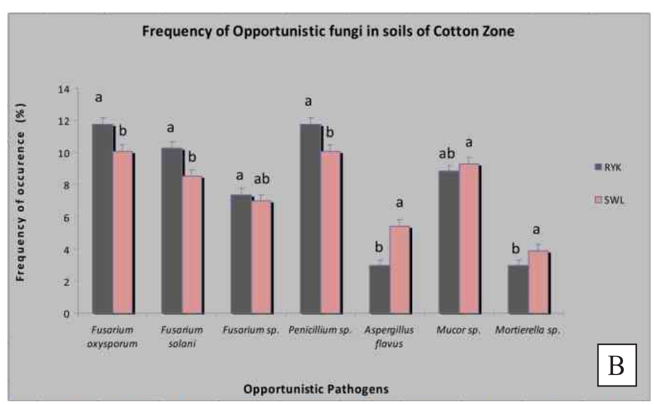
RESULTS AND DISCUSSION

Twenty soil samples from Cotton zone (Rahim Yar Khan and Sahiwal) were analyzed for occurrence frequency and distribution of entomopathogenic fungi.

Four species of Entomopathogenic fungi found in Cotton zone were *Verticillium lecanii, Beauveria bassiana, Metarhizium anisopliae* and an unknown species with different frequencies in each district. Occurrence frequency of Entomopathogenic fungi among all types of observed fungal species was the highest in District Rahim Yar Khan that was found to be 7.35 % in comparison with that of 6.21 % in District Sahiwal. The frequency of occurrence of individual Entomopathogenic fungi species was variable among Districts. The frequency of *V. lecanii* and *B. bassiana* was the highest in Rahim Yar Khan with the rate of 2.94 % for both and *Metarhizium anisopliae* with 3.10 % in Sahiwal. *Beauveria bassiana* and unknown sp. were lacking in the soil of Sahiwal and Rahim Yar Khan respectively. A significant difference was observed in the occurrence frequency of all isolated entomopathogenic fungi between both the districts of cotton zone (Fig. 01)

Seven species of opportunistic fungi found in cotton zone were *Fusarium oxysporum, F. solani, Fusarium* sp.*, Penicillium* sp.*, Aspergillus flavus, Mucor* sp. and *Mortierella* sp. with different frequencies in each district. Total frequency of occurrence of these fungi among all types of observed fungal species was the highest in District Rahim Yar Khan that was found to be 55.86 % in comparison with 54.28 % in District Sahiwal. The frequency of occurrence of individual Entomopathogenic fungi species was variable among Districts. The individual occurrence frequency of opportunistic fungi species, *F. oxysporum* and *Penicillium* sp. was the highest both in Rahim Yar Khan and Sahiwal with the rate of 11.76% and 10.08% respectively. A significant difference was observed in occurrence frequency of *F. oxysporum, F. solani, Penicillium* sp. *A. flavus* and *Mortierella* sp. On the other hand, there was no significant difference in the occurrence frequency of *Fusarium* sp. and *Mucor* sp. between both the districts of cotton zone (Fig. 01).





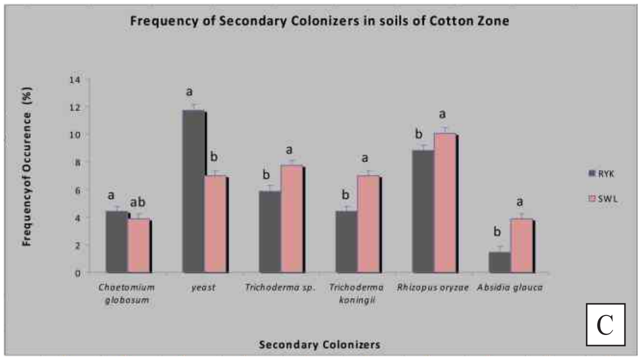


Fig. 1

Occurrence frequency of insect associated fungi in cotton zone. (A) Frequency of entomopathogens. (B) Frequency of opportunistic fungi. (C) Frequency of secondary colonizers

Six species of secondary colonizers found in cotton zone were *Chaetomium globosum,* yeast*, Trichoderma* sp.*, T. koningii, Rhizopus oryzae,* and *Absidia glauca* with varying frequencies in both the districts. Total occurrence frequency of these fungi was the highest in district Sahiwal that was found to be 39.55% among all types of observed fungal species in comparison with that of 36.75% in district Rahim Yar Khan.

The absolute value of beta diversity (β) of entomopathogenic fungi was 2 in cotton zone while in secondary colonizers and opportunistic fungi; it was zero in cotton zone (Table 1).

Table 1.

β Diversity of insect-associated fungi in soils of cotton zone.

	Fungal Community	RYK Present/ Absent	SWL Present/ Absent	Absolute value of Beta Diversity (β)
Entomo-Pathogen	*V. lecanii*	+	+	02
	M. anisopliae	+	+	
	B. bassiana	+	--	
	Unknown	--	+	
Opportunistic Pathogen	*F. oxysporum*	+	+	0
	F. solani	+	+	
	Fusarium sp.	+	+	
	Penicillium sp.	+	+	
	A. flavus	+	+	
	Mucor sp.	+	+	
	Mortierella sp.	+	+	
Secondary Colonizer	*C. globosum*	+	+	0
	Yeast	+	+	
	Trichoderma sp.	+	+	
	T. koningii	+	+	
	Rhizopus oryzae	+	+	
	Absidia glauca	+	+	

RYK=Rahim Yar Khan, SWL=Sahiwal

In our results the entomopathogens are relatively less than opportunistic and secondary colonizers in all localities of zone.

This low frequency may be due to diverse cropping systems because plant mixtures within crops minimize colonization by pest species. Push-pull strategy can lure the pest insects away from the crop (Hooks and Johnson, 2003; Cook *et al*., 2007). In agroecosystems, entomopathogenic fungal dispersal may also affected by insect behavior because fungal inoculums can be dispersed by insect activity. Reduction of bare ground area between crop plants by mulching may also reduce the population sizes of pests by enhancing conditions for ground dwelling predators (Hellqvist, 1996; Schmidt *et al*., 2004). Beta diversity was observed in case of entomopathogens while there was no absolute value of diversity in the case of Opportunistic fungi and secondary colonizers. This may be because increasing evidence suggest that heterogeneity is critical for the maintenance of species diversity in agriculture landscape and ecologically functional groups of organism that are relevant for pest management for sustainable agriculture in future (Benton *et al*., 2003; Weibull and Ostman, 2003). In cotton zone of Punjab different factors like temperature fluctuation around the year and different cropping system affect the diversity and occurrence of insect associated fungi so details study considering all these factors is necessary to determine the exact behavior of insects and their fungal pathogens before this these fungal pathogens are used as biological control.

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