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Application of entomopathogenic fungi for insect pests control

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Abstract
The grasshoppers and locust are one of the very familiar groups of insects to mankind. They cause significant damage to crops and cultivators protect their crops by using different chemical pesticides which put harmful effects on the human as well as on environment. During current era biological control is recommended to reduce the numbers of insects in field. In the complex of biological control agents entomopathogenic fungi are more effective and most successfully utilized insect pathogen. In this respect *Aspergillus, Beauveria, Metarhizium, Lecanicillium* spp. are gaining importance in the crop pest control in recent years due to the simpler, easier and cheaper mass production techniques. Many entomopathogenic species registered world-wide for insect's control but this practice is currently under consideration as a potential alternative to chemical insecticides for insects control in Pakistan. Present study focused on control of acridid because member of this group are major agricultural pests. They destroyed the rice, sugarcane, wheat, maize and fodder crops in everywhere. For control of this pest several pesticides of billion rupees are used indiscriminately in every year. So, there should be suitable, beneficial and cheap alternative of these poisonous chemical. For this purpose the biological control is very important therefore, an attempt was made to introduce pathogenic fungi, against the reduction of acridid population in Sindh.

Keywords: Insects, Microbial, Application, Entomopathogenic Fungi, Acridid

1. Introduction
Entomopathogenic fungi are regarded as bio-pesticides and expected to have a significant and increasing role for the control of locust and grasshopper in world including Pakistan [60]. This microbial agent commonly famous as myco-insecticides that has a great potential to kill locust and grasshopper species beside this, it also beneficial to control flies, beetles and aphids in field [62]. Pathogenic fungi penetrate into host external surface after utilization of pathogenic fungi large No. of grasshopper and locust were killed this finding suggests that this microbial agent is very useful against pest species. Microbial agents that include: bacteria, virus, nematodes, protozoan and pathogenic fungi are good bio-control agents [45] stated that pathogenic fungi are very important and interesting bio-control discipline due to its observed capacity that lead to formation of epizootics. Earlier, many workers have done research on this i-e [18, 19, 54, 70, 20, 26, 55, 60]. About 35 genera comprise on 400 species/sub-species of pathogenic fungi have been identified. These identified species having close association with more than 1800 insect species in field and mostly killed the wide varieties of insect's population in their favorable season [25]. Pathogenic fungi are cosmopolitan in their distribution and diversity; they put cruel attack on the insect's population. Due to their eco-friend and bio-persistence behavior and easily preference to kill pest species at different developmental stages, their utilization increase day-by-day. Now large numbers of pathogenic microorganisms are available for evaluation against grasshopper and locust in the world. Microorganism's priority is given to the entomopathogenic fungi and entomopoxvirus that are stable for prolonged period of storage and application. This microbial agent is considered very useful in IPM [32].
Consequently present attempt has been made to adopt biological control measures against pest by using the myco-insecticides from this region. A detail study has been done by [15, 50, 23, 4] to assess the mortality ratio of target pest after treating with various entomopathogenic fungi. But still now, nothing has been published with exception of [28, 52, 17]. But, mostly these scientists worked under environment constant regions that are condition for more infection and could not consider how this behavior and the overall impact of pathogen might change under more realistic, variable condition experiment in the field.

The present study was aimed to improve the effeteness of pathogenic doses on the feeding and incubation of insects under controlled conditions where temperature was optimum.

2. Materials and Methods

2.1 *Insect sampling*

The stock of grasshoppers (mature and immature) was collected from various districts of Sindh. Most of specimens have been collected in month of April to November during the year (2014-2016). Insects were collected by swept net having (25×25) diameter while, 82cm in length (without diameter). The material was mostly captured by hand picking, sweeping, trapping, night trap, aerial netting and black light pan traps when ever found. Collected insects took to the laboratory where two cages of different measurement i-e (42cm in length, 30cm in width) and (35cm in length, 32.5cm in width) were maintained. All collected individuals equally divided and put into cages. Fresh leaves of *Zea mays* serve to rearing insects before this leaves and twigs were sterilized in 5% solution of Sodium hypochlorite (NaOCl). This methodology has been adopted from [59, 60]. For identification of samples scheme given by [61] was followed.

2.2 Infected sampling

For capturing of insects contaminated with pathogenic fungi carefully observation has been made in field and only those insects were collected which having clear symptoms of mycoses viz: (i) insect don't move fast, (ii) de-coloration not original (iii) fungal mycelia fully spread on cuticle (iv) insects look sluggish/inactive and was very easy to capture. Infected specimens easy captured with large forceps after collection material transferred into glass jar and brought to laboratory for further analysis. All were sorted out into different host species and kept in clean cages. Fresh *Zea mays* leaves were provided to insects. Food plant was changed daily and following observations have been noted which include: food consumption, through analysis of faecal material and mortality of insect after every 24hrs.

2.3 *Incubation in laboratory*

Different species of Acrididae divided into group of about 50 individuals for each treatment. However, there was no differentiation in age, sex and developmental stage. All collection placed into wooden cages under laboratory conditions where temperature range was between (28±2 ºC to 41±2 ºC) and Relative humidity (RH) was (26.5% to 60.5%). Population of grasshoppers comprising on all developmental stages which were collected from field maintained in the laboratory, Entomology and Bio-Control Research Lab. (EBCRL), Department of Zoology, University of Sindh, Jamshoro (25º-23/N, 68º-24/E).

2.4 Fungal isolation and sporulation test

The sporulating fungi separated in pure culture on SDA (Sabouraud Dextrose Agar), after that it was formulated into oil (coconut) after preparing the oil formulation this fresh suspension was kept in sonicator for 60 sec to break the conidial chain. After breaking conidial was counted with the help of haemocytometer, this method has been adopted from [58, 34].

2.5 Identification of fungal isolates

Various species of *Aspergillus* have been identified on the basis of conidia shape and size. Beside this, for detail and authentic identification element concentration has been determined under SEM microscope. For reorganization of fungi terminology given by [21, 13, 24, 1, 22] was followed.

2.6 Pathogenicity Bioassay

Different fungi species were isolated and then isolates was grown at 28 ºC where photoperiod ratio: 12hrs light, and 12hrs darkness about 15 days [2, 34]. Sterile spatula after incubation was used to harvest the conidia from fungal culture. This harvested conidia shifted into small McCartney bottle (fully sterilize and contained coconut oil) fungal spores suspension prepared in oil and spore concentration measured with Neuberger [43].

2.7 Formulation of Aspergillus conidia

Two different formulations were selected in order to know that which formulation is more effected. Before starting the experiment different part of *Zea mays* (consist on leaves and stem) were broken shake into tap water than dire and put vertically into cage as well as in jars but before this, weight of food plants were taken i-e (2.5gm) put in small jars (26gm) kept in cages respectively. The insects were reared into small jars as well as in captivity. 10 insects were reared in 4 liters plastic jars, while 50 individuals were kept in cages. Oil and Water formulation was used.The conidial oil distilled water formulation was sprayed on the insects using a hard held sprayer. Each insect was directly and individually sprayed with 3.5ml of the appropriate concentration. After 15 to 20 minutes the treated insects were transferred to the jars as well as in cages. Control groups received the water formulation but, without conidia. The insect in each replicate were fed on *Zea mays* (30gm every 48hrs).

2.8 Applications

Before the commencement of bioassay test insects were reared in cage for one week. After that 0.1ml of conidial oil suspension was carefully applied beneath the pronotum shield of the insect by the help of (Sterile Pasteur Pipette). Beside this, in control replicant blank oil with spores was applied on the pronotum shield of hopper that was reared in jars individually. While in second replicant the conidial (mix in distilled water) formulation were sprayed on the insects (reared in captivity) using a hard held sprayer. Each insect was directly and individually sprayed with 3.5ml of the appropriate concentration. After 15 to 20 minutes the treated insects were transferred to the cages. Control groups received the same water formulation without conidia. The insect in each replicate were fed on *Zea mays* (30gm after every 48hrs). Insect feeding was assessed by measuring consumption of food and then assessing their faecal production. Food consumption of insets for every 48hrs was measured after treatment the faeces production from each cage and jars were also collected every 48hrs. After this insect contaminated with *Aspergillus* and healthy grasshoppers were shifted into separate cages place in laboratory. After the transferring the insect their detail mortality was recorded.

3. Results and Discussion

During the present study large number of grasshoppers were captured from many climatic regions of Sindh the collected material was sorted out into 32 species belong to 06 sub-families i-e Acridinae, Calliptaminae, Gomphocerinae, Hemiacridinae, Oedipodinae and Oxyinae of family Acrididae. Four dominant species i-e *Hieroglyphus nigrorepletus* Bolivar, 1912, *Oxya velox* (Fabricius, 1787), *Acrida exaltata* (Walker, 1859) and *O. hyla hyla* Serville,

1831 accounted for maximum number compare to other collection (Fig. 1). It was noticed that representative of family Acrididae, including grasshoppers and locusts are the most voracious pests known, with the fifth or sixth instars and adults capable of eating their own body weight in various vegetations. In result of extensive survey a total of 2520 specimens have been collected from different districts of Sindh. Grasshopper having great economic important due to its geographically distribution and wide pest status (Table I) numerous species reported as major pest of earning crops like rice, sugar-cane, maize, wheat and cotton they, destroy the important vegetables, fruits and fodder crops as well. Beside this, their targeted habitats were also highlighted in (Table 2).

During this study it was also noticed that infected insects altered their thermoregulatory behaviour and showed a *behavioral fever* response to the pathogen their body temperature was raised as a means of literally toasting a fungal invader. Further, these behavioural responses may result in enhanced spore diffusion and fungal fitness. After the pathogenic application it was also noticed that the production of cuticulur antimicrobial lipids, protein, and metabolites. Shedding of the cuticle during development and behaviour environmental adaptation that include: fever, burrowing and growing was also effective significantly. It was also noted that after the application of oil and water based formulation of *Aspergillus* acridid species undergo in very interesting behaviour modification after infection mostly prior to death. Beside this, insect become thin, sluggish, abnormally, climb a stalk of grass (put into jars and cages) they clasp their legs around the stem and die in this position in majority of cases. Reduction in feeding due to fungi infection may affect body fat accumulation at sexual maturity and consequently reproductive potential of insects is infected.

Table 1: The insect along with their major and minor target habitats

Taxonomic status of insects		Targeted Habitat		Total No. of insects (N= 2520)
		Major	**Minor**	
Acridinae	*Acrida exaltata*	Maize, Wheat	Fodder crops	148
	A. gigantean	Grass, Alfalfa	Cotton	164
	Duroniella laticornis	Maize	Sugarcane	36
	Gelastorhinus semipictus	Wheat	Millet	56
	Phlaeoba infumata	Maize	Sun hemp	37
	P. tenebrosa	Alfalfa	Grass, Fodder crops	57
	Truxalis exmia exmia	Meadow grass	Thorn weed	69
	T. fitzgeraldi	Millet, Maize	Maize	24
Calliptaminae	*Acorypha glaucopsis*	Rice	Mustard	73
	Sphodromerus undulatus undulates	Jawar, Millet	Bahamas grass	22
Gomphocerinae	*Chorthippus indus*	Rice, Cotton	Crab grass	16
	Ch. dorsatus	Grasses	Fodder crops	36
	Gonista rotundata	Sugarcane	Vegetable	18
	Ochrilidia geniculate	Maize	Fodder crops	8
	Oxypterna afghana	Rice	Cabbage	38
Hemiacridinae	*Hieroglyphus banian*	Rice, Sugarcane, Maize	Thorny vegetation	91
	H. nigrorepletus	Rice, Sugarcane	Maize, Wheat	207
	H. oryzivorus	Rice, Cotton	Sugarcane	146
	H. perpolita	Surrkanda	Grass, Maize, Sugarcane	91
	Spathosternum prasiniferum	Maize, Rice, Sugarcane	Vegetable, Fruits	23
Oedipodinae	*Acrotylus humbertianus*	Grasses, Vegetable	Maize	185
	A. longipes longipes	Vegetable	Cotton	64
	Aiolopus thalassinus thalassinus	Bajra	Wheat	128
	Hilethera aelopoides	Jawar, Maize	Cauliflower	61
	Locusta migratoria	Rice, Sugarcane	Wheat, Cotton	142
	Oedaleus rosescens	Grain	Vegetable	14
	O. senegalensis	Grasses, Maize	Flower	20
	Trilophidia annulata	Rice, Grasses	Bermuda grass	9
Oxyinae	*Oxya bidentata*	Rice, Maize	Bind weed	52
	O. fuscovittata	Rice, Maize	Cereal plant	51
	O. hyla hyla	Rice, Maize	Wheat, Grasses	247
	O. velox	Rice, Maize	Maize, Jower	187

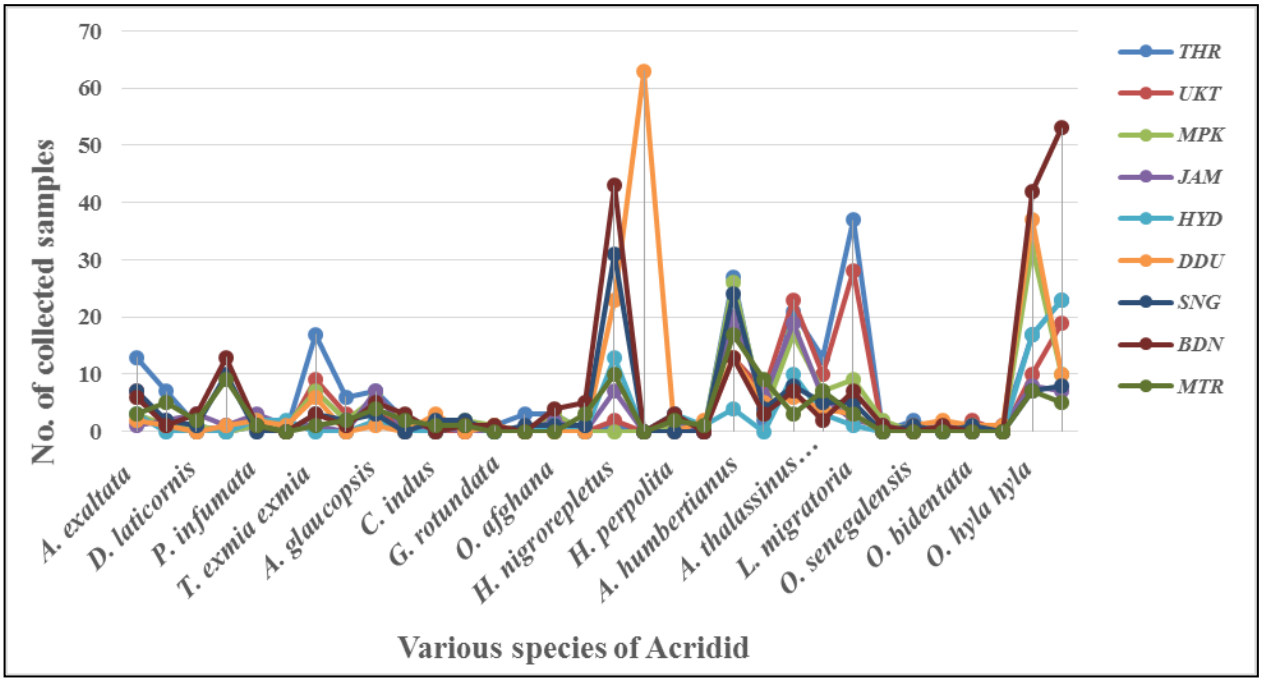

Fig 1: Showing the collection of various species from different districts of Sindh

Table 2: An overview of the entomopathogenic fungi developed for microbial control of insects pests in world-wide

Species name	Targeted insects	Produced in	Selected reference
Aschersonia aleyrodis	Hemiptera (Aleyrodidae)	Russia	Fransen [16], Meekers *et al.*,[48], Lacey *et al.*,[40], [41], McCoy *et al.*,[47]
Aspergillus sp.	Orthoptera	Pakistan	Kumar and Riffat [32, 33], Kumar *et al.*,[34-38]
Beauveria bassiana sensu lato	Acari, Coleoptera, Diplopoda, Diptera, Lepidoptera, Orthoptera, Siphonoptera, Thysanoptera	Africa, Asia, Australia, Europe, South & North America	Rosa *et al.*,[63], Wraight *et al.*,[72, 74], Brownbridge *et al.*,[5, 6], Chandler *et al.*,[9], Wekesa *et al.*,[71], Brownbridge, *et al.*,[6], Labbe *et al.*, [39]
Beauveria brongniartii	Coleoptera (Scarabaeidae)	Europe, Colombia, Reunion Island	Zimmermann [75], Keller *et al.*,[30], Dolci *et al.*,[12], Townsend *et al.*,[68]
Conidiobolus thrombodies	Acari Hemiptera, Thysanoptera	Colombia, India, South Africa	Papierok and Hajek [53], Nielsen and Hajek [51], Hajek *et al.*,[20]
Hirsutella thompsonii	Acari	India	McCoy *et al.*,[47], Chandler *et al.*,[8, 9]
Isaria fumosorosea	Acari, Diptera, Coleoptera, Hemiptera, Thysanoptera	Belgium, Colombia, Mexico, USA, Venezuela	Wraight *et al.*,[72-74] Lacey *et al.*,[40-42], Zimmermann [76]
Lagenidium giganteum	Diptera (Culicidae)	USA	Kerwin and Petersen [31], Skovmand *et al.*,[64]
Lecanicillium longisporum	Hemiptera	Brazil, Netherland	Bird *et al.*,[3], Down *et al.*,[14], Kim *et al.*,[29]
Lecanicillium muscarium	Acari, Hemiptera, Thysanoptera	Netherland, Russia	Chandler *et al.*, [9], Cuthbertson and Walters [11], Burges [7], Goettel *et al.*[19]
Metarhizium anisopliae sensu lato	Acari, Blattoidea, Diptera, Coleoptera, Hemiptera, Isoptera, Lepidoptera, Orthoptera	Africa, Asia, Australia, Europe, South Central & North America	Rosa *et al.*,[63], Chandler *et al.*,[9], Wekesa *et al.*,[71], Jaronski and Jackson [27], Lacey *et al.*,[42]
Metarhizium acridum	Orthoptera	Australia, South Africa, USA	Lomer *et al.*,[44, 45], Thomas [67]
Nomuraea rileyi	Lepidoptera	Columbia, India	Moscardi and Sosa-Gomez [49], Thakre *et al.*,[66]

Note: Fair No. of entomopathogenic species along with their targeted insects have been enlisted by various workers from all over the world but, any single reference is not yet avaible from Pakistan present attempt is being carried out for the first time (after [40-41])

Aspergillus is one of the most important groups of fungi exhibiting immense ecological and metabolic diversity [46]. Beside this, it is a large group with 180 accepted species belonging to different genera [57, 69] recommended that Orthopteran species are classified according to their reproductive strategies, micro-habitat and micro-humidity niche preference. Phipps [56] describe *Xerophilous*, *Mesophilous* and *Hydrophilous* preferences for dry, medium and humid habitat and [65] propose that *Hieroglyphus daganensis* and *C. fuscocreruleipes* were reported as more dominant species infesting millet, sorghum and rice crops in the Malanville area Benin. They treated these species with entomopathogenic fungi and got significant result at the present my obtained data closely relative with them. With a view to determine prevalence of grasshoppers species in certain area of Sindh investigation was undertaken in the upper Sindh comprise on 9 regions and later on in the lower Sindh under different ecological condition on grasses and other host plants. From control point of view, these investigations provided useful information's in establishing prevalence of grasshoppers species in different seasons in aforesaid area which did not seem to have been reported from Sindh.

Keeping the objective in view, studied on seasonal incidence of grasshoppers was initiated in the Sindh province. This province is known to have precaution topography. The major portion of the province was a part of desert areas. Present study recommends that, information pertaining to seasonal occurrence of grasshopper in a given habitat with different ecological conditions i-e cropped/non-cropped area will be useful in understanding population development in these areas.

4. Conclusion

During the present study, it was noticed that order of prevalence of grasshopper species was varying in both selected upper and lower region of Sindh. It can be seen from the data that majority of species having dominant and moderate pest status. The population pattern of these species suggest that grasshoppers are available throughout the season both in cropped and non-cropped areas due to these polyphagous habits particularly wide range of host plants. The activities of grasshopper, however, vary season to season in both selected regions. It may be recommends that control measures during monsoon (i-e June to July) may prove effective when grasshopper population started building up. It is particularly suggests that adjoining non-cropped areas of wild-flora be treated where development of grasshopper population is continued in parallel to cropped areas. The specimens have been captured during present survey all having great importance. Earlier, [10] gave overall assessment of many importance species of locust and grasshopper in Agricultural Manual; they indicated their pest status by rating different letters. Presently all minor and major importance insects were come in collection.

Present study suggests that these microbial insecticides are not caused any harm to non-target organisms which are available in field. This research is an initiative step towards the utilization of pathogenic fungi in Pakistan. It is recommends that grasshoppers that contaminated with fungi assist the raising of body temperature which don't permit insect for longer survival-ship.

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