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Review Article

Novel Fungal and Bacterial Species exploited for the control of Locust

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ABSTRACT

Insects like locusts and grasshoppers are one of the most dangerous bio-pests of cash crop. The locust control requires constant attentiveness. They could cause around 20 million people to be left without products of agriculture and that is only in Asia. Locusts attacked Khyber Pakhtunkhwa and Baluchistan provinces of Pakistan first in June 2019 moving towards Sindh and Southern Punjab. According to the Food and Agriculture Organization (FAO) 2020 the financial damages are range from 353 billion to 464 billion Pakistani rupees. The current environmental issues and high price of insecticides are increasing the demand of biological control. In this paper we have reviewed the microbes that can be effectively used to control locust attack in Pakistan.

INTRODUCTION

The animal and plant that is harmful to humans or human concerns like agriculture and livestock is called a pest [14]. The pests, weeds, and other pathogens are the factors on which the quality of crops for human use depends on and is currently at risk. About 10,000 years ago numerous applications of agricultural processes came to light and since then farmers have to protect their crops from plant pathogens, weeds, animals, and other pests, including insects, mites, nematodes, rodents, slugs, snails, and birds [16]. There are approximately 10,000 species of insects and pests, more than 30,000 species of weeds, up to 1000 species of nematodes, and over 100,000 associated diseases that cause damage to the world of crops [3]. Locusts, grasshoppers, termites, and cattle ticks are the pests that are responsible for vast economic and agricultural loss in most parts of the world for instance, China, Japan, Australia, Malaysia, Africa, Brazil, and Mexico [1]. The family Acrididae's include pests like grasshoppers and locusts are present in grasslands and crops for the entire dry zones of the world [7]. Grasshopper and Locust are similar in appearances but differ in behaviour. The locust demonstrates two kinds of social behaviours; a) solitary and b) gregarious, but grasshoppers do not show this behaviour. The excessive growth of vegetation increases the level of neuro-transmitter serotonin, after a drought, that causes changes in their brain and they start to reproduce exceptionally [23]. The most dangerous of all locust species is considered to be desert locust (Schistocerca gregaria) [17]. The breeding sites of desert locust (Schistocerca gregaria) are present in Pakistan. The breeding starts in the deserts of Baluchistan in winter and then proceeds to the desert areas of Sindh, Pakistan for

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second breeding. These breeding grounds makes the control and prevention methods a bit difficult [21]. The desert locust is the main concern because it has the capability to travel long distances and reproduce rapidly while the migratory locust (Locusta migratoria) is the most widespread specie of locusts. The desert locust's life cycle consists of 3 – 6 months and a mature locust may lay over 100 eggs a day. The larva requires only 20 days for maturation as it is a fast growing pest. With the maturation of larva, the next generation of pests initiates. The 25 – 32°C temperature ranges are optimal for its growth with humidity of about 85 – 92%. The desert locust could travel 10 hours a day covering 150kms because of their ability of powerful and long flights (Figure: 1) [17].

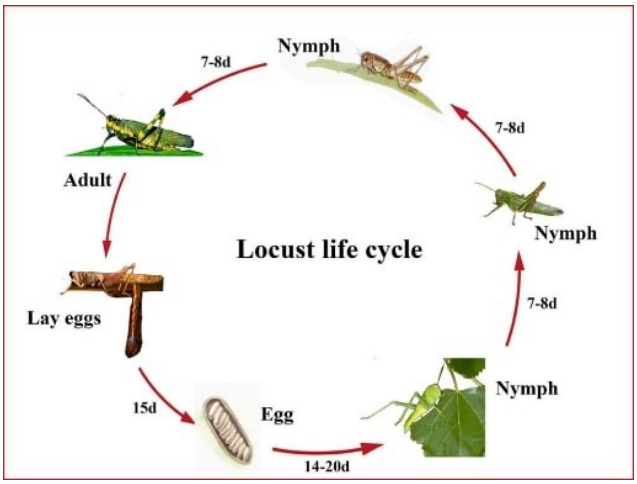

Figure 1: Life cycle of Locust

The traditional migratory routes of locust may change due to climate change which causes the locust to look for the new breeding and propagation areas [20]. More precipitation and high temperature speed up the growth and increases the number of locust population [5]. The outbreak of desert locust has been seen in 2019 – 2020 in Africa, South Asia, and Arabian Peninsula (Figure 2). This is causing a thread to food supply across the region. The locust found in Pakistan and Iran came from India [23].

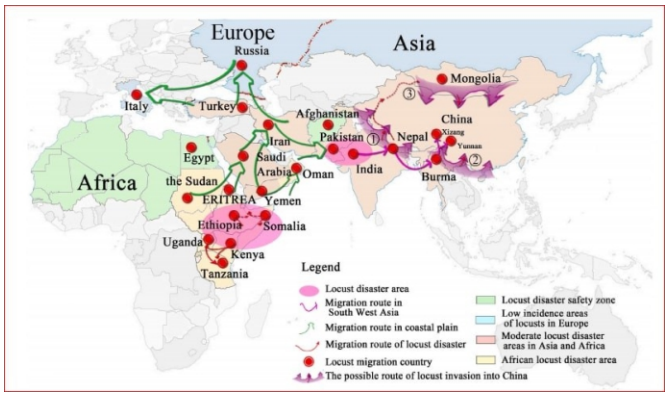

Figure 2: Chronology of the great locust outbreak in 2020

The desert locust consumes many kinds of crops and plants which includes a wide range of vegetable and cereal crops, banana, citrus, groundnuts, fruit trees, coffee, and many others. Locust also attack economically important crops like wheat, sunflowers, beans, cotton, potatoes, and sugar cane [12]. In order to minimize the effect of chemical pesticides, the spray methodology is being used widely since mid-20th century [13]. According to Ilboudo et al, (2014) [8], the chemical pesticide Diazinon risks mostly aquatic invertebrates then crustacean, fish, and algae. His findings suggested that pesticides that are used in the control of locusts create a potential danger to aquatic organisms. The replacement of Dieldrin and BHC pesticides, due to their banning, organophosphates, and synthetic pyrethroids are being used either separately or in combination for locust and grasshopper control. The organophosphates and synthetic pyrethroids have toxic effects on mammals and adverse effect on aquatic organisms and insects that are beneficial [10]. 300,000 litres of Malathion pesticide which is an organophosphates pesticide, has been given to Pakistan by China. This pesticide is being used since March 2020 as reported by DAWN [4]. Banik et al., (2020) [2] reported that Silafluofen pesticide is more toxic to fish as compared to Biphenthrin and Diafenthiuron. It may also be toxic to birds while it shows positive AMES toxic test level, meaning that it could cause DNA mutations. The locust control requires constant attentiveness. They could cause around 20 million people to be left without products of agriculture and that is only in Asia [9]. Locust attack can cause damage to crops in a short period of time that could possibly lead to famine and starvation conditions [22]. But the locust and grasshopper are also important in ecosystem of grassland and are crucial in the nutrient cycle [23]. Locusts attacked Khyber Pakhtunkhwa and Baluchistan provinces of Pakistan first in June 2019, moving towards Sindh and Southern Punjab. It was stated that this attack would cause a financial damage of PKR 688.5 billion ($4.1 billion) of kharif crops and PKR 705.8 billion ($4.2 billion) of Rabi crops by FAO [25]. The use of parasites of insects is very common to be used as bio-pesticides against many of the insect species [11]. In this regard the affective microbes found by researchers are mostly fungal species, *Aspergillus spp., Metarhizium spp., Beauveria spp., and* novel bacterial specie *Bacillus thuringiensis and Bacillus cereus.*

FUNGAL SPECIES

Kumar and Sultana, 2015 [11] collected 1075 Insects from different crops like Rice, Maize, and Sugarcane. Only mycoses infected insects were collected. These insects were kept in clear cages and Zea mays (maize) leaves were provided. For the isolation of fungal species, dead insects were picked up from cages with mycelia. The fungi specie is

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isolated with film methods and placed on slides, which are stained with lacto-phenol cotton blue to observe the hyphae and conidia. These slides were studied under Stereoscopes Binocular Microscope. Using that specific microscope, the shape and size was noted. For the identification of isolated fungal species, the observations made of shape and size of fungal isolates were identified according to description provided by previous studies. The results of experiment showed that 438/1075 were observed to be infected by *Aspergillus* and *Beauveria* infection. It was observed that fungal infections increase and could been seen on the side of pronotum and thorax. The insects begin to die slowly by the 3rd day and by the 7th day all insects died. They recommend that earlier stages are more vulnerable to fungal infection. The lateral stages have slight resistance to fungal infections but with increased pathogenic dispersal all stages die in approximately 6 days. They reported that a pathogenic fungus helps in repression of insect population. ***Metarhizium anisopliae;*** Kh et al., 2020 [9] collected Moroccan Locust (*Daciastaurus moroccanus*) from pastures of Guzar and Nishan provinces of Kashkhadarya region of the Republic of Uzbekistan. Two experiments were performed, one with *Metarhizium anisopliae* in laboratory experience and used hand sprayer of 120 l/ha. Second experiment with *Metarhizium anisopliae* with addition of 1% betacypermetrhin (chemical pesticide) in laboratory and hand sprayer of 120 l/ha. For the control The Green Guard, SC is used in measurement of 0.5 l/ha. In case of *Metarhizium anisopliae,* the results suggested that experimented bio-pesticide if used in 1l/ha and 1.5 l/ha gives maximum results of biological efficiency i.e. 88.4% and 96.8% respectively. *Metarhizium anisopliae* and 1% betacypermetrhin; the results suggested that experimented bio pesticide if used in 1 l/ha gives 100 % results of biological efficiency. Sabbour, 2014 [19] researched locust beneath laboratory circumstances for numerous generations on semi-artificial diet. The *Metarhizium anisopliae,* fungus isolated from screening experiments by means of 200 samples. The isolates were inoculating in 50mL Potato Dextrose Broth (PDB) medium for destruxin manufacture. For the bioassays; the fresh citrus leaves containing CLM larvae be collected every day and once counting at least 10 early larvae used in experiments. The extracted destruxin as well as 10, 15, and 20-fold dilutions be used in bioassays. The ready leaves be dipped in concentration for 10sec and dehydrate for about one hour. The treated leaves were then placed in petri dishes and were subjected in an incubator at 27°C. The probit and T-test options of SPSS software were used for analysing time mortality and comprising means of mortality, correspondingly. The extracted destruxin were ready to be subjected for nanoparticles via National Centre Microbiological Team. Then, scanning microscopy was performed and the results show that the laboratory experiments indicate that the Nano-destruxin is the mainly efficient against the locust *S. gregaria* compared to the destruxin and the control. The infestation of the locust *S. gregaria* under semi-conditions in comparison with destruxin and Nano-destruxin which demonstrate that the infestation present was considerably decreased following treated with Nano-destruxin.

BACTERIAL SPECIES

Bacillus thuringiensis has 2 classes of endotoxins; a) Diptera-specific cytolytic (Cyt) proteins and b) Crystal (Cry) proteins. Crystal (Cry) proteins are insect-specific insecticidal and is encoded by cry genes but it has a narrow range of target insects. It has 3 domains (from N terminus to C terminus). Wu et al., 2011 [24] collected *Bacillus thuringiensis* from Chinese soil sample. The vector plasmid pGEM-T Easy was purchased from Promega and Ziniu Yu of Huazhong Agricultural University provided Vector pQE30 and *Escherichia coli* M15. For cloning of Cry genes, they first amplified a fragment 1.2kb of cry gene form genomic DNA using two step PCR and then this fragment was sequenced. Based on this sequenced 1.2kb fragment, primers were designed and the flanking DNA regions were amplified using genome walking. Purification of PCR product using 1.2% agarose gel was completed. Purified product was then ligated into pGEM-T Easy vector. The recombinant plasmid was transformed into the *E. coli*. For analysis of the Cry gene, the primers were again utilized based on sequenced 1.2kb fragment and amplification was done of strain BTH-13 using PCR. The full cry gene was then sequenced. Structure analysis of Cry protein of BTH-13 was done by NCBI BLAST, Clustal W, Predict Protein, Swiss-model, and Strap. For the expression of Cry protein in *E. coli,* 3 plasmids were prepared; a) pQE-30/7Ca1 (gene encoding the whole cry protein), b) pQE-30/toxin1 (including three domains but without N-terminal and C terminus), and c) pQE-30/toxin2 (three domains and C terminus). Positive transforms were selected on the bases of kanamycin and ampicillin. The cells were lysed and harvested by centrifugation at 13,400 x g for 10min at 4°C and supernatant was stored at -20ºC. The crystal protein is prepared and activated by trypsin. This activated protein is then solubilized in 0.1 M NaOH at temperature of 37°C for 30 min. It was then digested by trypsin at a final concentration of 10 mg/ml. The Analysis of δ-Endotoxin obtained from transformed cells was done by western blotting, where they prepared crystal proteins from wild type BTH to immune a rabbit and then obtained the anti-serum. The protein isolated was separated by SDS-PAGE and western blotting was performed. Bioassay preparation of δ-Endotoxin: the expressed toxin 1 & 2 protein + prepared novel endotoxin and crystal protein was

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diluted with distilled water in concentrations of 20, 10, 5, 2.5, and, 1.25 g/ml. These concentrations were applied to 30 migratory locusts (*Locusta migratoria manilensis*) /dilution. For application inoculated corn seedling were fed to locusts. These locusts were kept at 28 °C at 65 to 75% relative humidity. Observations were recorded from 2-7 days. This experiment was repeated 3 times. Observation of δ-Endotoxin on the mid-guts of locusts was done by selecting random insects and dissection was performed to get the mid-guts. Phase-contrast microscope was used for the observations. Results of the experiments explained that the segment of 1.2kb and final plasmid vector pGEM-T Easy, ORF has 3432bp, the gene encodes for a protein of 1144 amino acids with a molecular mass of 129kDa. The sequence was then submitted to GenBank. The designed δ-Endotoxin was named Cry7Ca1. For the analysis of Cry7Cal Structure the wild type domains were compared with designed domains. For Domain I there was difference of 2 loops, for Domain II 3rd loop is 4 residues longer, for Domain III 5 loop difference was noted. The results of expression of Cry proteins in *E. coli* suggested that pQE-30/toxin1: Cry7Ca1 and pQE-30/toxin2 were activated by Cry7Ca1. The expressed product has a weight of 129, 64, and 72kDa. The toxin activated by trypsin was 64kDa and was expressed by toxin 1 which was activated by Cry7Ca1. The bioassay was applied on second instar and results showed that CryCa1 and activated toxin 1 and 2 have significant effect on the locust. The lowest concentration requirement of CryCa1 is 8.98 g/ml, toxin 1 is 0.87 g/ml and toxin 2 is 4.43 g/ml. The examination of mid-gut of locust showed disruption of epithelial cells and it is by action of toxin 1 which is activated by CryCa1. They concluded that in expression of Cry7Ca1, lack of certain molecule chaperons may affect the expression. N-terminus may inhibit toxins action and C-terminus it is not important for the toxins action. So if the terminus is cleaved then activity of Cry protein can be enhanced. Cry7Ca1 activated toxins act on mid-gut only and therefore killing the locusts. The Cry7Ca1 has potential application for an insecticide and transgenic plants that have the activity against locust. ***Bacillus cereus;*** Reda et al., 2018 [18] acquired the nymphs and adults of desert locust (*Schistocerca gregaria*). The dead were separated from infected ones and bacteria were collected from body surface, internal swap for mid-gut and cavity, and dead locust paste were swabbed and directly streaked on nutrient agar plates. Plates were incubated at temperature of 28 °C for 72 h. In order to identify the bacteria demonstrating activity against locusts. The isolated bacteria were centrifuged at 4000 rpm for 15 min at 4 °C after they were grown overnight at a temperature of 30 °C with 150RPM agitation. The pellets were collected and adjustment of cell density for each isolate was done and mixed in a beaker of 50ml. The clover leaves were treated with these solutions and dried at room temperature for 10 minutes. These treated leaves were fed to 4th nymph instar locust. 10-day observation was made and 2/30 bacterial isolate showed the activity against the locust. Bacteria were identified morphologically and biochemically. For the preparation of Bioassay 4 dilutions of stock solutions were prepared. Two techniques were opted for the application of bioassay on the locust. (a) Leaf dipping technique; stock solutions of DL3 and DL4 539 × 10^6, 23 × 10^6 CFU/ml were prepared respectively. The 4 dilutions were prepared of each stock solution. DL3 stock solution was diluted as; Dilution 1 (269 × 10^6 CFU/ml), Dilution 2 (134 × 10^6 CFU/ml), Dilution 3 (67 × 10^6 CFU/ml) and Dilution 4 (33 × 10^6 CFU/ml). DL4 stock solution was diluted as; Dilution 1 (11.5 × 10^6 CFU/ml), Dilution 2 (5.7 × 10^6 CFU/ml), Dilution 3 (2.8 × 10^6 CFU/ml) and Dilution 4 (1.4 × 10^6 CFU/ml). Fresh clover leaves were dipped in each dilution for 3min then then allowed to dry at room temperature for 10 minutes. These treated leaves were fed to 3 sets having 180 insects of 4th instar nymph. (b) Per OS technique: For this the stock solutions of DL3 and DL4 1078 × 10^6, 467 × 10^6 CFU/ml were prepared respectively. The 4 dilutions were prepared of each stock solution. DL3 stock solution was diluted as; Dilution 1 (539 × 10^6 CFU/ml), Dilution 2 (270 × 10^6 CFU/ml), Dilution 3 (135 × 10^6 CFU/ml) and Dilution 4 (67 × 10^6 CFU/ml). DL4 stock solution was diluted as; Dilution 1 (233 × 10^6 CFU/ml), Dilution 2 (117 × 10^6 CFU/ml), Dilution 3 (58 × 10^6 CFU/ml), and Dilution 4 (29 × 10^6 CFU/ml). The dilutions made from stock solution were applied through 1 cm Hamilton syringe with a needle of 24 gauge. For each isolate 1 set of 30 insects were selected of 4th instar nymph. The results of evaluation of toxicity regression were obtained by using probit analysis. Results of experiment suggested that 30 isolates were founds and 2 isolates were selected because of their activity against the locust. Bacterial identification by morphologically, biochemically and sequencing rRNA all resulted that isolates that showed activity against locust were DL3 and DL4. 100% mortality results were observed for cell density of 539 × 10-6 DL3 and 23 × 10-6 DL4 after 48 h and 3 days. Leaf dipping technique was found to be effective. Throughout the treatment the body colour of locusts changed from red to dark red and lastly black. DL4 was found to be virulent as compare to DL3. *Bacillus cereus* produces enteric toxins. The 2 isolates can be potentially used as bio pesticides and decrease the use of chemical pesticides. Novel endotoxins from *Bacillus thuringiensis - 13* preparations are laborious but can be useful in killing locust. *Bacillus cereus DL3* and *DL4* gives results in just 3 days. It is also present ubiquitous in nature (Figure 3).

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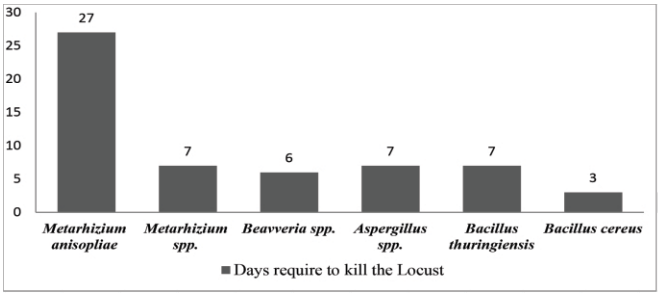

Figure 3: Comparison of time period required to kill Locusts [11, 9, 19, 24, 18]

CONCLUSION

The microbes which can be used are Aspergillus spp., Metarhizium spp. and Beauveria spp. and novel bacterial specie Bacillus thuringiensis. Aspergillus spp., Metarhizium spp. and Beauveria spp. would kill in 6 – 7 days the locust while Metarhizium anisopliae would require 20 – 35 days. The novel endotoxins from Bacillus thuringiensis-13 would also give results in 6-7 days. Aspergillus spp., Metarhizium spp. and Beauveria spp can easily be isolated from nature as they are readily available in soil and only require 6 – 7 days to give results. Metarhizium anisopliae despite being easily available requires a time period of 20 – 35 days to give results. According to FAO there in the month of November they do not find any locust in any of the 4 provinces. They predicted that there could some adult locusts present in Baluchistan but the number would not be harmful.

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