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Entomopathogenic Potential of *Hirsutella spp*. Against Guava Fruit Fly (*Bactrocera Zonata*): A Step Toward Sustainable Agriculture

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Guava (*psidium gujava* L.*)* belonging to the Myrtaceae family, is an important fruit crop of tropical and subtropical regions. Production of guava is affected by many biotic or abiotic factors. Biotic factors, particularly fruit flies (Diptera), cause heavy losses. This research aimed to control fruit fly infestations. The entomopathogenic fungus *Hirsutella* spp. was utilized as a biocontrol agent against the fruit fly, *Bactrocera zonata*. *In vitro* bioassays of bio-control agent *Hirsutella* against *Bactrocera zonata* was performed. Data were statistically analyzed. The mycelial growth and entomopathogenic activity of fungus against *B. zonata* were observed under optimal laboratory conditions maintained at 23±1°C for 10-12 days. *Hirsutella spp.* achieved significant control of *Bactrocera zonata* (Diptera: Tephritidae). Under lab conditions, the concentrations of *Hirsutella* culture was applied as protective treatment which was more efficient as compared to curative one. *Hirsutella spp.* demonstrated significant potential as a biological control agent for effectively suppressing fruit fly population. *Hirsutella* showed significant results as a preferable substitute for both the environment and human health. Different concentrations of *Hirsutella* was applied against *B. zonata* and significantly different effects showed at mortality percentage. Different concentrations of *Hirsutella* was applied against *B. zonata* and significantly different effects showed at mortality percentage. Significant mortality in adult 93.4% and in pupae 62.9% was observed at high amount of fungal concentration ($1x10^{20}$ conidia/mL). The least mortality was showed at minimum concentration applied ($1x10^{10}$ conidia/mL). Mazximum mortality was observed at ($1 x10^{20}$ conidia/mL) concentration at adult stage. These results indicate *Hirsutella spp.* an effective, environmentally safe biocontrol agent for *B. zonata*, supporting its integration into fruit fly management strategies.

Keywords: Biocontrol, *Bactrocera Zonata*, *Hirsutella spp*, insect bait method, entomopathogenic fungi.

INTRODUCTION

Guava (*Psidium guajava* L.) belongs to the Myrtaceae family, which consists of about 133 genera and 3800 species (Kapoor et al., 2020). Guava is an essential crop grown in subtropical regions. It is a resilient crop that may be grown effectively even on decaying soil. Guavas are thought to have originated in Mexico or Central America. Commercially, guava cultivars are predominantly grown in India, Pakistan, and Brazil globally. Pakistan ranks 4th among guava-cultivating countries, with the fruit grown on 62.3 thousand hectares nationwide (Ayon-Reyna et al., 2017). Guava is known to have a high nutritional profile carrying necessary amino acids and nutrients needed by the human system.

One of the most economically significant pests attacking on fruit crops worldwide is fruit fly (Diptera: Tephritidae) (Mun et al., 2003). Damage attributed to the fruit flies has a wide range attacking fruits and fleshy vegetables. Most of the yield losses related to this pest are caused by fruit that has decayed, become inedible or dropped prematurely as a result of larvae feeding on the flesh. Because of its remarkable adaptability, this species has expanded rapidly over the globe (Liu et al., 2013).*Bactrocera zonata* is the most devastating pest in the genus *Bactrocera* (Wang, 1996; Kitthawee, 2000). *B. zonata* currently occurs in China and a large portion of South and Southeast Asia (Drew & Raghu, 2002; Zingore et al., 2020). In Pakistan, *B. zonata* fruit fly species have been responsible for 30 to 100% of the reported damage. Even when hosts are accessible, it can also be found on a wide variety of other wild

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and cultivated fruits such as Citrus, peach, guava and mango (Fletcher, 1987). It can outcompete other fruit fly pests like *Ceratitis capitata*, *C. rosa* and *B. dorsalis* because of its quicker larval growth, larger eggs, longer lifespan and better reproductive output (Duyck et al., 2006). Through the infestation of the guava fruit fly, severe damage occurs, which reaches about 30-100% through *Bactrocera Zonata*.Entomopathogenic fungi are significant because they are noxious, transmit by contact, survive in the environment for a long time, and are typically mass-produced. They are considered natural mortality biocontrol agents and safe for the environment. They have received worldwide interest in the utilization and manipulation of insects for biological control (Santos et al., 2022).

Due to their ability to infect their host through the cuticle, fungi are among the agents that are essential in the biological management of insect pests. Entomopathogenic fungi have a wide spectrum of insect hosts and a global distribution (Zimmermann, 2008). It is crucial for them to be able to control insect populations in tropical and temperate areas. (Tiganomilani et al., 1995; Mangan, 2014).

Hirsutella is a genus of fungi that reproduces asexually and belongs to of the ophiocordycipitaceae family. It was first described by the French mycologist Narcisse Theophile Patouillard in 1892 and is used to manage insect pests. *Hirsutella thompsonii*, *Hirsutella gigantean*, and *Hirsutella citriformis* are the three most significant species. This genus comprises pathogens of insects, mites and nematodes (Sung et al., 2007). Among the major genera of fungal entomopathogens, this genus has been one of the most challenging to identify. The citrus rust mite is managed with the help of *Hirsutella thompsonii*. Moreover, the Acarida, Lepidoptera and Hemiptera groups of insects are also managed by *Hirsutella thomsonii* (Reddy et al., 2020).

The use of EPF as mycoinsecticides has a variety of advantageous properties, including the removal of toxic residues from crops and a general low level of toxicity to beneficial and other non-target insects. In the case of pesticides, they pose negligible hazards to people and animals and cause no harm to the natural environment (Zimmerman, 2008). Their host-specificity increases their potential for use in IPM since it protects natural enemies, who thus contribute significantly to overall pest control (Brodeur et al., 2012). Entomopathogenic fungi *(Hirsutella*), which are biological pest controller, have various benefits including high efficacy, simple production and environmental safety. There are several strains of various entomopathogenic fungus species. It is essential to collect and characterize fungi (Roberts & Yendol, 1971) and asses the efficacy against the fruit fly.

MATERIALS AND METHODS

***Acquisition of Fruit Fly Population*:** Larvae, pupa, and adults of *Bactrocera zonata* was acquired from the Insect Chemical Ecology Laboratory, Department of Entomology, UAF. The fruit flies were reared on a natural and artificial diet in the laboratory. A susceptible population of pupa of *Bactrocera zonata* was acquired and purified for laboratory bioassays.

***Fungal Acquisition*:** Pure cultures of *Hirsutella spp.* were obtained from the Fungal Molecular Biology Laboratory, Department of Plant Pathology, University of Agriculture, Faisalabad. These isolates had been previously maintained and preserved under standard laboratory conditions. Morphological characterization of the fungal isolates was conducted using macroscopic and microscopic features, including colony color, texture, spore shape, and hyphal structure, following standard.

***Mass culturing*:** Mass culturing of *Hirsutella* culture was done by inoculating in Sabouraud dextrose broth in a culture flask. Inoculated culture flasks were incubated in a shaking incubator for approximately one week. After that a spore suspension with different concentrations was prepared. Neubauer hemocytometer was used for quantification to achieve the desired concentrations. After obtaining pure culture of entomopathogenic different concentrations ($1x10^{10}$, $1x\ 10^{14}$, $1x\ 10^{16}$ and $1x\ 10^{20}$ conidia/ml) were made, along with a control one using only distilled water.

***Bioassays*:** The efficacy of different spore concentrations of *Hirsutella spp.* was evaluated in the laboratory. The behavioral assay was performed on the developmental stages of larvae and Pupae. The bioassay was performed in plastic boxes containing 25g of sterilized soil. One ml of water solution containing 1×10^{10},$1x10^{14}$, $1x10^{16}$ and $1x10^{20}$ viable conidia per mL was poured and mixed into the soil. After this, pupae were released into soil contaminated with EPF. Fifteen pupae were added in plastic box. Regular inspection was done by maintaining soil moisture up to 75%. All treatment boxes were covered and incubated at 23±1°C. Pupal mortality was assessed by counting the number of adults that emerged and subtracting them from the total number of pupae used for each treatment. Each treatment was replicated three times. Pupal mortality data was recorded on the 7^{th} day after emergence. At different time intervals of 24hrs, 48 hrs, 72 hrs, and 168 hrs.

***Data analysis*:** Data was recorded, and experiments were done by Factorial under CRD (completely randomized Design). Then the data was analyzed statistically using the basic statistical method. The mortality rate of the experiment was calculated using a statistical tool (Tuckey test). The potential of different conidial concentrations of *Hirsutella*

spp. on pupae and adults was compared by using ANOVA (analysis of variance).

RESULTS

***In Vitro bioassays of Hirsutella for the control of Bactrocera Zonata*:** Mass culturing of *Hirsutella* culture was done by inoculated in Sabouraud dextrose broth in a culture flask. That inoculated culture flasks were incubated in shaking incubator for approximately 1 week. After regular inspection a spore suspension of *Hirsutella* culture was prepared. By using that suspension required suspension with different concentrations were then prepared. Four concentration s were used for further processing. Those concentrations were mixed in soil in plastic boxes and 15 pupae and adults were added to the plastic boxes.

***Percent Mortality calculation of pupae and larval stage of Bactrocera Zonata*:** For calculating mortality, the total number of adults that emerged was subtracted from total number of pupae

Percent pupal mortality =

$$\frac{\text{Total number of pupa} - \text{number of adults emerged}}{\text{Total number of pupa}} \times 100$$

***Percent mortality fruit fly adult stage by applying Hirsutella spp. with different concentrations and time intervals*:** The results pertaining the analysis of variance of different concentrations of *Hirsutella spp*. and time intervals regarding the percent pupae mortality of fruit fly are presented in the (Table No. 1). All concentrations have significant difference from each other and time intervals also showed significantly different effect on percent mortality. The interaction between concentrations and time intervals were significant effects also.

The maximum pupae mortality (62.92%) were recorded in 1 x 10^{20} conidia/mL concentration significantly different from all others. After 1 x 10^{16} conidia/mL the most mortality (44.67%) which follow the 1 x 10^{14} conidia/mL (34.26%) and 1 x 10^{10} conidia/mL (27.27%). The minimum mortality (1.36%) in the control treatment was significantly different from all other *Hirsutella spp*. concentrations.

During the time intervals the highest mortality of pupae stage (37.248%) was measured after 168 hrs. significantly different from all others time intervals. During the time interval of 72 hours the mortality was recorded (34.48%) followed by 48 hrs. (30.58%). The lowest fruit fly pupae stage mortality (30.58%) was observed after 48 hrs. Which showed significant differences from all other time intervals (Figure 1). The data regarding the interaction between concentrations and time intervals showed in (Figure 2). The highest percent mortality (70.66%) was recorded in 1x10^{20} conidia/mL concentration after 168 hours, significantly different from all other concentrations and time intervals. Non-significant difference were observed between 1 x 10^{20} conidia/mL (48.30%) after 48 hours and 1 x 10^{16} conidia/mL (46.94%) after 72 hours significant different from all other concentrations and time intervals. The lowest mortality at pupae stage was found in control treatment (36%) after 24 hours which was non-significant from also control one (1.9%) after 48 hours which were significantly different from all other concentrations and time intervals which showed in (Figure 2).

Table 1. Analysis of Variance for percent mortality fruit fly pupal stage by applying *Hirsutella sp*. with different concentrations and time interval.

Source	DF	MS	F	P
Replication	2	23.04	4503.20	0.0000*
Concentration (C)	4	5390.42	140.20	0.0000*
Time interval (T)	2	167.82	25.46	0.0000*
T×C	8	30.48		
Error	31	1.20		
Total	47			

Grand Mean 34.101; CV 3.21; * mean highly significant

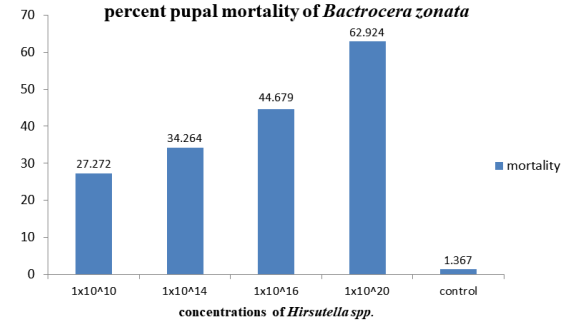

Figure 1. Percent mortality fruit fly pupal stage by application of *Hirsutella* with different concentrations.

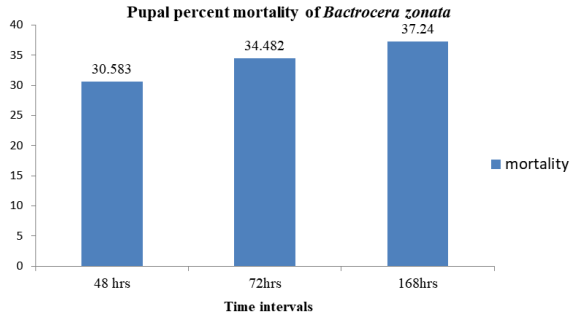

Figure 2. Percent mortality fruit fly pupal stage by application of *Hirsutella sp*. at different time interval.

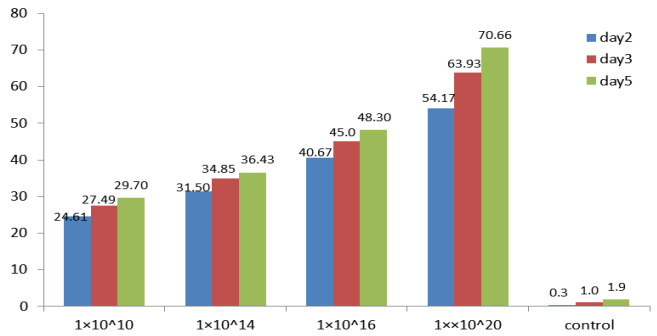

Figure 3. Percent *mortality* fruit fly pupal stage by application of *Hirsutella* with different concentrations and time intervals.

***Percent mortality fruit fly adult stage by applying Hirsutella spp. with different concentrations and time intervals*:** The results of the analysis of variance of different concentrations of *Hirsutella spp*. and time intervals regarding the percent adult mortality of fruit fly are presented in the (Table No. 2). All concentrations have significant difference from each other and time intervals also showed significantly different effect on percent mortality. The interaction between concentrations and time intervals were significant effects also.

The maximum adult mortality (93.40%) were recorded in $1x10^{20}$ conidia/mL concentration have significant differences from all others concentrations. At 1×10^{16} the most mortality (54.17%) which follow the $1x10^{14}$ conidia/mL (38.96%) and $1x10^{10}$ conidia/mL (28.17%). The minimum mortality (0.517%) in control treatment, which was significant different from all others concentrations of *Hirsutella spp.* which showed in (Figure 3).

During the time intervals the highest mortality of adult stage (47%) was measured after 168 hours, significantly different from all other time intervals. During the time interval of 72 hours the mortality was recorded (47%) followed by 28 hrs. (37%)., showing significant differences from all other time intervals shown in (Figure 4).

Table 2. Analysis of Variance for percent mortality fruit fly adult stage by applying *Hirsutella sp*. with different concentrations and time interval.

Source	DF	MS	F	P
Replication	2	76.8	3596.29	0.0000*
Concentration (C)	4	11861.2	162.74	0.0000*
Time interval (T)	2	536.8	65.27	0.0000*
C×T	8	215.3		
Error	31	3.3		
Total	47			

Grand Mean 43.048 ; CV 4.22; * mean significant

Table 3. Different concentrations of *Hirsutella* showed different percent mortality.

No.	Concentrations	Mortality
1	$1x10^{20}$	93.406
2	$1x10^{16}$	54.172
3	$1x10^{14}$	38.969
4	$1x10^{10}$	28.177
5	Control	0.517

The data regarding the interaction between concentrations and time intervals showed in (Figure 5). The highest percent mortality (58%) was recorded in $1x10^{20}$ conidia/mL concentration after 168 hours, significantly different from all other concentrations and time intervals. Non-significant difference were observed between $1x10^{16}$ conidia/mL (49%) after 48 hours and $1x10^{14}$ conidia/mL (40%) after 72 hours significant different from all other concentrations and time intervals. The lowest mortality at adult stage was found at $1x10^{10}$ conidia/mLtreatment (30%) after 24 hours which was non-significant from also control one (2.4%) after 48 hours which were significantly different from all other concentrations and time intervals which showed in (Figure 5).

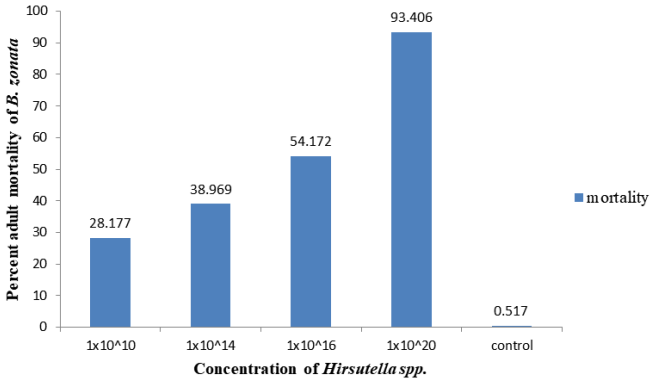

Figure 4. Percent mortality fruit fly to adult stage by applying *Hirsutella spp*. with different concentrations.

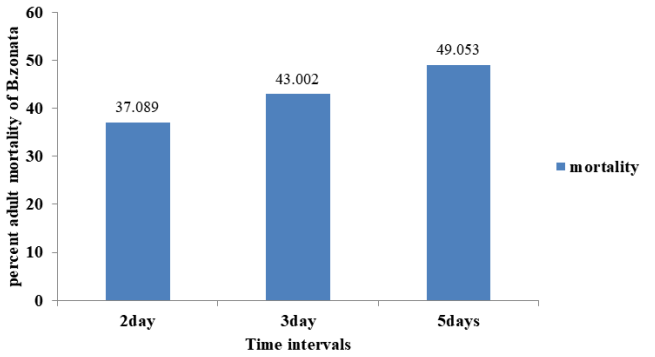

Figure 5. Percent mortality fruit fly to adult stage by the applying *Hirsutella spp*. with different time intervals.

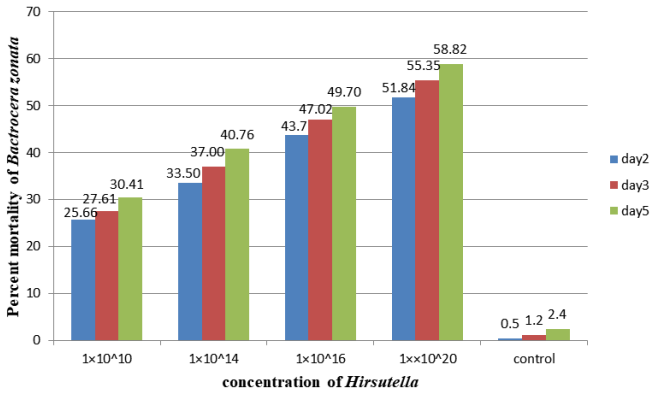

Figure 6. Percent mortality fruit fly adult stage by the applying *Hirsutella* with different concentrations and time intervals.

DISCUSSION

Insect pathogenic microorganisms offer an alternative for use as biocontrol agents (Lacey et al., 2015). The bacteria are not only economical but also safe for the environment, human health and non-target animals. The main pathogen that naturally influence insect pests is microsporidium fungi, so they are employed to control pest populations, especially when chemical insecticide applications are inefficient or unfeasible (Bjørnson and Oi, 2014). Technology is available to use entomopathogens in the Area-Wide Integrated Pest Management (AW-IPM) strategy to manage economically significant fruit flies. (Hendrichs et al., 2007; Boulahia-Kheder, 2021). It is now essential to investigate alternate pest management methods that can reduce the use of these synthetic chemicals (Pino et al., 2013). To control the pest population mostly farmer rely on the use of synthetic insecticides. These insecticides have some serious drawback such as pesticides confrontation, toxic residues, collective cost of application, and environmental contamination and health hazards in human being and native animals due to long time use and repetition. Consequently, it is desirable to explore the alternative methods to control pest population largely by use of biological control. In organic control different natural enemies like fungi and certain plant extracts are involved for the managing of fruit fly. Plant extract can potentially be eco-friendly substitute to synthetic insecticides in IPM of fruit fly population (Usha et al., 2019).

Among natural enemies microorganism have prominent position in different control measures against insect pests. Different entomopathogens is used in insect management strategies include; *Hirsutella sp*. *Pseudomonas fluorescens, Metarhizium anisopliae, Isaria fumosorosea, Beauveria bassiana* and *nucleo polyhedro virus* (Gul et al., 2014).

One of the most prevalent and significant entomogenous fungi, Hirsutella, may be crucial in the natural regulation of pest insects. The original species *H. entomophila Pat.*, which described from a specimen discovered on a beetle in Ecuador, served as the basis for the development of the genus *Hirsutella*. Based on the presence or lack of synnemata, this entomopathogenic genus is divided into two sections: Synnematous and Mononematous. The majority of *Hirsutella* species produce synnemata on certain occasions, but a small number are mononematous (Reddy*et al.*, 2005).

Entomopathogenic fungi have been effectively control the lepidopterous pest and utilized in biological control, classical, conservation and augmentative (Kidanu & Hagos, 2020). Entomopathogenic fungi direct penetration into hosts by which is the mostly barrier between the microbial attack. Fungal have biological control by sucking and defoliator insect pest (Butt et al., 2016).

Lately developed entomopathogenic microbial organisms are also involved in biological control by way of an alternative to synthetic pesticides (Van Lenteren et al., 2018). One advantage of EPF is that they can distress their host by contact, by epicutical integument rather than other entomopathogenic microbes (Sharma & Sharma, 2021). In integrated pest management (IPM) EPF are measured contagious against inclusive spectrum of plant beetle. In contradiction of *B. cucurbitae* fruit fly, *Metarhizium anisopliae*used as EPF are highly contagious and effect on adult and pupae mortality phases (Onsongo et al., 2022). EPF are considering safe with low impact on environment which make them attractive for IPM (Zimmermann et al., 2008).

Our experiment results of different concentrations of *Hirsutella* and time intervals showed that maximum pupal mortality (92.47%) were recorded in 1 x 10^{20} concentration after 5 days and lowest mortality at pupal stage was found in control treatment (0.33%) after 2 days.

The results pertaining the different concentrations of x $1x10^{20}$ Hirsutella and time intervals regarding the percent pupal mortality of fruit fly showed that highest mortality (90.14%) were recorded in 1 x 10^{20} concentration after 168 hours and lowest mortality at pupal stage was found in control treatment (0.67%) after 24 hours.

***Conclusion*:** The present study highlights the significant entomopathogenic potential of *Hirsutella spp*. against *Bactrocera zonata*, one of the most destructive pests of guava in subtropical regions. Laboratory-based bioassays using various concentrations of *Hirsutella spp.* demonstrated a clear dose-dependent response in both pupal and adult stages of the guava fruit fly. The highest mortality was observed at $1×10^{20}$ conidia/mL, indicating strong pathogenic activity of the fungus at elevated concentrations. Both time intervals and fungal concentrations were found to have statistically significant effects on mortality, with longer exposure durations resulting in increased insect mortality. Notably,

protective treatments were more effective than curative treatment, suggesting that early application of *Hirsutella spp.* can suppress the pest population. Unlike chemical pesticides, *Hirsutella spp.* is host-specific, eco-friendly and compatible with integrated pest management (IPM) strategies. Further field trials and formulation development are recommended to optimize its practical use and assess its performance under natural environmental conditions. Overall, *Hirsutella spp.* presents a promising biological tool to enhance sustainable agriculture and mitigate the economic impact of fruit fly infestations.

***CRediT author statement*:** Saba Sajjad performed the experiments, collected and analyzed data, and drafted the manuscript. Muhammad Tauseef Tariq Kisana conceptualized and contributed to data interpretation and reviewed the manuscript. Abdul Manan assisted in manuscript editing. Nasir Ahmad Khan provided technical support, helped and reviewed the final draft.

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***Conflict of interest*:** We Clarify that the submitted manuscript is our original Research work. We declare No conflict of interest.

***Ethical statement*:** All experiments included in this research paper have been planned keeping in the mind of ethics of research. No studies involving animal and human participants were included in the experiments.

***Availability of data*:** We declare that the work we have submitted is original, has never been published and is not being considered for publication anywhere.

***Consent for publication*:** The Final draft of this reserach paper has been carefully reviewed and approved by all authors to published this paper in Phytopathogenomics and Disease Control.

***SDGs addressed*:** Zero Hunger; Good Health and Well-being; Responsible Consumption and Production

***Policy referred*:** National Food Security Policy of Pakistan (2018); Pakistan's National Agricultural Policy; International Plant Protection Convention (IPPC).

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