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POTENTIAL OF *P. AERUGINOSA CD6C* AS ENVIRONMENT FRIENDLY BIO-FUNGICIDE

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ABSTRACT

Practicing fungicides on crops is an effective method of controlling fungal diseases, but overuse or misuse can deteriorate the ecosystem. Whereas past scientific studies revealed that biological control produces reliable and rewarding results. The Pseudomonas spp. efficiency proved to be important plant growth promoting and effective biocontrol agent. The research study was conducted at Microbiology Laboratory, Auriga Group of Companies, Lahore duing 2019, aimed for screening and isolating a biocontrol agent that inhibit *Macrophomina phaseolina* (*M. phaseolina*) and *Fusarium incarnatum* (*F. incarnatum*) growth by reducing disease incidence and increase maize agronomic performance. A plate culture assay was used to screen a biocontrol agent for fungal pathogens such as *M. phaseolina* and *F. incarnatum* responsible for charcoal foot rot and stalk rot. Among 37 isolates, CD6C isolated from press mud, demonstrated a substantial antifungal potential. The 16S rRNA sequence analysis showed 100% similarity between CD6C and *Pseudomonas aeruginosa*. The growth inhibition % during plate culture assay for *M. phaseolina* and *F. incarnatum* were was 46.5±1.9 and 49.8±1.1, respectively. The supernatant also had the best antifungal activity against charcoal rots caused by *M. phaseolina* in comparison to the control during the pot experiment. *P. aeruginosa* CD6C treatment resulted a decline in maize foot rot caused by *M. phaseolina*. Research findings showed that *P. aeruginosa* CD6C could impede *M. phaseolina* growth and exacerbate maize plant growth parameters. The data supports discovering more antifungal compounds from *P. aeruginosa* CD6C, which can lead to competent and successful pathogen biocontrol treatment. Moreover, study suggested that *P. aeruginosa* CD6C was found to be auspicious for maize plant and should be promoted for plant growth enhancement and suppression of fungal diseases by scaling down chemical pesticides for pollution safety.

KEYWORDS: Biocontrol; fungicide; *Fusarium incarnatum*; *Macrophomina phaseolina*; *Pseudomonas aeruginosa;* Pakistan

INTRODUCTION

Modern agro-economics is challenged by multiple issues, including insufficient land, saline soil, poor water quality, higher residual abiotic contaminants and lower crop productivity needs to improve on a sustainable basis (Srivastava *et al.,* 2016). Among cereal crops, maize is the most crucial crop after wheat and rice with a cultivation area of about 1,418 thousand hectare in Pakistan (GoP, 2021). It is cultivated all over the world and susceptible to various diseases. Crop rotation, resistant crop varieties and chemical fertilizers are commonly used, for successful crop production but biological control distracts attention due to its cost-effectiveness and environmental friendly approach (Li *et al.,* 2018; Liu *et al.,* 2018; Sun *et al.,* 2015). Some bacterial strains are reported to enhance plant growth and diseases suppression (Rahman *et al.,* 2016; Shafique *et al.,* 2016). Among them, *Bacillus and Pseudomonas* are common biocontrol agent which might enter through aerial parts and plant roots and generate resistance to plant pathogens (Korejo *et al.,* 2019; Ek-Ramos *et al.,* 2019). Some more examples of successfully applied biocontrol agents include *Streptomyces spp*. (Abbassi *et al.,* 2019) *Pseudomonas spp.* (Islam *et al.,* 2018) and Bacillus spp. (Jangir *et al.,* 2018).

Maize is exposed to nearly 112 diseases all over the world including seedling growth, root rot, charcoal rot and stalk rot etc. (Kozdroj *et al.,* 2004; Li *et al.,* 2016; Pal *et al.,* 2001). Among them, *M. phaseolina and F. incarnatum* are playing a crucial role by scaling down the crop yield. Maize charcoal rot is one of the most

economically important diseases of maize worldwide. The mechanism of infection involves seed dark bruises that cause seedling cessation due to the discontinuation of xylem vessels. Red to brown lesions appear on stems and roots followed by defoliation, wilting and perishing. Signs of charcoal rot commonly look at the end of the season, followed by flowering (Jordan *et al.,* 2019). *M. phaseolina* is considered as the causative agent for charcoal rot of sunflower (Khan, 2007) soyabean, cotton (Jana *et al.,* 2005), peanut (Gupta *et al.,* 2002), cluster bean and sorghum etc. (Purkayastha *et al.,* 2005). Fusarium species involved in kernel and stalk rot as well as mycotoxins contamination that leads to health risk for humans and animals (Tsehaye *et al.,* 2017). Gai *et al.* (2016) initially reported stalk rot caused by *F. incarnatum* on maize in China.

This research aimed at evaluating the ability of press mud isolated biocontrol agent to inhibit fungal disease and assessing the performance of agronomic parameters of maize in a pot experiment.

MATERIALS AND METHODS

Bacterial biocontrol strain isolation &collection of pathogenic fungal strain

The press mud was used to isolate bacterial biocontrol strain collected from the Macca Sugar Mill, Manga Road (31°15'34.12 N, 74°9'19.72" E) Lahore, Pakistan**.** Serial dilution technique was used to isolate distinct colonies on luria bertani (LB) agar medium and incubated at 28±2 °C for 48 hours. Aseptically picked single colonies were streaked on LB and subcultured to get pure culture. The bacterial isolation, screening, virulent *M. phaseolina* & *F. incarnatum*strain collection and all experimentation was performed in the Microbiology Laboratory of Auriga Group of Companies, Lahore during 2019. Fungal strains were sub-cultured and maintained on potato dextrose agar (PDA).

Identification

Gram staining was used for preliminarily characterization. Strain was identified by 16S rDNA. The sequencing primer name and primer sequence were 785F 5' (GGA TTA GAT ACC CTG GTA) 3' and 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'. PCR Primer sequence were 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3 and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'.

In- vitro antifungal assay

The antagonism assay was conducted against *M. phaseolina* & *F. incarnatum* using the plate culture technique. Briefly, the biocontrol strain was inoculated at the center of PDA petri-plate and fungal pathogen was aseptically streaked at equidistance on each side of biocontrol strain. Biocontrol plates were incubated at 28±2 °C for 5 days. After incubation, both fungal and bacterial growth was observed. The strain was selected and stored, displaying a higher antifungal effect. Growth inhibition was used to express antifungal activity by using the following formula (Cui *et al.,* 2019).

$$GI\% = \frac{PCD(C) - PCD(T)}{PCD(C)} \times 100$$

Where,

GI% = Growth inhibition

PCD(C) = Pathogen colony diameter (control)

PCD(T) = Pathogen colony diameter (treatments)

Preparation of *P. aeruginosa CD6C* and *M. phaseolina* inoculum

The methodology adopted for the pot experiment following the method of (Pal *et al.,* 2001; Xu *et al.,* 2020) with minor modifications. For inoculum preparation, *P. aeruginosa* CD6C was inoculated in nutrient broth on shaker at 180 rm^{-1} at 28+°C for 48 hours. The serial dilution technique was used to determine CFU/ml and the concentration was adjusted to 1×10^9 CFU/ml. After incubation, 50 ml suspension of *P. aeruginosa* CD6C was thoroughly mixed with 450 g of soil. The moisture content of the mixture was retained at 40–45% at room temperature for 72 hours to endorse *P. aeruginosa* CD6C growth (Xu *et al.,* 2015). PDA petri plates were used to get mature growth of *M. phaseolina*. To remove fungal pathogen conidia, petri-plates were dipped in sterile distilled water in a beaker, and the conidia were separated with the help of brush. The cheesecloth was used to filter the suspension to remove mycelial fragments (Xu *et al.,* 2015).

Biological control pot experiment and assessment of disease incidence

M. phaseolina was selected based on its virulence for in vivo analysis under natural light and temperature conditions. Maize (*Zea mays* L.) seeds (variety Pak afgoi) were raised in the small pots before two cotyledons grow up. Twelve maize seedlings/pot were transferred at a depth of 3 cm to other earthen pots (25 cm × 25 cm) comprising 450 g of soil with 1.1 ± 0.1% organic matter, 0.06 ± 0.03% N, 6.5 ± 0.1 mg/kg available phosphorous, and 100 + 1.2 mg/kg available potassium. The electrolytic conductivity (EC) was 1.5 ± 0.05 d/Sm and the pH was 7.1 ± 0.02. The study includes three treatments: Treatment I: Control (Auriga group farmland soil, seedlings were not suspended in

M. phaseolina suspension). Treatment II: Pathogen control (seedling roots were dipped into a suspension of *M. phaseolina* for one hour and then transplanted into other pots containing 450 g of soil) and; Treatment III: Pathogen with isolate (seedlings were immersed into a suspension of *M. phaseolina* for two hours and then transferred into other earthen pots containing 450 g of 7 days before *P. aeruginosa* CD6C inoculated soil. Pots containing maize plants were randomly arranged with three replicates. Maize plants were allowed to grow up to 2 months in the open air with an average day and night temperature of 33 ± 2 °C and 25 ± 2 °C, respectively. The soil water content was controlled by weight.

The disease incidence was indicated as a number of diseased plants % divided by the total number of plants % (Xu *et al.,* 2020). When the maize plant began to develop the disease, the samples of the maize plant were collected and analyzed for disease incidence. Agronomic parameters like stem height, fresh and dry maize plant weight were used as stress indicators.

Statistical analysis

The collected data were statistically analyzed by "Statistics version 10" using the least significant difference (LSD). Analysis of variance was applied at P < 0.05 probability level, Used for the measurement of significant differences between treatments.

RESULTS AND DISCUSSION

Forty-four plant growth promoting rhizobacterial strains (PGPR) were screened from press mud. *P. aeruginosa* was screened and selected for its PGPR characteristics. Nitrogen fixation, phosphorous, zinc and potassium mobilization were already examined in another component of the study. The strain also haD the best antifungal activity against *M. phaseolina* and *F.incarnatum* was selected for biocontrol Table 1, Fig 1. Commonly the plate culture assay decides the preliminary characterization of the biocontrol bacterial strain against fungal pathogens (Xu *et al.,* 2020).

Globally, crop yields are widely affected by phyto-pathogens. Fungal pathogens are widely studied as crops are more susceptible to fungal pathogens. Biocontrol bacteria gained attention due to their environmentally friendly and economically productive nature. The most practical approach to fungal control involves the use of chemical fungicides to reduce crop infection, leading to water pollution, soil degradation, and resistant pathogens (Ndoumbe and Sache, 2003). Different letters above the bars indicate that the differences are significant ($P < 0.05$) using LSD analysis of variance.

Identification

Strains was identified by 16S rRNA genetic results obtained from Macrogen, Korea. A phylogenetic tree was constructed showing percent similarity to closest known bacterial strain (Fig 2).

Growth inhibition % plate assay

The growth inhibition of *P. aeruginosa* CD6C towards fungal pathogens was assayed by plate culture. Results revealed that *P. aeruginosa* CD6C suppressed fungal pathogens and growth inhibition % for *M. phaseolina*and *F. incarnatum* were 46.5 ± 1.9, 49.8 ± 1.1, respectively Table 1. Lim *et al.* (2017); Xie *et al.* (2018) reported that antifungal activity caused by diffusible and volatile

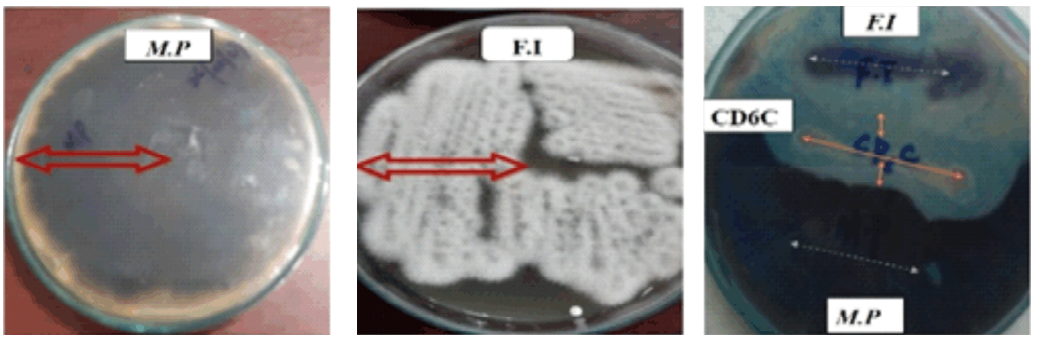

Fig. 1. (a) control plate for *macrophomina phaseolina*; (b) control plate for Fusarium incarnatum; © Biocontrol qualitative assay; *Pseudomonas aeruginos*a gainst M.P. and F.I.

Table 1. Growth Inhibition% by *P. aeruginosa* CD6C against *M. phaseolina*& *F. incarnatum*

Bacterial isolate	Fungal pathogen	Growth inhibition%
P. aeruginosa CD6C	*M. phaseolina*	46.5±1.9[b]
	F. incarnatum	49.8±1.1[a]

Fig. 2. Phylogenetic tree constructed based on *16S rDNA* and chromatogram of indigenously isolated CD6C from pressmud

Table 2. Growth parameters of maize plants noted after 40-days of seedling plantation

Treatment	Stem height (cm)	Leaf length (cm)	Fresh weight (g)	Dry weight (g)
Control	40 ± 0.1[b]	22.9 ± 1.1[b]	3.05 ± 0.04[b]	0.52 ± 0.01[b]
Pathogen control	33.2 ± 1.5[c]	17.8 ± 0.9[c]	2.1 ± 0.05[c]	0.40 ± 0.05[c]
Pathogen with isolate	49 ± 1.2[a]	28.2 ± 0.5[a]	4.1 ± 0.09[a]	0.9 ± 0.03[a]

organic compounds. Current research confirms that *P. aeruginosa* CD6C had better antifungal action against *M. phaseolina* and *F. incarnatum.* Chenniappan *et al.* (2019); Islam *et al.* (2014) also reported that *P. aeruginosa* is a well-known PGPR strain with an exciting performance in plant growth competence and biocontrol against various phytopathogens. Bacterial isolate with antifungal activity was selected and characterized by 16S rRNA gene sequencing. The homology analysis of *P. aeruginosa* CD6C showed 100% similarity with *P. aeruginosa*.

Incidence of maize *charcoal rot*

In vivo analysis was performed against the charcoal rot of *M. phaseolina*. The rate of disease incidence was 53.2 ± 2.1% in the pathogen control treatment and it significantly lowers down (14.1 ± 1.5%) in the treatment (pathogen with isolate). Incidence of maize charcoal rot affected by *P. aeruginosa* CD6C was not detected in control plants Fig 3. Treatment receiving bacterial isolate enhances growth of maize by 22.5% stem height, 23.1% leaf length, 27.7% fresh weight, and 26.8% dry weight as compared to control. The treatment (pathogen control) reduced the 17% stem height, 22.2% leaf length, 31.1 % fresh weight and, 23% dry weight as compared to control. In vivo analysis showed that *P. aeruginosa* CD6C is capable of improving maize growth by building up stem length and above ground fresh and dry weight of the plant Table 2. The *P. aeruginosa* CD6C treated plants resulted in higher agronomic characters as compared to other treatments. These promoting effects on maize plant growth due to the ability of *P. aeruginosa* CD6C to increase soluble mineral nutrients (Abaid and Yusuf 2019; Kumari *et al.,* 2018). The findings are consistent with (Cui *et al.,* 2019) that antagonistic biocontrol agent affects crop pathogens suppression as well as increased maize growth parameters and yield. Different letters above the bars indicate that the differences are significant ($P < 0.05$) using LSD analysis of variance.

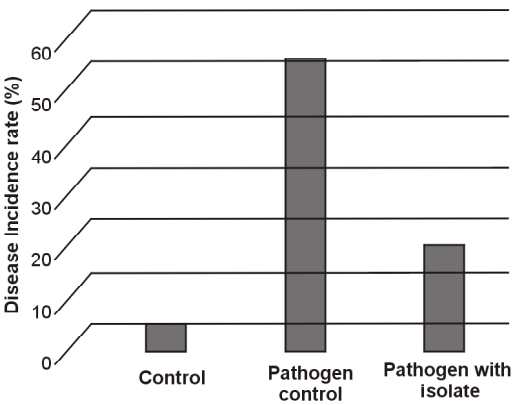

Fig. 3. Charcoal rot disease incidence by *M. phaseolina*

CONCLUSION

Phylogenetic 16S rRNA sequence analysis, indigenously isolated CD6C was identified as *P. aeruginosa* that exhibited remarkable antifungal activity against *M. phaseolina* and *F. incarnatum*. Besides *P. aeruginosa* CD6C not only suppressed disease incidenced but also enhances agronomic

growth parameters including stem height, leaf length, fresh and dry weight above ground in maize plants. From this research, it can be inferred that biocontrol agents can be integrated as a fungicide to combat fungal pathogens in the crop production management strategy to save the environment from using chemicals as fungicides.

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3.	Irfan ul Haq	Helped in wite up the manuscript	
4.	Muhammad Afzaal	Co-supervisor	
5	Imtiaz Ahmad Warraich	Proof read the manuscript