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Effect of quinoa biomass and biocontrol fungi on expression of *IPER* gene in mung bean in *Macrophomina phaseolina* contaminated soil

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Abstract

Background: Mung bean is a pulse crop of economic significance, grown in Pakistan for its edible seeds. Its production is severely affected by *Macrophomina phaseolina*, a necrotrophic pathogen. This study was carried out to investigate the effect of different biocontrol fungi on expression of *IPER* gene in mung bean plants inoculated with *M. phaseolina.*

Methods: Pot trials were carried out by adding different concentration of dry biomass of quinoa (DBQ) and six antagonistic fungi *viz. Aspergillus flavipes*, *Aspergillus versicolor*, *Penicillium digitatum*, *Penicillium italicum*, *Trichoderma pseudokoningii* and *Trichoderma viride* in *M. phaseolina* infected soils. After four weeks of germination of mung bean seeds, RNA was extracted from roots and leaves by using TRIzol method and cDNA was prepared by using SuperScript™ IV First-Strand Synthesis Kit. *IPER* gene expression was studied on qRT-PCR and *ACT* was used as a housekeeping gene.

Results: The expression of *IPER* gene was higher in positive control (only inoculated with *M. phaseolina*) than in negative control (no amendment). Moreover, 1 and 2% doses of DBQ showed slightly higher Ct values than in 3% dose where it was dropped down indicating the allelopathic stress of DBQ treated soils. The treatments either with sole application of antagonistic fungi or together with DBQ showed much higher Ct values indicating no stress at all.

Conclusion: Plants only inoculated with *M. phaseolina* showed the highest disease incidence as compared to the negative control. The soils amended with DBQ and antagonistic fungi significantly alleviated the effect of *M. phaseolina.* The treatments with increased pathogen stress showed reduced Ct values and vice versa.

Introduction

Mung bean is an economically significant pulse crop in Asian agricultural system including Pakistan [1]. It is a warm season leguminous crop that is cultivated for its edible seeds and sprouts in the form of vegetable salad. It is not only a source of protein but also contains iron and folate in higher amounts than in most of other legumes [2]. Although a considerable improvement in its yield has been made yet its production is low in Asian farming systems due to several fungal diseases [3]. Among various pathogenic fungi, *M. phaseolina* has an important position as a pathogen as it inflicts damage to the mung bean plant at different growth stages [4]. It is globally known as a devastating pathogen that infects more than 500 plant species. It is mainly a soil- and seed-borne pathogen whose growth is favored under low moisture condition coupled with high temperature (30-35 °C) [5]. It generally causes charcoal rot disease in various plant species including mung bean [6]. At the start, the fungal hyphae invade cortical tissues of mung beans followed by the development of spindle shaped lesions with minute microsclerotia and pycnidia [7]. The production of sclerotia in infected areas exhibits charcoal rot disease symptoms. Under unfavorable conditions, microsclerotia are responsible for survival of the fungus for prolonged time period in soil and plant debris making its management a challenging task [8].

To date, different disease management approaches such as physical, cultural, regulatory and use of chemicals have been implemented to eradicate *M. phaseolina* populations in the soil but each has some limitations [9]. Moreover, the conventional use of fungicides has drastic effects as it disrupts the balance of beneficial microbes in the soil and these are economically not feasible to low-income farmers [10]. Apart from health hazards, the regular use of fungicides may give rise to the appearance of resistant pathogenic strains [11]. To reduce the dependence on chemicals, application of biological agents such as *Aspergillus* spp. [12], *Penicillium* spp. [13] and *Trichoderma* spp. [14] offers good alternatives to the growers for control of plant diseases. They have some interesting properties for instance, they are cost effective, do not pollute the environment and are target specific [15]. The mechanism through which antagonists retard the pathogen growth is not always clear. However, they might show a direct parasitism, release of toxins by means of antibiosis, and competition for available resources [16,17].

In recent past, the use of natural plant products has also gained importance. These products have broad host range, safe, easily biodegradable, cheap, non-phytotoxic, environment friendly and exhibit a structural diversity. Several plant families such as Asteraceae [18], Meliaceae [19], Chenopodiaceae [20], Magnoliaceae, Acanthaceae [21] and Amaranthaceae [22] are known for their antifungal properties. Recent studies have demonstrated that purified compounds isolated from plant extracts [23,24], and plant dry biomass can efficiently be used for the control of plant diseases [25,26]. *C. quinoa* is native to Andean regions since 3000 BC. Recently, it has been introduced in Africa, North America, Europe, Asia and South East Asia due to its tolerance to frost, drought and salinity [27]. Moreover, it contains saponins, triterpenoids, glycosides, phenolic acids, kaempferol, quercetin, tannins and flavonoids that contribute to diverse biological properties [28]. Hence, considering the adverse impact of fungicides the use of beneficial antagonistic fungi and natural plant products may play a key role in eco-friendly sustainable agriculture system.

In response to pathogenic fungal attacks, plants defend themselves with an arsenal of defensive mechanisms. These involve the formation of structural barriers by the release of antimicrobial proteins, which prevent the pathogen colonization into the host plants [29]. Among the proteins induced during plant defense, peroxidase precursor (*IPER*) belongs to class Ⅲ is well known [30]. It plays a critical role during the pathogen colonization either by development of stiff plant structures or by creating a more oxygenated environment for the pathogens [31]. It is generally involved in many physiological processes such as wound healing, degradation pathways, cell wall metabolism, removal of H_2O_2, auxin catabolism, host defense mechanism, oxidation of toxic reductants, and cell growth [32]. In addition, *IPER* can generate highly reactive oxygen species (ROS) that can possess an intrinsic activity during diverse hypersensitive responses. It also acts as a part of signal transduction pathway during biotic and abiotic stress responses, cell division and programmed cell death [33]. In pathogenic attacks, peroxidase gene expression is triggered to repair the damaged tissues and to provide a protective mechanism [34]. Therefore, the present study was undertaken to examine the effect of biocontrol agents namely *Trichoderma* spp., *Aspergillus* spp. and *Penicillium* spp. and dry biomass of quinoa plants on expression of *IPER* gene in mung bean plants in *M. phaseolina* contaminated soil.

Methods

Pot trials

A pot study was carried out during March 2017. For this, sandy loam soil was fumigated and filled in 30 cm diameter earthen pots followed by the application of pearl seed-based *M. phaseolina* inoculum (10 g kg^{-1} of soil) prepared on pear millet seeds, except in pots of negative control where only same amount of boiled pearl millet seeds were added. After one week, inoculum

of the antagonistic fungi (10 g kg^{-1} of soil) *viz. A. flavipes*, *A. versicolor*, *P. digitatum*, *P. italicum*, *T. pseudokoningii* and *T. viride* were added alone and in combination with *C. quinoa* dry plant biomasses at different concentrations of 1%, 2% and 3% (w/w). The experimental pots were left for one week and watered when required for the establishment of antagonists as well as the release of allelochemicals by *C. quinoa* added biomass. Ten seeds of mung bean were sown in all the pots, having three replicates of each. After four weeks of germination, sampling was done from each pot. Harvested root and leaf samples were immediately crushed in liquid nitrogen and stored at −80 °C for further analysis.

Isolation of total RNA and synthesis of cDNA

Total RNA from mung bean roots and leaf samples was extracted by using TRIzol method. For this, 2 mL of TRIzol was added into each falcon (15 mL) containing homogenized plant samples and left for 20 min. After that, 3 mL of chloroform: isoamyl alcohol (24:1) were added and the mixture was centrifuged at 13000 rpm for 20 min. Supernatant was taken in a new falcon, added the same mixture and centrifuged for another 20 min. Again, supernatant was separated followed by the addition of isopropanol and 3 M sodium acetate, and kept overnight at −20 °C. After that, RNA pellet was obtained by centrifuge at 13000 rpm for 10 min, washed with 70% ethanol and mixed in double distilled water. The quality and quantity of RNA were measured through Nanodrop spectrophotometer and processed for cDNA by using SuperScript™ IV First-Strand Synthesis Kit following the manufacturer protocol.

Gene confirmation with specific primers

The ORFs of mung bean were amplified by using a set of primers (Table 1) already used for the amplification of ACT (a house keeping gene) and *IPER* (peroxidase precursor gene). The obtained PCR products were then run on 1% agarose gel for genes confirmation (Fig. 1).

Quantitative real-time PCR assay

The qRT-PCR experiment was done on the StepOnePlus™ RealTime PCR System (Thermo Fisher Scientific) in a 48 well microtiter plates using SYBR® Green PCR Master Mix. A total of 25 µL reaction mixture was prepared by adding 1 µL cDNA template, 1 µL of each forward and reverse primers (10 µM) and 12.5 µL of master mix followed by the addition of 9.5 µL nuclease free water. Three independent biological and technical replicates of each treatment were used to carry out the gene expression (Fig. 2 and 3).

Statistical analysis

All the data were analyzed by ANOVA followed by application of LSD test at P = 0.05.

Gene	Functional Annotation	Sequences	Size Bp	Annealing Temp. (°C)	Accession Number
Act II	Actin-II	TGCATACGTTGGTGATGAGG AGCCTTGGGGTTAAGAGGAG	190	55	XM017553520
IPER	Peroxidase Precursor gene	GGCAAGCATTATATGGTTGAAA GATGGCAACATCCATCACTTTA	196	55	XM007138446

Table 1: Detail of primers used for gene expression studies.

Results

Disease incidence (DI)

The highest incidence of disease (39%) was recorded in the positive control while no disease was noted in the negative control. In other treatments, where the soil was treated with DBQ and the six species of biocontrol fungi, a significantly ($P \leqslant 0.05$) decrease in DI was recorded. In these treatments, DI ranged from 4 to 8%.

IPER gene expression in response to *M. phaseolina*

The Ct values were 31.54 and 31.84 in leaves and roots of negative control, respectively. *M. phaseolina* application in positive control reduced Ct values to 15.65 and 13.71 in leaves and roots, respectively, showing the significant increase in *IPER* gene expression in the positive control as compared to negative control treatment (Fig. 4 A & B).

IPER gene expression in response to quinoa biomass

In leaf, Ct values in 1% and 2% DBQ were a little higher than those in the negative control. Conversely, the Ct value was slump to 21.29 in 3% DBQ showing allelopathic effect of the added plant biomass that resulted in 48% higher gene expression than in the negative control. In root, the effect of DBQ on Ct values was generally similar to that recorded in leaf (Fig. 4 A & B).

IPER gene expression in response to *Aspergillus* spp.

In leaves of mung bean, generally the Ct values in *A. flavipes* (AF) or *A. versicolor* (AV) inoculated treatments, separately or combined with 1% or 2% DBQ, were a little higher or near to that in the negative control (31.54). By contrast, AV + 3% DBQ and AF + 3% DBQ treatments exhibited Ct values of 24.24 and 24.38, showing light stress on the mung bean plants due to allelopathic effects of added plant material causing a slight expression of *IPER* gene in these treatments over the negative control (Fig. 4 A). A similar trend in Ct values in roots was recorded due to application of *Aspergillus* species, and DBQ (4 B).

IPER gene expression in response to *Penicillium* spp.

In treatments where the two *Penicillium* species were applied singly or in combination with 1% DBQ, the Ct values were much higher than in negative control and were in the range of 35.71–39.19 in leaf and 35.31–39.41 in root (Fig. 5 A and B).

IPER gene expression in response to *Trichoderma* spp.

Treatments with either of the two *Trichoderma* spp. alone or together with 1% DBQ showed significantly higher Ct values than the negative control showing no gene expression. The *IPER* gene was expressed in those treatments where 3% DBQ was added alone or together with *T. pseudokoningii* (TP) and *T. viride* (TV) with Ct values in the range of 20.97–23.45 in leaf and 21.62–23.28 in root (Fig. 6 A and B).

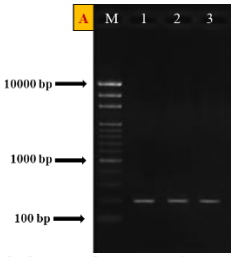

Figure 1: Agarose gel electrophoresis of mung bean amplified products. **1:** ACT-II (~190 bp); **2 & 3:** IPER (~196 bp) isolated from plant leaf and root; **M:** 1 Kb marker.

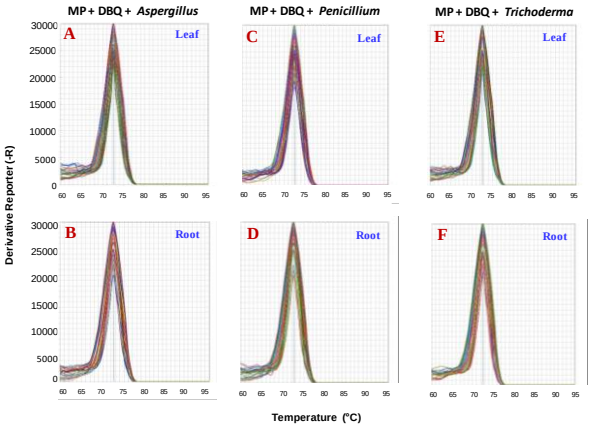

Figure 2: Melting curves of *IPER* gene in leaves and roots of mung bean grown under different treatments of *Macrophomina phaseolina* (MP), dry biomass of quinoa (DBQ), and biological control fungi.

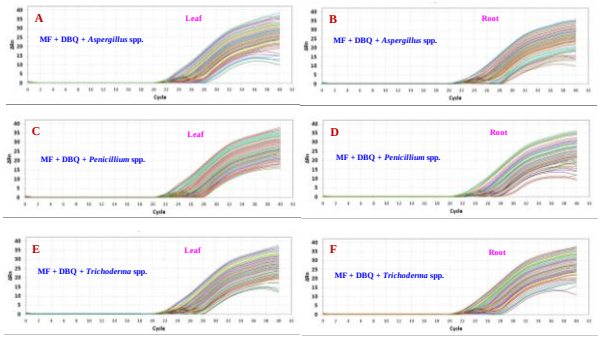

Figure 3: Amplification plots of *IPER* gene in leaves and roots of mung bean grown under different treatments of *Macrophomina phaseolina* (MP), dry biomass of quinoa (DBQ), and biological control fungi.

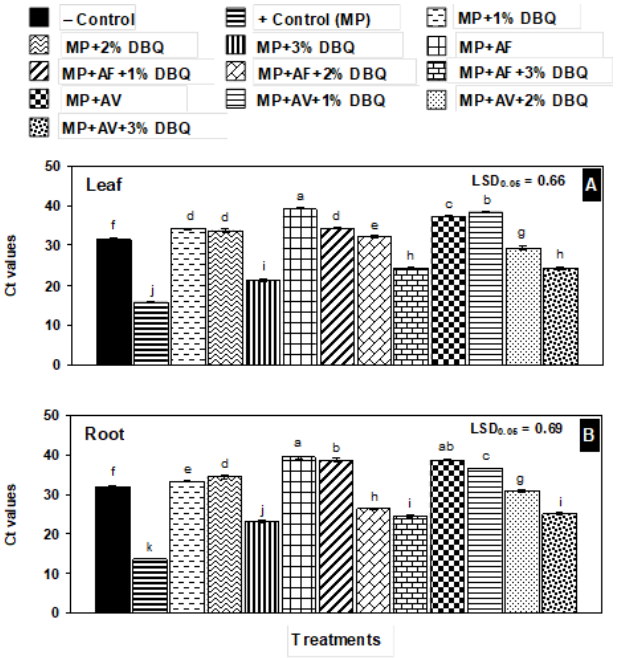

Figure 4: Effect of *Macrophomina phaseolina* (MP), different doses of dry biomass of quinoa (DBQ) and inoculation of two *Aspergillus* spp. *viz. A. flavipes* (AF) and *A. versicolor* (AV) on expression of *IPER* gene in leaves and roots of spring-sown mung bean. Vertical bars show standard errors of means of three replicates. Different letters indicate significant differences ($P \leq 0.05$) among the treatments as determined by LSD Test.

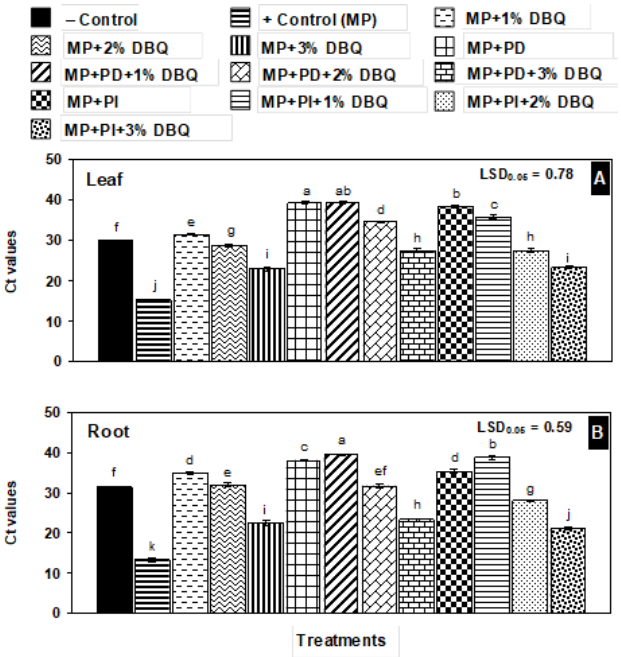

Figure 5: Effect of *Macrophomina phaseolina* (MP), different doses of dry biomass of quinoa (DBQ) and inoculation of two *Penicillium* spp. *viz. P. digitatum* (PD) and *P. italicum* (PI) on expression of *IPER* gene in leaves and roots of spring-sown mung bean. Vertical bars show standard errors of means of three replicates. Different letters indicate significant differences ($P \leq 0.05$) among the treatments as determined by LSD Test.

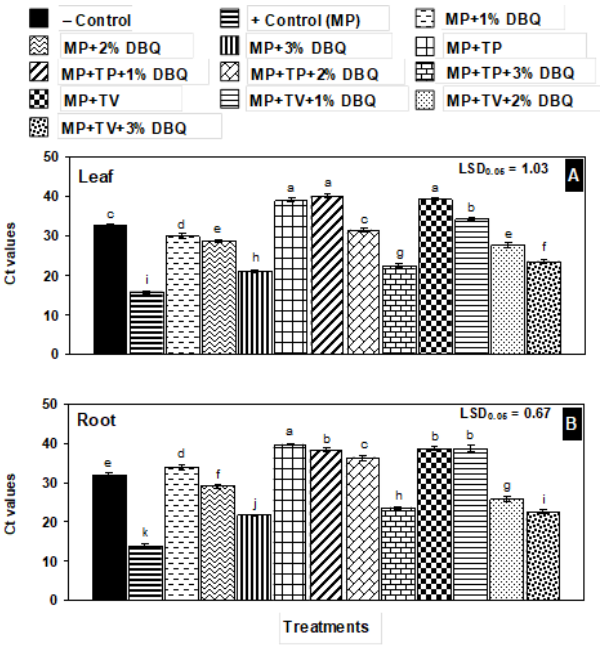

Figure 6: Effect of *Macrophomina phaseolina* (MP), dry biomass of quinoa (DBQ) and inoculation of two *Trichoderma* spp. *viz. T. pseudokoningii* (TP) and *T. viride* (TV) on expression of *IPER* gene in leaves and roots of spring-sown mung bean. Vertical bars show standard errors of means of three replicates. Different letters indicate significant differences (P≤0.05) among the treatments as determined by LSD Test.

Discussion

qRT-PCR analysis was used to reveal the different patterns of *IPER* gene expression and its role in resistance mechanism after *M. phaseolina* infection. The soil amendments with antagonistic fungi and quinoa dry plant biomass generally increased the resistance in mung bean plants against the charcoal rot pathogen in comparison to positive control. Moreover, different concentrations of quinoa resulted in the distinctive regulation of peroxidase precursor gene in mung bean plants. The present findings showed that the expression of *IPER* gene was interestingly modulated according to the treatments and increased strongly in positive control root and leaf samples. In 3% amendment of quinoa, it was found to be overexpressed, which was might be due to the release of excessive allelochemicals from the decomposing material. The increased expression of *IPER* gene after the infection has also been confirmed by previous studies. Bela et al. [35] reported that the attack of fungal pathogens triggered the expression of peroxidase gene and it was proved in *Arabidopsis thaliana*. Peroxidase precursors perform different biochemical activities and functions under pathogenic stresses by modulating the reactive oxygen species [36]. Peroxidase precursors play a critical role in apple plants defense mechanism infected with *Valsa mali* [37]. It is a defense related enzyme and an exponentially higher expression was also observed in tomato plants infected with *Meloidogyne javanica* in comparison to *Trichoderma harzianum* treated plants [38]. Singh et al. [39] also revealed a positive correlation between *IPER* activity and resistance to *Brassica napus* to *Verticillium longisporum* attack. Previously, it has been reported that *IPER* is induced exponentially in response to fungal and bacterial infections [30]. Sharma et al. [40] found that the expression pattern of *IPER* is correlated with phenols oxidation that is dependent on H_2O_2 availability and is increased exceptionally under tissue damage. To regulate the gene expression, *IPER* is acquired to perform activities in order to facilitate the opposing plant reactions that are initiated in response to pathogens. Gayoso et al. [41] also described a comparable increase in *IPER* activity that was parallel to H_2O_2 production in diseased tomatoes. This might be the result of functions performed by a number of enzymes in susceptible plants. Furthermore, Lanubile et al. [42] reported a rapid induction of *IPER* gene in maize plants infected with *Fusarium verticillioides*. In response to *Botrytis cinerea,* an enhanced expression of *IPER* has been observed in transgenic tomatoes [43]. Jogaiah et al. [44] also found that the expression of *IPER* gene was significantly higher in tomato plants when infected with *Ralstonia solanacearum* whereas in *Trichoderma harzianum* and *Penicillium chrysogenum* treated plants, the expression was found to be very low. This change might be due to the presence of antagonistic fungi which arrested the growth of bacterial wilt pathogen. Recently, a finding concluded that soybean plants inoculated with *Fusarium virguliforme* exhibited an increased expression of *IPER* gene but in *T. harzianum* treated plants, a reduced potential of pathogen infection along with defense related gene was observed [45].

Mung bean plants grown only inoculated with *M. phaseolina* showed the highest expression of *IPER* gene. Soil amendments with different concentration of DBQ, *Aspergillus* spp., *Penicillium* spp. and *Trichoderma* spp., singly or combined, had very positive effective in lowering the stress of *M. phaseolina.* The higher Ct values indicated that the pathogen's stress was very low in these treatments as compared to positive control treatment.

Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

Iqra Haider Khan did experimental work and wrote initial manuscript, Arshad Javaid supervised the work and finalized the manuscript, Farman Ahmad Chaudhury did final editing of the manuscript.

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