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RESEARCH ARTICLE

Effect of *Macrophomina phaseolina* on growth and expression of defense related genes in *Arabidopsis thaliana*

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Abstract: Infection caused by the necrotrophic fungal pathogen *Macrophomina phaseolina* leads to changes in the growth, development, and metabolism in plants. Plants produce defense responses as a result of infection. The present study was designed to evaluate the defense responses in the model plant *Arabidopsis thaliana* inoculated with *M. phaseolina*. Reduction in shoot length, root length, photosynthetic pigments, relative water content (RWC) and increase in sugar and proline contents in leaves were recorded as defense responses in the inoculated plants. Analysis of gene expression showed that among defense related genes, the expression of mitogen-activated protein kinases and thaumatin proteins increased while chitinase and beta-1,3-glucanase showed little increase compared with control plants. It is, therefore, concluded that defense related genes along with increased sugar and proline may play a role in the development of resistance against *M. phaseolina*.

Keywords: *Arabidopsis thaliana*, gene expression, *Macrophomina phaseolina*, necrotrophic fungus, pathogenesis related proteins.

INTRODUCTION

Macrophomina phaseolina is a globally distributed necrotrophic fungal pathogen which belongs to the class Deuteromycetes. It can cause infection in more than 500 plant species of more than 100 families including economically important crops such as maize, sorghum (Su *et al*., 2001), common bean (Mayek-Perez *et al*., 2001), gram (Raguchander *et al*., 1993), cotton (Aly *et al*., 2007), sunflower (Khan, 2007) and groundnut (Sobti & Sharma, 1992). The disease is named as charcoal rot, root rot, stem rot, seedling damping-off, seedling blight ashy or stem blight depending on the plant species and the part it infects. *M. phaseolina* infects plants at almost all growth stages. Diseases caused by *M. phaseolina* become more severe under high temperature and in dry environments and can severely reduce the yield (Bashir & Malik, 1988). It is very difficult to control this fungus because it can remain viable in soil and plant debris for many years as sclerotia (Short & Wyllie, 1980). *M. phaseolina* is distributed globally from tropical to subtropical and arid to semi-arid regions of Asia, Africa, Europe and America (Wrather *et al*., 2001). Despite having a wide geographical distribution and broad host range, *Macrophomina* is a monotypic genus (Purkayastha *et al*., 2006).

Fungal pathogen invasion interferes with several physiological processes such as reduction in the growth of plants, alterations in total chlorophyll content (chlorophyll a and b) and reduction in photosynthesis which consequently decreases biomass (Pazarlar *et al*., 2013). Proline is a kind of protein that confers tolerance to plants against pathogens (Qamar *et al*., 2015).

In response to pathogen infection, plants also start producing pathogenesis-related (PR) proteins because they are important weaponry of the whole plant against the pathogen and have the ability to inhibit fungal growth (Ebrahim *et al*., 2011). PR proteins are evolutionarily conserved in the plant kingdom and broadly classified into 17 families based on their biochemical functions

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and sequence similarity. These include chitinases (Cletus *et al*., 2013), glucanases (Singh *et al*., 2014), thaumatin-like proteins (Acharya *et al*., 2013), mitogen-activated protein kinases (Beckers *et al*., 2009) and osmotin-like proteins (Subramanyam *et al*., 2012). Chitinases are the enzymes that hydrolyse the main structural components of the fungus cell wall and release the cell wall elicitors of defense reactions (Souza *et al*., 2017). *Arabidopsis thaliana* has about 25 open reading frames that have chitinase or chitinase-like sequences. The average length of these chitinases in *A. thaliana* is 308 amino acids (Akenaka *et al*., 2009). These chitinases also have been involved in other plant physiological processes such as growth and development, programmed cell death and resistance to different environmental stresses (Grover, 2012). Therefore, chitinases are very important genes for increasing biotic stress resistance in plants. Another PR protein is β-1,3-glucanase, also referred as PR2, which hydrolyses β-1,3-linked glucans (non-starch polysaccharide), a structural component of fungal cell walls. It indirectly releases the elicitors that initiate a chain of reactions by reducing hydrolysis of fungal cell walls (Moravcikova *et al*., 2016).

In *Arabidopsis*, a defense response against pathogens also includes the activation of the mitogen-activated protein kinase (MPK) cascade (Eschen-Lippold *et al*., 2012). Those are mainly involved in the stress responses and activated by both biotic and abiotic stresses (Alzwiya & Morris, 2007). Thaumatin-like proteins (TLPs) showed great sequence homology with PR-5 proteins that are activated in plants upon fungal infection (Ahmed *et al*., 2013). TLPs have the ability to rupture the fungal membrane by forming a pore and inhibiting further spread (Roberts & Selitrennikoff, 1990). In *A. thaliana*, these TLPs showed antifungal activity against *Verticillium alboatrum, V. dahliae* and *Fusarium oxysporum* (Hu & Reddy, 1997). Osmotin is also a stress responsive, multifunctional basic protein. These proteins show antifungal activity by permeabilising the fungal plasma membrane and resulting in dissipation of the membrane potential of the fungi (Subramanyam *et al*., 2012).

Not much research has been conducted to determine the effects of *M. phaseolina* on defense responses of *A. thaliana*. The main objective of the current study was to evaluate the role of physiological parameters (chlorophyll, sugar, protein and proline contents) and major defense related genes in the defense response of the model plant *A. thaliana* in the presence of *M. phaseolina*.

METHODOLOGY

Collection of fungus

The strain of fungus (*Macrophomina phaseolina*) used in this research project was obtained from the National Agricultural Research Council (NARC), Islamabad, Pakistan.

Systemic inoculation of the fungus

Preparation of fungal inoculum on Sorghum seeds

Sorghum seeds were sterilised with ethanol (70 %) and then washed with distilled water thrice and soaked overnight in distilled water. Seeds were dried and autoclaved. The autoclaved seeds (100 g) were placed in each 500 mL flask and inoculated with one 4 mm diameter mycelial disk per flask with the help of a cork-borer. The seeds were mixed and the flasks were placed in an Isotemp Standard Lab Incubator (Thermo Fisher Scientific®, USA) at 30–32 ^{o}C for 15 ds. Flasks were shaken every day. After 15 ds, maximum spore concentration was obtained and fungal mycelial growth could be observed with the naked eye. Two grams of inoculated sorghum seeds were mixed with 1 kg of peat soil. The seed-inoculated soil was used for sowing *Arabidopsis* seeds.

Plant material and growth conditions

Seeds of Col-0 ecotype plants of *A. thaliana* were obtained from the National Agricultural Research Council (NARC), Islamabad, Pakistan in 2016. Seeds were surface sterilised with sodium hypochlorite (NaClO) for 5 min and washed with distilled water thrice. The seeds were sown in Petri dishes containing half strength MS Murashige and Skoog medium (Murashige & Skoog, 1962). For stratification, the Petri dishes were placed at 4 °C in darkness for 3 ds and transferred to a controlled climate greenhouse (with 9/15 h light/dark cycle, 22 °C and 200 l mol m^{-2} s^{-1} light intensity). After 2 wks, half of the young seedlings were transplanted into 600 mL plastic pots. Three seedlings were transferred to each pot containing garden peat soil and these plants were used as the control. The remaining half were transplanted to the soil containing fungal inoculum. Both control and treated plants were kept in a greenhouse with the above mentioned conditions. Plants were irrigated with water weekly up to saturation of soil.

Harvesting

Both treated and control plants were harvested four weeks after germination for expression profiling of defense related genes. Plants were frozen in liquid nitrogen immediately after harvest and stored at -80 °C.

Determination of physiological and biochemical parameters

Sugar content of leaves

By following the procedure described by Dubo *et al*. (1956) and amended by Johnson *et al*. (1966), total sugar content of the leaves of treated and control plants were measured. In this assay, 0.5 g of one month old fresh leaves were homogenised by adding 10 mL of distilled water and then centrifuged for 5 min at 3000 rpm. The supernatant (0.1 mL) was mixed with 1 mL of phenol (80 %) and the mixture was incubated at room temperature for 1 h. Concentrated sulphuric acid (H_2SO_4) was added to the mixture and incubated at room temperature for 4 h and the absorbance was measured at 420 nm in a spectrophotometer (Bio-Tek Instruments Inc. USA), using water as blank. By using the standard curve, sugar content of unknown samples was calculated in three replicates.

Protein content

Leaves were harvested for the determination of protein content by following the method of Lowry *et al*. (1951). Protein extraction was done in phosphate buffer. For the preparation of phosphate buffer, 2.76 g of dibasic sodium phosphate was dissolved in 100 mL distilled water. Monobasic (16 mL) and dibasic (84 mL) sodium phosphate were mixed and the pH was adjusted to 7.5. For protein extraction, 0.1 g of leaves was taken and homogenised with 1 mL phosphate buffer with the help of mortar and pestle. The mixture was centrifuged at 3000 rpm for 10 min. The supernatant (0.1 mL) was taken in a test tube and 0.9 mL distilled water was added to make a total volume of 1 mL. Other reagents were prepared as follows;

Reagent A: 2 g of sodium carbonate (Na_2CO_3), 0.4 g of NaOH and 1g of N-K tartarate dissolved in 100 mL of water.

Reagent B: $CuSO_4.5H_2O$ (0.5 g) was dissolved in 100 mL of distilled water.

Reagent C: Reagent A (50 mL) and reagent B (1 mL) were mixed together.

50 % folin phenol reagent was named as reagent D.

One milliliter of reagent C was shaken for 10 min and then 0.1 mL of reagent D was added. The mixture was incubated at room temperature for 30 min. The absorbance of each sample was recorded at 650 nm. Buffer was used as the blank. Protein contents of leaves were determined by the following formula;

$$\text{Protein content} = \frac{k \times \text{Absorbance} \times \text{dilution factor}}{\text{sample weight}}$$

Proline content of leaves

The procedure of Bates *et al*. (1973) was used for the measurement of proline content of the leaves. In this method fresh leaves of 0.1 – 0.2 g were homogenised with 4 mL of 3 % sulfosalicylic acid using mortar and pestle. The mixture was centrifuged for 5 min at 2000 rpm, supernatant (2 mL) was collected and 2 mL of ninhydrin solution was added. The ninhydrin solution was prepared by adding 1.25 g of ninhydrin into a mixture of 30 mL of glacial acetic acid and 20 mL of 6M phosphoric acid. The mixture was incubated at 100 ^oC in a water bath for 1 h. Then 4 mL of toluene was added and shaken for 15–20 s. From the aqueous phase, toluene was aspirated. Toluene was used as the standard and absorbance was recorded at 530 nm. From the standard curve, proline concentration of all samples were determined.

Chlorophyll content

Chlorophyll content of leaves was determined by the method described by Arnon (1949). Fresh leaves (0.3 g) were ground using mortar and pestle with 5 mL of 80 % acetone. The mixture was centrifuged at 3000 rpm for 12 min and the supernatant was collected. The absorbance of supernatant was measured at 650 nm. Acetone (80 %) was used as the blank for all measurements. To calculate milligrams of chlorophyll per gram fresh weight, the following formula described by Arnon (1949) was used;

Total chlorophyll $mgmL^{-1}$ = A650/34.5

Shoot and root length

Using a measuring tape, length of the freshly harvested shoots and roots were measured in three replicates.

Relative water content of leaves

Relative water content (RWC) of leaves was determined by following the method described by Weatherly (1950) and using the following formula;

RWC % = (FW – DW)/(FT – DW) × 100

where FW = fresh weight, DW = dry weight, FT = fresh turgid weight

RNA extraction, quantification and cDNA synthesis

In order to study the expression level of six PR genes (Table 1), RNA was extracted from the control and inoculated plants. RNA extraction was done by Gene JET plant RNA purification kit (Thermo scientific® Qiagen, Germany) by using 0.5 g of fresh leaves. The mRNA concentration was estimated by spectrophotometer (Bio-Tek Instruments Inc. USA). M-MLV, a reverse transcriptase (Invitrogen, Life Technologies) was used to synthesise first-strand cDNA according to manufacturer's instruction. The cDNA was stored at -20 °C until further use.

Primer designing, PCR and RT-PCR

Real time PCR (RT-PCR) was performed to check the expression of six defense related genes. Total cDNA was used as a template and respective genes were amplified using specific primers (Table 1). Primers were designed by using Oligo 6. To standardise cDNA samples of each PCR, actin was used as an internal control.

Table 1: Real-time PCR primer sequences used for amplification of different PR genes

Gene name	Accession No.	Forward primer	Reverse primer	Product size (bp)
Chitinase (CH)	NM_122314.3	'CCAAAACGGAAACGAAGGTA'	'AACCTTGATGCCACGAGACT'	199
Beta-1,3-glucanase (GLU)	NM_123575.4	'AACATGAAGCCTGGACCAAC'	'GTTACCCGTGACTGGCTGAT'	210
EDR1	NM_100745.2	'AATCGGATTCACGTTTCTGC'	'GCTCGGATCTGATGCTTCTC'	230
Mitogen-activated protein kinase 4 (MPK4)	NM_116367.3	'GCATGGTTTGAGCTGATGAA'	'TGGGGATTCTTTGATCTTCG'	177
Osmotin (OSM)	NM_117234.3	'ACGGTCAGGGATCATGTAGC'	'CACCCTCACACACACACACA'	245
Thaumatin (TH)	NM_106230.4	'TCCAATTCAATGGCTCTTCC'	'CAGAATCTTCCTCCCCATGA'	230
Actin (ACT2)	NM_001338358.1	'TGCCAATCTACGAGGGTTTC'	'TTCTCGATGGAAGAGCTGGT'	226

Real-time PCR analysis

Applied Biosystems 7300 real-time PCR system (Thermo Fisher Scientific®, USA) was used to perform quantitative real time PCR. For qPCR, 3µl of cDNA and 5µL of SYBR Green PCR Master Mix (Thermo Fisher Scientific®, USA) was used for thermal cycling with the following conditions: initial denaturation at 95 °C for 1 min, 40 cycles of 95 °C for 15 s, 54 °C for 15 s, and 72 °C for 45 s and final extension at 72 °C for 10 min. Actin was used as housekeeping gene (Pathan *et al*., 2017). This experiment was repeated thrice.

Statistical analysis

The experiment was carried out with three replicates unless otherwise stated (represented with n value). The data were subjected to one-way analysis of variance (ANOVA), using STATISTICA (version 5.5 a) at 0.05 level of significance.

RESULTS AND DISCUSSION

Plants are sessile organisms, therefore they have evolved self-defense systems to protect themselves against different environmental stresses. Interactions between plants and fungal pathogens involve alterations in physiological and biochemical parameters as well as induction of genes that confer resistance to plants.

Growth parameters

When pathogens infect a plant, they interfere with physiological functions of the plant that result in the appearance of different symptoms (Pazarlar *et al*., 2013). In this study infection of *M. phaseolina* resulted in significant reduction in the growth parameters such as shoot length, root length, and relative water content in *A. thaliana*. Infected plants showed deleterious effects on the overall growth of the plants (Table 2) as indicated by Eastburn *et al*. (2011).

Table 2: Effect of inoculation of *M. phaseolina* on shoot length, root length, relative water content and chlorophyll content of 4-week-old seedlings of *A. thaliana* seedlings. Values represent mean ± SEM of 3 replicates. Means sharing different letters in a column are significant at $p < 0.05$.

Treatments	Shoot length (m)	Root length (m)	Relative water content (%)	Chlorophyll content (mg/g fresh matter)
Control	$18.2^{a} \pm 1.3$	$2.5^{a} \pm 0.3$	$86^{a} \pm 6.2$	$1.1^{a} \pm 0.11$
Inoculated	$15.7^{b} \pm 0.9$	$1.7^{b} \pm 0.2$	$57^{b} \pm 5.6$	$1.57^{ab} \pm 0.18$

Sugar content

Pathogen infection is one of the drastic environmental stresses that plants face. As a result of this stress, a series of reactions activate to enhance the defense. To accomplish these defense responses, energy is required due to up- and down-regulation of several genes from multiple defense pathways (Scheideler *et al*., 2002). Energy can be derived from different metabolic activities, i.e. by increasing the respiratory metabolism. In many plant species, sucrose is the main transport sugar. In *A. thaliana* tolerance to biotic stress is strongly enhanced by the accumulation of sucrose (Verslues & Sharma, 2010). The results of the present study were consistent with the findings that total sugar contents were higher in inoculated seedlings compared to the control (Morkunas & Ratajczak, 2014). However, the increase was not statistically significant (Table 3). Increased sugar content is one of the adaptive strategies of plants for the maintenance of structure and functions during biotic stress (Hirabayashi, 1996).

Protein and proline content

The results presented in Table 3 show that *Arabidopsis* plants subjected to *M. phaseolina* infection have increased the protein and proline contents in leaves as compared to the control. Protein content of inoculated leaves showed 43 % increase as compared to the control. Inoculated *Arabidopsis* leaves had 71 % increase in proline content than that of the control (Table 3). When higher plants are under stress they accumulate greater amounts of proline (Pazarlar *et al*., 2013). As a result reactive oxygen species (ROS) are produced causing programmed cell death around the point of infection and terminate the disease progress (Qamar *et al*., 2015).

Table 3: Effect of inoculation of *M. phaseolina* on sugar content, protein content, and proline content of leaves of 4-week-old seedlings of *A. thaliana*. Values represent mean ± SEM of 3 replicates. Means sharing different letters in a row or in a column are significant at $p < 0.05$.

Treatments	Sugar content (µg/g fresh weight)	Protein content (µg/g fresh weight)	Proline content (µg/g fresh weight)
Control	$121^{a} \pm 5.1$	$203^{a} \pm 10.3$	$557^{a} \pm 25.1$
Inoculated	$225^{ab} \pm 11.2$	$251^{b} \pm 12.5$	$697^{b} \pm 31.7$

Chlorophyll content

Fungal infection damages the ultra-structure of chloroplasts that affect chlorophyll pigments a and b and hence reduces the rate of photosynthesis (Lobato *et al*., 2009). The results suggest that plants subjected to *M. phaseolina* infection exhibited significantly lower chlorophyll content as compared to the control (Table 2).

Gene expression

Chitinase is a hydrolytic enzyme that belongs to the parthenogenesis-related (PR) protein family and plays a crucial role in defense against biotic stress. During infection, chitinase enzyme degrades the chitins in the cell walls of fungi and inhibits the growth of the pathogen (Sela-Buurlage *et al*., 1993; Jayaraj & Punja, 2007). In

the present investigation transcript level of chitinase gene was up-regulated as a response to *M. phaseolina* infection (Figure 1). Grover (2012) and Cletus *et al*. (2013) also reported the up-regulation of chitinase gene in plants as a defense mechanism under fungal infection.

Table 4: Analysis of variance (ANOVA) of shoot length, root length, relative water content, chlorophyll content, sugar content, protein content and proline content of 4-week-old seedlings of *A. thaliana* inoculated with *M. phaseolina*

Source	Shoot length	Root length	Relative water content	Chlorophyll content	Sugar content (µg/g fresh weight)	Protein content (µg/g fresh weight)	Proline content (µg/g fresh weight)
Treatment	1811.3***	1309.64***	1954.29***	3737.39***	899.01 ns	1497.5***	2367.5***

* = significant ($p < 0.05$); ** = significant ($p < 0.01$); *** = highly significant ($p < 0.00$); F values are given; ns = not significant

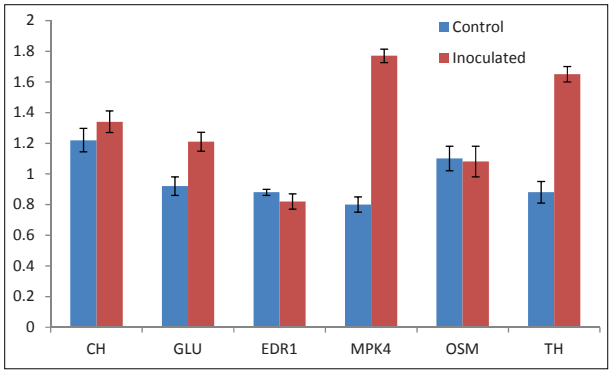

Figure 1: Relative expression of PR gene transcripts in *A. thaliana* plants infected by *M. phaseolina* and non-infected control plants. The data was obtained from real-time RT-PCR analysis. Bars represent means ± standard deviations of five replications. Abbreviations- CH: chitinase; GLU: beta-1,3-glucanase; EDR1: enhanced disease resistance 1; MPK4: Mitogen-activated protein kinase 4; OSM: osmotin; TH: thaumatin

β-1,3-glucanase is a well-known pathogenesis-related protein and induction of this protein is mostly associated with the induction of chitinase during biotic stresses (Liu *et al*., 2010). In the present study expression of β-1,3-glucanase was highly stimulated in *Arabidopsis* after inoculation with *M. phaseolina* compared to un-inoculated controls (Figure 1). This suggests that the up -regulation of this gene may play an important role in plant defense. β-1,3-glucanase can degrade the cell wall of the pathogen or release the oligosaccharide elicitors to induce defense reactions and both of these mechanisms are very effective against fungal infections. Therefore β-1,3-glucanase play an important role in plant defense because the cell wall of an organism is the protective barrier against infection mechanisms and survival of the organism depends on its integrity (Moravcikova *et al*., 2016).

Treatment of plants with microorganisms leads to changes in the phosphorylation of proteins. Among protein kinases, the MPK family is very important and MPKs have been associated with various stress responses, especially in pathogenesis. The present study showed that in *A. thaliana,* MPK4 was activated in the plants infected by *M. phaseolina*. The strong up-regulation of this gene in the infected plants enhanced the resistance in *Arabidopsis* against the fungal infection (Beckers *et al*., 2009).

Osmotin is an antifungal protein that has a broad spectrum of antifungal activities. It recognises the target site by interaction with the pathogen cell surface that has glycoproteins or phosphomannan components that either enhance or reduce the fungal activity (Subramanyam *et al*., 2012). In the present study, the expression level of osmotin and enhanced disease resistance 1 (EDR1) was down-regulated in the inoculated plants as compared to normal plants although this down-regulation was not significant (Figure 1). Similar results were reported by Parkhi *et al*. (2009).

Thaumatin proteins also have a contributory role in increasing the tolerance against both biotic and abiotic stresses (El-kereamy *et al*., 2011). In this study inoculated plants showed resistance to *M. phaseolina* infection due to high induction of thaumatin. This gene was up-regulated almost double as compared to un-inoculated control plants (Figure 1). Similar results were presented by Hu and Reddy (1997) and Ahmed *et al*. (2013).

CONCLUSION

Arabidospsis thaliana was found susceptible and responded to the infection of *Macrophomina phaseolina* by stimulating the accumulation of different defense related compounds like sugar, proline, chlorophyll and total protein in the cell. Among defense related genes, the expression of MPK4 and thaumatin was the highest.

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