Original Research Article  

Macrophomina phaseolina, Cause of Stem Canker Disease in Pigeonpea: Physiological Response under Different Nitrogen Source

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A B S T R A C T

Macrophomina phaseolina (Tassi) Goid is a soil inhabiting fungus having a host range of about 500 plant species. The present study was conducted to investigate physiological variability among two different isolates, microsclerotial (MphP) and mycelial (MphM), of Macrophomina phaseolina in the presence of different nitrogen source. Variability in M. phaseolina offers fitness to the pathogen both in terms of pathogenesis and survivability. Therefore, it is hypothesized that MphP and MphM, might exhibit physiological variability. In this perspective, the present study was conducted by measuring the fungal biomass, total extracellular proteins (ECP), extracellular proteins protease (EP). The effect of different nitrogen on fungal morphology was studied microscopically by aniline blue staining. Two different isolates of M. phaseolina exhibit significant variations in biomass accumulation, ECP and EP. Interaction of Nitrogen source, molarity and molarity had a significant effect ($p \leq 0.001$) on fungal biomass, ECP and EP. The study may serve to increase the understanding that nitrogen might play an important function in fungal physiology that enables its survival as well as pathogenesis.

Introduction

Plants utilize nitrogen from soil in the form of nitrate. Inorganic nitrogen transforms to ammonia before assimilation in higher plants (Paungfoo-Lonhienne et al., 2008). Eventually, ammonia integrates into nitrogen-carrying molecules, such as glutamine, glutamate, asparagine, and aspartate in plants (Lam et al., 1996). Plant pathogens colonize plant tissues as nutrient source for their growth and development. According to Snoeijers et al., (2000), the nutritional status of plant affects the transcription of in planta-induced genes of pathogens. In general, filamentous fungi can efficiently utilize wide range of compounds as nutrients even under exceptionally varying environmental conditions. Uptake and metabolism of different nitrogen source is under complex regulatory control governed by many domains and pathway-specific regulatory genes that operate primarily at the transcriptional level (Wong et al., 2008). Several simple as well as complex macromolecules in living cells, such as proteins and nucleic acids are composed of nitrogen as a major element. Therefore, it is obvious that an extensive investment in cellular metabolic pathways, such as nitrogen catabolism, is necessary in order to guarantee
steady nitrogen supply for cellular growth and development.

Nitrogenous, both organic as well as inorganic, fertilizers influence the disease incidence and severity, plant resistance, plant growth, weeds, pest population and ultimately the microclimate. Different fertilizer might exert multifarious effect on plant disease due to the individual effect of different elements in the fertilizer (Huber, 1980). In the same way, the available nutrients might cause shift in the transcriptional profiling in microorganisms (Wong et al., 2008). Therefore, it is plausible to contemplate that nutritional limitations, particularly of nitrogen, also affect pathogenesis (Talbot et al., 1997). The utilization or assimilation of nitrogen in microorganism varies from necrotrophic to biotrophic and hemi-biotrophic fungi as well as from root inhabiting to aerial pathogens, depends on the source. The knowledge of nitrogen metabolism is limited to certain phytopathogenic fungi, such as *Cladosporium fulvum* (Solomon and Oliver 2001), *Fusarium oxysporum* (Divon et al., 2005), *Uromyces fabae* (Voegele et al., 2005), *Fusarium graminearum* (Guldener et al., 2006), *Magnaporthe grisea* (Wang et al., 2003). However, related studies are lacking in an emerging potential plant pathogen, *Macrophomina phaseolina*.

*Macrophomina* exhibits high degree of morphological, pathogenic, physiological and genetic variation (Jana et al., 2005; Kaur et al., 2013). According to Islam et al., (2012), *M. phaseolina* possesses a large repertoire of cell wall degrading enzymes such as cellulases, hemicellulases, pectinases, ligninases, and cutinases. The overall number of carbohydrate active enzymes is particularly high, primarily due to the abundance of glycoside hydrolases. Kaur et al., (2012) studied the ability of carbohydrate degrading enzyme production by two different isolates of *M. phaseolina*, microsclerotal (MphP) and mycelial (MphM) on apple pomace supplemented with 1% (w/w) rice husk as a substrate through koji fermentation. Activity of different hydrolytic enzymes, such as cellulases, hemicellulase and amylase was higher in MphP as compared to MphM. Therefore, it is hypothesized that these two isolates, microsclerotal (MphP) and mycelial (MphM), might also exhibit physiological variability in the presence of different nitrogen source. In this perspective, the present study was conducted to investigate the effect of different nitrogen source on the total extracellular proteins (ECP), extracellular protease (EP) and fungal biomass in *M. phaseolina*. The varying concentration of different nitrogen on the morphology of fungal cells was studied by microscopic imaging using aniline blue.

Materials and Methods

*Macrophomina* isolates microsclerotal, MphP and mycelial, MphM used in the study. Isolates MphP produces microsclerotia and mycelia in the culture media. However, isolate MphM grows as mycelial form without producing any apparent microsclerotia. The study conducted in a synthetic medium as described by Kaur et al., (2012) with slight modification. Nitrogen source, NH₄NO₃ was replaced by different nitrogen source, such as ammonium molybdate (AM), ammonium sulfate (AS), ammonium acetate (AA), ammonium chloride (AC), ammonium nitrate (AN), ammonium persulfate (AP), ammonium oxalate (AO) and urea at concentrations of 2.5, 5.0 and 10.0 mM, respectively. The pH was adjusted to 5.5 with 1N HCl and 1N NaOH. Inoculation method was described earlier (Kaur et al., 2012). The cultures incubated for seven days in incubator shaker at 30±1°C and 150 rpm. After seven days of incubation, the broth was
centrifuged at 12,000 × g for 15 min at 4±1 °C. The supernatant was separated from the mycelium pellet and stored at 4 °C for further analysis. The pellet was dried at 45±1 °C for 24 h or until a constant weight was obtained. Weight of the dried fungal biomass measured in gm/l.

The protein content of the crude extract was estimated (Lowry et al., 1951). In brief, sample (100 µl), distilled water (400 µl), and diluted Bradford reagent (2.5 ml) were added and incubated for 5 min. The protein content (mg/ml) estimated using a standard graph at 595 nm. Extracellular protease activity measured from the crude extract (Sudhakar et al., 2010). In brief, 3 ml of reaction mixture containing 2.5 ml casein (0.65%) in sodium phosphate buffer (50 mM), pH 7.5 and 0.5 ml of crude enzyme extract incubated at 37±1 °C for 10 min. The reaction stopped by adding 2.5 ml of 110 mM TCA and again incubated for 30 min at 37±1°C. The reaction mixture filtered through whatman no. 50 filter paper. The colour developed by adding 2.5 ml of sodium carbonate solution (500 mM) and 0.5 ml of Folin-Ciocalteu's phenol reagent and measured at 280 nm. Enzyme activity was calculated by measuring mg of tyrosine equivalent released and compared with the standard. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1 mg of tyrosine under standard assay conditions.

Fungal hyphae was stained with aniline blue (0.1 mg/ml in distilled water) to observe the cell morphology or any cellular damage in all treatments. Micrographs were taken using a Zeiss Axioplan light microscope with a 10X and 40X objective. Data were statistically analyzed by using the multiple analyses of variance (ANOVA) by SPSS, version 16.0. The test was performed at the level of p ≤ 0.05 to determine the significance of difference. The Duncan’s Multiple Range Test (DMRT), p ≤ 0.05, separated treatment means.

Results and Discussion

Fungal biomass

Fungal biomass was measured in all the treatments containing different nitrogen source at 2.5, 5.0 and 10.0 mM concentrations. Maximum biomass, 7.08 g/l was obtained in AC followed by AN (6.44 g/l), AP (4.46 g/l) and AM (4.35 g/l) in MphP. In MphM, highest biomass was obtained was obtained in AO (5.82 g/l) and AC (5.65 g/l) followed by urea (5.40 g/l) and AS (3.77 g/l) (Table 1). MphP and MphM had maximum biomass, 5.57 g/l and 3.97 g/l, at 5.0 mM, respectively. Fungal biomass reduced at the concentration above or below 5.0 mM i.e., 10.0 mM and 2.5 mM in the study. Nitrogen source, molarity and nitrogen source × molarity had a significant effect (p<0.001) on fungal biomass (Table 2).

Fungal biomass is an important parameter in modelling fungal growth (Lamour et al., 2000). Nutrient uptake in mycelial fungi occurs by active transportation across the plasma membrane. Development of turgor pressure due to the internal substrate concentration, nutrient utilization, as well as product formation and the development of the tip vesicles in mycelial fungi results in hyphal branching (Boswell and Hopkins, 2008). In the present study, biomass accumulation differed significantly between the different nitrogen source and concentration.

Decrease in fungal biomass with the increase in urea concentration to 10 mM suggests the adverse effects of urea on soil borne fungal pathogens due to the release of toxic ammonium ions during the degradation of urea (Oteifa, 1995). Maximum biomass in AC might be due to rapid diffusion of ammonium ions.
ions. However, the increasing concentration of nitrogen becomes a limiting factor for the growth and development of *M. phaseolina*.

The ability to survive well in the presence of relatively toxic compounds, such as AP, AN and AM, in MphP as compared to MphM, suggests less nitrogen utilization by sclerotia than lipid content thereby impairing their survival (Wassef *et al*., 1975). Sclerotia germinate rapidly with the dissipation of ammonia from the soil. Fungal mycelium has the potential to assimilate wide range of inorganic and organic nitrogen compounds (Finlay *et al*., 1989).

**Total extracellular proteins**

Maximum total ECP observed in MphP as compared to MphM. In MphP, maximum ECP was obtained in urea (0.85 mg/ml) and AN (0.66 mg/ml) while AP (0.49 mg/ml), AA (0.47 mg/ml) and AC (0.44 mg/ml) was non-significant. However, minimum ECP was 0.30 mg/ml (AO), 0.34 mg/ml (AS) and 0.35 mg/ml (AM) (Table 3). In MphM, highest ECP was obtained in AC (0.77 mg/ml) followed by AN (0.42 mg/ml), urea (0.35 mg/ml) and AM (0.34 mg/ml) (Table 2). Total ECP differed significantly (*p* ≤ 0.01) among different treatments (Table 2).

**Table 1** Fungal biomass in *Macrophomina phaseolina* in the presence of different nitrogen Source supplemented in synthetic medium

| Nitrogen source       | MphP (g/l) | Mean       | MphM (g/l) | Mean       |
|-----------------------|------------|------------|------------|------------|
|                       | 2.5M | 5.0M | 10.0M |          | 2.5M | 5.0M | 10.0M |
| Ammonium molybdate    | 3.90  | 4.24  | 4.92   | 4.35<sup>c</sup> | 1.37  | 0.46  | 1.16   | 1.00<sup>g</sup> |
| Ammonium sulfate      | 0.34  | 0.45  | 0.24   | 0.34<sup>e</sup> | 3.87  | 3.65  | 3.78   | 3.77<sup>c</sup> |
| Ammonium acetate      | 4.05  | 1.53  | 4.29   | 3.29<sup>f</sup> | 1.74  | 2.48  | 2.46   | 2.23<sup>e</sup> |
| Ammonium chloride     | 3.09  | 15.53 | 2.62   | 7.08<sup>a</sup> | 6.18  | 5.51  | 5.25   | 5.65<sup>a</sup> |
| Ammonium nitrate      | 4.09  | 11.03 | 4.19   | 6.44<sup>b</sup> | 2.84  | 3.72  | 1.55   | 2.70<sup>d</sup> |
| Ammonium persulfate   | 6.46  | 3.12  | 3.81   | 4.46<sup>c</sup> | 1.07  | 1.86  | 1.20   | 1.38<sup>f</sup> |
| Ammonium oxalate      | 3.94  | 4.09  | 3.84   | 3.96<sup>a</sup> | 5.64  | 5.91  | 5.90   | 5.82<sup>a</sup> |
| Urea                  | 3.58  | 3.76  | 2.16   | 3.17<sup>e</sup> | 4.70  | 8.14  | 3.37   | 5.40<sup>b</sup> |
| Mean                  | 3.68<sup>c</sup> | 5.47<sup>f</sup> | 3.26<sup>a</sup> | 4.14  | 3.43<sup>f</sup> | 3.97<sup>i</sup> | 3.08<sup>i</sup> | 3.49  |

**L.S.D** 0.19 (Nitrogen source) 0.12 (Molarity) 0.18 (Nitrogen source) 0.17 (Molarity)

The values with letters and digits in superscripts differ significantly (*p*≤0.05) for different nitrogen source and molarity, respectively.

**Table 2** Effect of different nitrogen source on *Macrophomina phaseolina* in synthetic media

| Source of variation | Dry weight | Extracellular proteins | Protease |
|---------------------|------------|------------------------|----------|
|                     | MphM       | MphP                   | MphM     |
|                     | MSS *      | F Value                | MSS *    | F Value | MSS *      | F Value | MSS *      | F Value | MSS *      | F Value |
| Nitrogen source     |            |                       |          |         |            |         |            |         |            |         |
| (NS)                |            |                       |          |         |            |         |            |         |            |         |
| Concentration (C)   |            |                       |          |         |            |         |            |         |            |         |
| NS°C               |            |                       |          |         |            |         |            |         |            |         |

<sup>*Significant at *p* ≤ 0.01</sup>
### Table 3
Total extracellular proteins secretion by *Macrophomina phaseolina* in the presence of Different nitrogen source in synthetic medium

| Nitrogen source          | MphP (mg/ml) | Mean | MphM (mg/ml) | Mean |
|--------------------------|--------------|------|--------------|------|
|                          | 2.5mM | 5.0mM | 10.0mM |       | 2.5mM | 5.0mM | 10.0mM |       |
| Ammonium molybdate       | 0.16  | 0.54  | 0.36   | 0.35c | 0.29  | 0.34  | 0.38   | 0.34bc |
| Ammonium sulfate         | 0.34  | 0.45  | 0.24   | 0.34c | 0.10  | 0.21  | 0.18   | 0.16d  |
| Ammonium acetate         | 0.37  | 0.46  | 0.58   | 0.47bc| 0.11  | 0.09  | 0.50   | 0.23cd |
| Ammonium chloride        | 0.33  | 0.66  | 0.32   | 0.44bc| 0.25  | 0.13  | 1.94   | 0.77a  |
| Ammonium nitrate         | 1.26  | 0.40  | 0.31   | 0.66ab| 0.03  | 1.02  | 0.20   | 0.42bc |
| Ammonium persulfate      | 0.58  | 0.51  | 0.39   | 0.49bc| 0.17  | 0.31  | 0.34   | 0.27cd |
| Ammonium oxalate         | 0.14  | 0.32  | 0.43   | 0.30c | 0.58  | 0.03  | 0.24   | 0.28cd |
| Urea                     | 1.52  | 0.49  | 0.54   | 0.85a | 0.09  | 0.17  | 0.79   | 0.35bc |
| **Mean**                 | 0.59  | 0.48  | 0.40   | 0.49 | 0.20  | 0.29  | 0.57   | 0.35   |
| **L.S.D**                |       |       |        |      | 0.27  | 0.17  | 0.13   | 0.08   |

The values with letters and digits in superscripts differ significantly ($p \leq 0.05$) for different nitrogen source and molarity, respectively.

### Table 4
Extracellular protease secretion in synthetic media by *Macrophomina phaseolina* in the presence of different nitrogen source

| Nitrogen source          | MphP (U/ml) | Mean | MphM (U/ml) | Mean |
|--------------------------|--------------|------|--------------|------|
|                          | 2.5mM | 5.0mM | 10.0mM |       | 2.5mM | 5.0mM | 10.0mM |       |
| Ammonium molybdate       | 11.11 | 15.05 | 33.11 | 19.76a | 11.95 | 22.15 | 44.84 | 26.31a |
| Ammonium sulfate         | 6.40  | 10.93 | 8.81  | 8.71bc| 4.00  | 1.94  | 7.84  | 4.59c  |
| Ammonium acetate         | 3.92  | 1.91  | 4.74  | 3.52bc| 0.83  | 3.12  | 4.64  | 2.86de |
| Ammonium chloride        | 3.11  | 4.04  | 0.62  | 2.59de| 0.33  | 0.81  | 4.41  | 1.85e  |
| Ammonium nitrate         | 8.75  | 9.54  | 5.69  | 7.99bc| 4.13  | 2.15  | 3.02  | 3.10de |
| Ammonium persulfate      | 6.42  | 8.51  | 12.70 | 9.21bc| 7.40  | 8.85  | 14.92 | 10.39bc|
| Ammonium oxalate         | 3.87  | 11.47 | 7.62  | 7.65c | 0.91  | 1.89  | 4.53  | 2.44de |
| Urea                     | 5.93  | 6.05  | 12.64 | 8.21bc| 0.84  | 1.43  | 7.48  | 3.25cd |
| **Mean**                 | 6.19  | 8.44  | 10.74 | 8.46 | 3.80  | 5.29  | 11.46 | 6.85   |
| **L.S.D**                | 1.30  | 1.35  | 0.83  | 1.70 | 0.08  | 0.13  | 0.20  | 0.08   |

The values with letters and digits in superscripts differ significantly ($p \leq 0.05$) for different nitrogen source and molarity, respectively.
Fig. 1 Effect of ammonium acetate (AA) and ammonium molybdate (AM) on *Macrophomina phaseolina* (MphM) at different concentrations (2.5, 5.0 and 10.0 mM) at 40X magnification
Fig. 2 Effect of ammonium chloride (AC) and ammonium nitrate (AN) on *Macrophomina phaseolina* (MphM) at different concentrations (2.5, 5.0 and 10.0 mM) stained with aniline blue at 40X magnification.
Fig. 3 Effect of ammonium oxalate (AO) and urea (U) on *Macrophomina phaseolina* (MphM) at different concentrations (2.5, 5.0 and 10.0 mM) stained with aniline blue at 40X magnification.
Fig. 4 Effect of ammonium sulphate (AS) and ammonium persulphate (AP) on *Macrophomina phaseolina* (MphM) at different concentrations (2.5, 5.0 and 10.0 mM) stained with aniline blue at 40X magnification.
**Fig.5** Effect of ammonium acetate (AA) and ammonium molybdate (AM) on *Macrophomina phaseolina* (MphP) at different concentrations (2.5, 5.0 and 10.0 mM) at 40X magnification.
Fig. 6 Effect of ammonium chloride (AC) and ammonium nitrate (AN) on *Macrophomina phaseolina* (MphP) at different concentrations (2.5, 5.0 and 10.0 mM) at 40X magnification.
Fig. 7 Effect of ammonium oxalate (AO) and urea (U) on *Macrophomina phaseolina* (MphP) at different concentrations (2.5, 5.0 and 10.0 mM) at 40X magnification.
Fig. 8 Effect of ammonium sulphate (AS) and ammonium persulphate (AP) on *Macrophomina phaseolina* (MphM) at different concentrations (2.5, 5.0 and 10.0 mM) at 40X magnification.
Protein secretion is a vital process in fungi and plays a vital role in their nutrition to the transportation of toxic molecules and overcoming desiccation. ECP secretion depends on both differentiation and compartmentalization, important steps in fungal growth cycle. In MphM, ECP was highest in AC followed by AN, urea and AM while it was lowest in AS (Table 3). It might be due to the concurrent process of ECP secretion in actively growing hyphal tips (Wosten et al., 1991; Moukha et al., 1993). Cell wall lysis, cell condensation and granulation in AC, AN, urea and AM might be the reason for the difference in ECP secretion (Figs. 1-4). The increasing concentration on AN cause cell wall weakening and cell lysis (Fig. 2) which facilitated the release of more ECP. In MphP, maximum increase in ECP was obtained in urea might be due to the increase in hyphal tips per unit length of hypha or the failure of growth polarity (Fig. 5-8), that tends to increase the secretion of ECP in the growth medium (Lee et al., 1998). The cell lysis, weakened cell wall, or slow hyphal growth increased ECP in mcb mutant of Neurospora crassa (Lee et al., 1998).

**Extracellular protease activity**

Secretion of EP was maximum, 26.31 U/ml and 19.76 U/ml, respectively in MphM and MphP in AM. Similarly, minimum EP, (1.85 U/ml) and (2.59 U/ml), was obtained in AC as nitrogen source in MphM and MphP, respectively. EP activity increased with the increase in nitrogen concentration from 2.5 mM to 10.0 mM (Table 4).

Maximum EP at 10.0 mM was 11.46 U/ml and 10.74 U/ml in MphM and MphP respectively. Nitrogen source, molarity and nitrogen source × molarity differed significantly ($p \leq 0.001$) between both the isolates (Table 2).

Proteolysis plays important physiological functions, such as protein digestion, hormone maturation, immune response, coagulation, fertilization, germination, and other morphological processes (Holzer and Heinrich, 1988). It is vital in coping extreme or stressed environmental conditions, such as nutritional stress that may lead to altered enzyme due to the reorganization of cellular metabolism (Mercado-Flores et al., 2003). Higher amount of EP production in the presence of AM and AP can be attributed to the fact that EP production are often repressed under the conditions of high ammonium levels in the medium (Wang et al., 2005). The complex growth media usually results in higher protease production (Joo et al., 2002). At the same time, the decrease in EP production in the presence of AA, AC and AN in both the isolates might be due to the catabolite repression in the presence of ammonium ions responsible for protein degradation (Ward et al., 2005). The expression of protease genes in Aspergillus niger, pepA and pepB, were repressed by the addition of ammonia or urea in the presence of glucose and BSA (Jarai and Buxton, 1994). The significant difference among different treatments in the two isolates suggested that EP production in *M. phaseolina* might be under the control of several regulatory genes.

Various nitrogen at different concentrations have a significant effect on fungal biomass accumulation, total extracellular proteins and extracellular protease secretion. The knowledge of physiological metabolism and/or nutritional signals in phytopathogenic fungi needs to be dealt in detail in order to understand their potential phytopathogenesis. To best of our knowledge, this is the first study reporting the effect of different nitrogen source on the physiology of *M. phaseolina*. Furthermore, it is envisaged that by physiological role of nitrogen at molecular level in *M. phaseolina* and drawing its
applicability in the management program at the field level will certainly aid the plant disease management program. The knowledge gained from this research may enable the remodeling and designing of new antifungal compounds targeting fungal metabolism. The study may serve to increase the understanding that nitrogen might plays an important function in fungal physiology that enables its survival as well as enhanced pathogenesis during physiological maturity of the plant. However, the apparent fundamental difference in the two different isolates of Macrophomina and their response to different nitrogen source could not be cleared. Molecular approaches, such as studying various structural and regulatory genes, might offer an important piece of information regarding this concept.

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