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*Full Length Research Paper*

# Biochemical response and host-pathogen relation of stalk rot fungi in early stages of maize (*Zea mays* L.)

**Nirupma Singh*, Ambika Rajendran, Meena Shekhar and Girish Mittal**

Directorate of Maize Research, Pusa Campus, New Delhi, 110012-India.

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**Stalk rot is a destructive disease in maize caused by *Fusarium and Macrophomina* species. A study was carried out to understand the mode of infection, host biochemical response and comparison of inoculation techniques in *Fusarium verticillioides* and *Macrophomina phaseolina* in maize. In seed inoculation experiment, high mycelia growth on seed surface lead to rotting in 36.6% of seeds inoculated by *F. verticillioides* and 10.0% seeds in *M. phaseolina.* In seedling inoculation experiment, twenty one days old seedlings raised in glasshouse were inoculated with spore suspension of both pathogens, respectively in two sets, resulting in symptoms like tip drying, necrotic lesions, chlorotic bands, pale green leaves and yellowing of margins in varying numbers. Significant result was the appearance of asymptomatic seedlings in *F. verticillioides* infection which was confirmed by the increase in total soluble phenols (9.39 mg/g) and total sugars (5.33 mg/g) content in comparison to the control (2.84 mg/g total soluble solid (TSS) and 2.18 mg/g total soluble phenols) and symptomatic ones. While in *M. phaseolina*, total contents of sugar and soluble phenols were on part in asymptomatic and control (uninfected), depicting disease escape to be the possible cause of this phenotypic expression. The study concludes that inoculation techniques for screening of genotypes play a major role. The appearance and non appearance of symptoms in infected host can mislead the identification of resistant genotypes.**

**Key words:** Maize, *Fusarium verticillioides*, *Macrophomina phaseolina*, total soluble sugar, total soluble phenols.

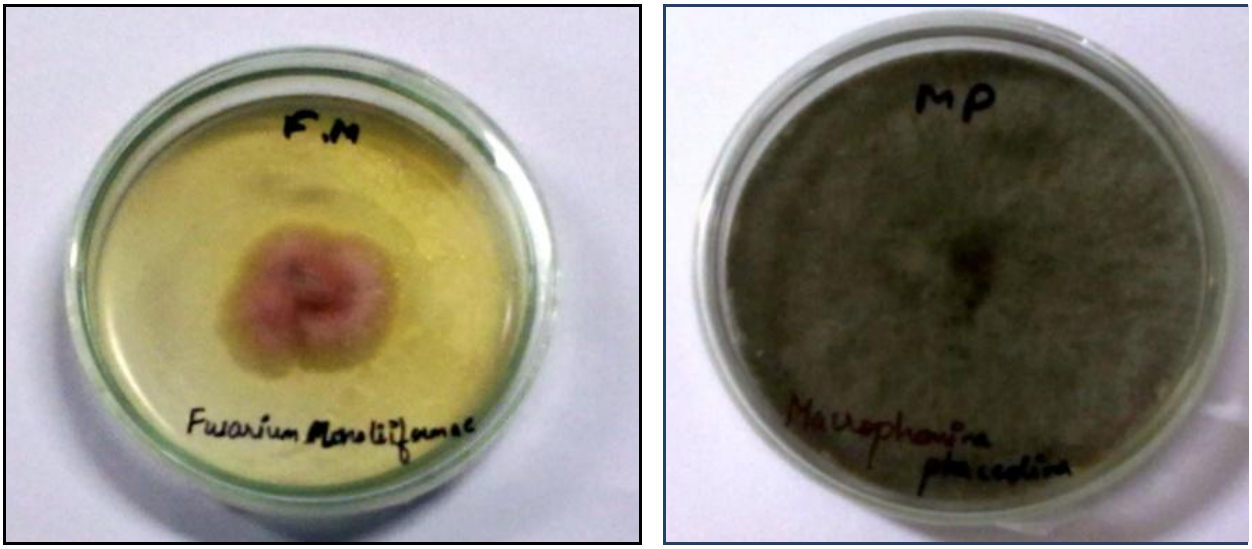
## INTRODUCTION

Stalk rots are among the most destructive diseases in maize (*Zea mays* L.) throughout the world (De Leon and Pandey, 1989). Pathogen such as *F. verticillioides* and *M. phaseolina* are commonly associated with stalk rot. Stalk rots cause yield losses by increasing lodging of the crop or by cutting off the supply of water and nutrients from the roots. It causes comparatively more damage in tropical as compared to temperate regions/countries (Christensen and Wilcoxon, 1966). In India, the disease is prevalent in most of the maize growing areas, namely Jammu and Kashmir, Punjab, Haryana, Delhi, Rajasthan, Madhya Pradesh, Uttar Pradesh, Bihar, West Bengal, Andhra Pradesh, Tamil Nadu and Karnataka. In recent years, maize workers have been quite concerned with this problem, especially in kharif (rainy) season. Maize stalk strength is determined by two main factors: the mechanical structure of the stalk and abiotic stress factor, fusarium stalk rot. The degree of stalk rot infection depends greatly on environmental factors, the genotype × environment interaction and the resistance of the given maize genotype to the pathogens (Kommedahl and Windels, 1981).

*F. verticillioides* is a pathogen causing disease of ears, stalks and seedlings. It can also survive as an endophyte of maize, causing no visible symptoms. *Fusarium* infection occurs through infected seeds, through silk channels or wounds, causing grain rot during both the pre and post harvest periods (Munkvold and Desjardins, 1997). *M. phaseolina* (Tassi) Gold., is a soil-borne, microsclerotia producing fungus, common in tropical and

*Corresponding author. E-mail: nirupmasingh@rediffmail.com. Tel: 91-11-25849725, 25841805. Fax: 91-11-25848195.

**Abbreviations: PDA,** Potato dextrose medium; **OD,** optical density; **FC reagent,** folin and ciocalteu.




(a) (b)

**Figure 1.** Plates of fungal pathogens cultured in PDA media. (a) Culture of *F. verticillioides;* (b) culture of *M. phaseolina.*

subtropical regions, affecting the fibrovascular system of the roots and basal internodes, impending the transport of nutrients and water to the upper parts of the plant. The pathogen is also responsible for seedling blight, damping off, root rot and basal stem rot. Plants produce a number of secondary metabolites as a defensive biochemical compounds to resist the pathogen invasion. Phenol compounds are essential for the growth and reproduction of plants, and are produced as a response for defending injured plants against pathogens. Sugars also play an important role in defence mechanism of plants against invading pathogens. In general, the infection by some pathogens bring changes in respiratory pathway and photosynthesis, which are the vital processes taking place inside the plant, leading to wide fluctuations in sugars (Klement and Goodman, 1967). The estimation of these compounds help in understanding the extent of host resistance to the pathogen.

Life cycle of pathogen plays an important role in investigating the expression and severity of disease symptoms and suitable control measures. Keeping this in view, the following experiments were therefore carried out to study the pathogenicity and biochemical host-pathogen interactions of *F. verticillioides* and *M. phaseolina.*

## MATERIALS AND METHODS

### Inoculum production

Delhi isolate of *F. verticillioides* Sheldon, causal organism of stalk rot of maize, was established from diseased sample from Delhi (India) experimental field. Isolations were made by plating surface sterilized (4% sodium hypochlorite) pieces of infected tissues on Potato dextrose agar (PDA) medium (Figure 1). Purification of cultures was made by single spore/sclerotia method. Temperature for the growth of the fungus was maintained at 27 ± 2°C, in Biological oxygen demand (BOD) incubator for seven days. The seven days old fungal culture was carefully scrapped off using sterile spatula and transferred to sterilized flask containing 10 ml of distilled water. To this, one drop of Tween-20 was added and spore suspension was stirred continuously to avoid clumping/sticking together of spores on the upper surface of the water. The suspension was kept on rotary shaker for 5 min and filtered carefully using muslin cloth. The spore concentration was adjusted $10^8$ spores/ml with a haemocytometer. The same procedure was followed for inoculums production of *M. phaseolina* (Tassi) Gold.

### Experiment I (seed experiment)

Seeds of experimental inbred (JCY 2-2-4-1-1-1-3-1-3-1-1-1-2) were surface sterilized by giving twenty minutes incubation in 5% (w/v) sodium hypochlorite solution. Surface sterilized seeds were soaked overnight in spore suspension of both the pathogens (*F. verticillioides and M. phaseolina*) (Figure 2). Three sets of autoclaved Petri plates with wet sterile tissue papers were taken. Ten soaked seeds per plate were placed in three Petri plates and sealed with paraffin film to avoid contamination and incubated at 28°C and 70% relative humidity for seven days. Seeds soaked with sterile water were maintained as control. On the 7th day, the observations were recorded for the extent of germination, mycelia growth and seed rotting.

### Experiment II (seedling experiment)

Surface sterilized seeds were planted in six replications with five seedlings per pot, and allowed to grow for two weeks in glass house conditions. The suspension spray was prepared by taking 200 ml of distilled water and 1 ml of Tween-20, and stirred vigorously. To this, culture of both the pathogens was added and put on shaker for 10 to 15 min. Spore concentration was $10^8$ spores/ml. Suspension was filtered with muslin cloth and


**Figure 2.** Incubation of seeds in spore suspension.

transferred to spray bottles. The same was sprayed carefully over seedlings at 2 ml per seedling. For control, seedlings were sprayed with sterile water. The experiment was conducted in controlled conditions of glass house. After 21 days, observations on above ground disease symptoms were recorded.

**Experiment III (biochemical assay)**

The comparative biochemical study of symptomatic, asymptomatic and control (uninfected) seedlings was done to understand biochemical factors which are, total soluble phenols and total soluble sugars and their relationship with progress of disease. Leaf samples (100 mg) were extracted with 5 ml of 80% ethanol, and centrifuged at 3000 rpm for 10 min. Extraction was repeated four times with 80% ethanol, and supernatants were collected into 25 ml volumetric flasks. Final volume of the extract was made to 25 ml with 80% ethanol. This extract was used for total soluble sugar and total soluble phenols estimation.

**Estimation of total sugar**

The extract (0.3 ml) was pipette from treatments into separate test tubes and the tubes were placed in a boiling water bath for 3 min to evaporate the ethanol. One millliter (1 ml) of Millipore water and 4 ml of 0.2% anthrone reagent (200 mg in 100 ml $H_2SO_4$) was added in each test tube and placed in ice cold water. Reagent blank was prepared by adding 1 ml of distilled water and 4 ml of anthrone reagent. The intensity of colour was read at 600 nm on spectrophotometer. A standard curve was prepared using 10 mg glucose per 100 ml distilled water (Franscis et al., 1971).

Total soluble sugar (mg/g) = Sample optical density (OD) × Standard OD × Dilution factor

**Estimation of total soluble phenols**

The ethanol extract (1 ml) was evaporated to dryness in water bath. One millliter (1 ml) of Millipore water in each test tube and 0.5 ml of Folin and Ciocalteu (FC) reagent (1:1 with water) was added and kept for 3 min. After this, 2 ml of 20% $Na_2CO_3$ was added and mixed thoroughly. The tubes were placed in boiling water for exactly 1 min and cooled in ice water. The absorbance was read at 650 nm against a reagent blank (Malik and Singh, 1980). A standard graph was prepared using Gallic acid ranging between 0 to 25 μg concentrations. The amount of total soluble phenols present in the sample was calculated as: Phenol (mg/g) = Sample OD × Standard OD × Dilution factor.

**Statistical analysis**

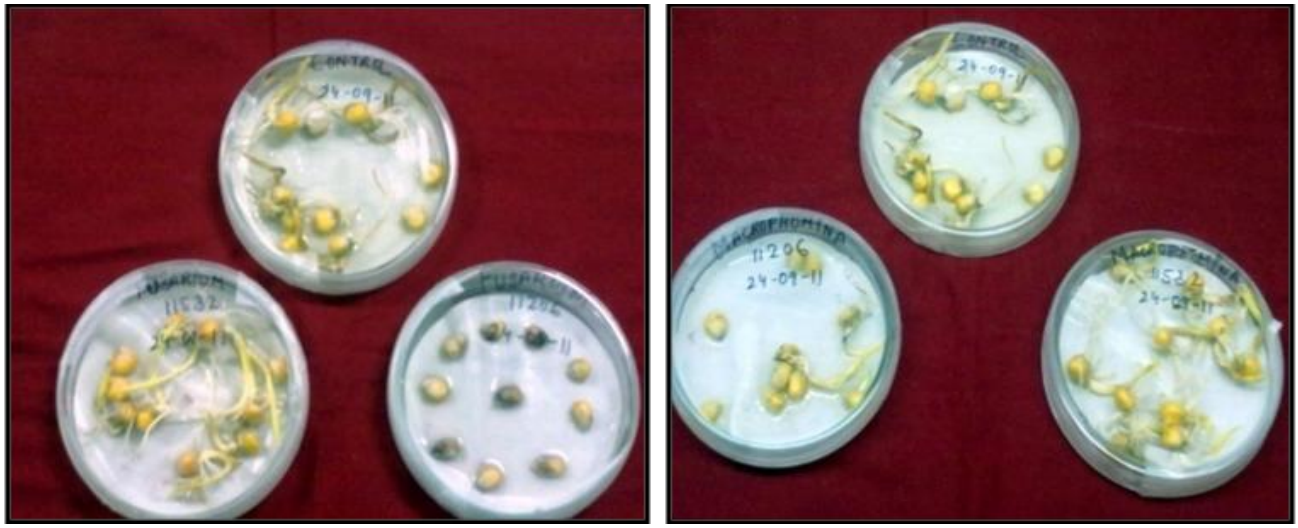
Duncan's multiple range test (DMRT) analysis was done to note the significant difference between concentration of total soluble sugars and total soluble phenols among control and treatments.

## RESULTS

Stalk rot disease caused by *F. verticillioides* and *M .phaseolina* was studied at two stages which were, seed and seedling. The symptoms associated with disease

**Table 1.** Percentage and number of seeds showing symptoms after *F. verticillioides* and *M. phaseolina* inoculation.

| Symptoms | | *Fusarium verticillioides* | | *Macrophomina phaseolina* | |
|---|---|---|---|---|---|
| | | Number of seeds | Percentage of total seeds (%) | Number of seeds | Percentage of total seeds (%) |
| Number of seed germination | Higher surface infection leading to seed rotting | 11 | 36.6 | 3 | 10.0 |
| Seed germination | Low surface infection | 5 | 16.7 | 4 | 13.3 |
| | No surface infection | 14 | 46.7 | 23 | 76.7 |
| Total | | 30 | 100.0 | 30 | 100.0 |



**(a)** **(b)**

**Figure 3.** Seed germination experiment. (a) *F. verticillioides;* (b) *M. phaseolina.*

were observed. The salient findings of the experiments were presented below.

**Experiment I (seed inoculation)**

Seeds inoculated with *F. verticillioides* and *M. phaseolina* cultures were observed after seven days incubation in ambient conditions. The observations on germination, surface infection and rotting were recorded (Table 1 and Figure 3). In *F. verticillioides* inoculated seeds, 63.4% germination was recorded. Higher growth of mycelia on seed surface led to seed rotting, and ultimately no germination in eleven seeds (36.6% of total number of seeds). Among nineteen germinated seeds, five seeds showed low surface infection (16.7% of total seeds) and fourteen seeds expressed no infection at all (46.7% of total seeds). When inoculated with *M. phaseolina*, 90% germination was observed along with no occurrence of seed rotting. However, 13.3% of germinated seeds showed low surface infection and 76.7% had no mycelia growth on surface. In control, 100% germination was recorded. From the study, at seed stage, it is observed that the experimental inbred was more resistant to *M. phaseolina* than *F. verticillioides* in terms of mycelia growth on the surface on seed and coleoptiles.

**Experiment II (seedling inoculation)**

Two weeks old seedlings obtained from fresh seeds sown in glass house were sprayed with suspension of *F. verticillioides* and *M. phaseolina*, and expressions of air borne symptoms of disease were observed. The symptoms caused by *F. verticillioides* and *M. phaseolina* were common, varying in number of seedlings exhibiting the symptoms which are, tip drying, necrotic lesions, chlorotic bands and yellowing of margins (Figure 4). Out of the thirty seedlings infected by *F. verticillioides*, nine (30%) seedlings showed yellowing of leaf margins, followed by six (20%) seedlings which showed longitudinal chlorotic bands. In case of *M. phaseolina*,

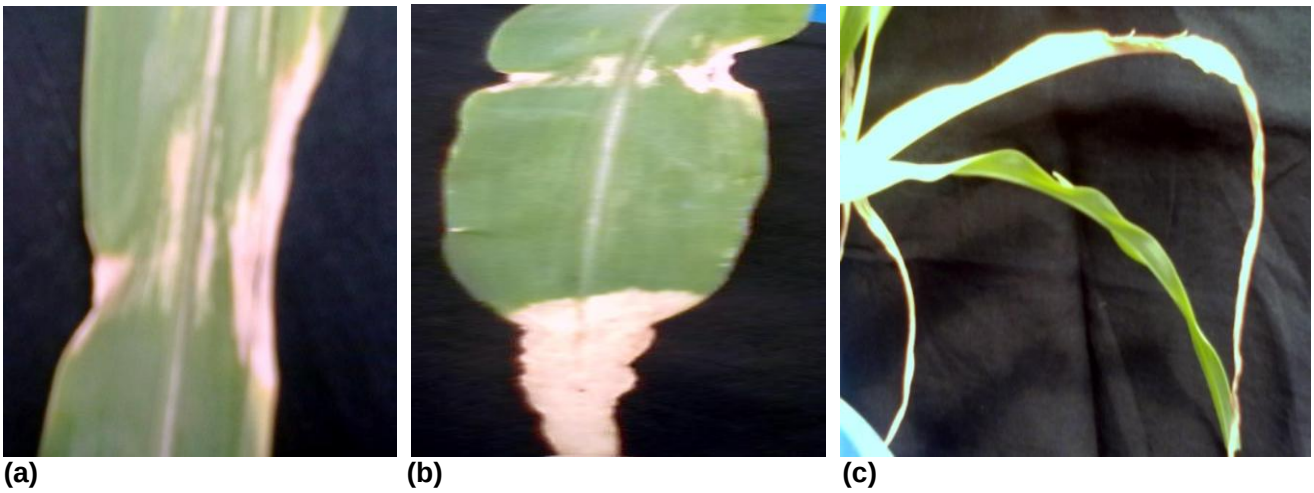



**Figure 4.** Symptoms observed in seedlings sprayed with spore suspension of *F. verticillioides* and *M. phaseolina* in experiment II. (a) Yellowing of leaf area; (b) tip drying; (c) necrosis.

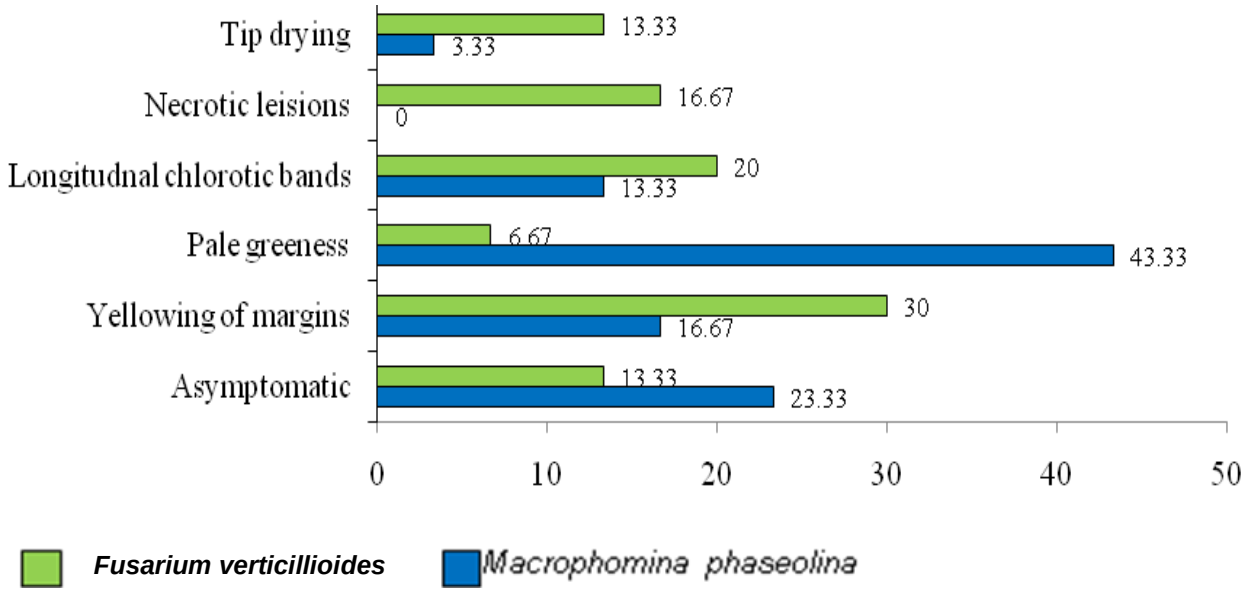



**Figure 5.** Symptoms of *F. verticillioides* and *M. phaseolina* on seedlings in experiment II.

16.67% seedlings exhibited yellowing of leaf margins and 13.33% showed longitudinal chlorotic bands. Necrotic lesions were observed in 16.67% (*F. verticillioides*) seedlings. Pale green leaves were the major symptom observed in *M. phaseolina* (43.33%) seedlings as compared to *F. verticillioides* (6.67%). In *F. verticillioides*, four (13.33%) and in *M. phaseolina* one (3.33%) seedling exhibited tip drying of leaves. It was observed that in four seedlings (*F. verticillioides*) and seven seedlings (*M. phaseolina*), there was no morphological disease symptoms resembling asymptomatic types, although similar concentration of suspension was used for all seedlings (Figure 5).

**Experiment III (biochemical analysis)**

Further biochemical assays in these seedlings was done to determine the content of total soluble phenols and sugars and relate it to observed symptoms. The total soluble phenols content estimated using FC reagent was 2.18 mg/g of fresh weight in the control seedlings. Anthrone reagent was used to estimate total soluble sugar content in mg/g of fresh weight and the control recorded 2.8364 mg/g of fresh weight. The phenol and total sugar contents obtained in the biochemical assay is presented in Table 2. All values were recorded on fresh weight basis. The values of total soluble sugars and total

**Table 2.** Total soluble phenols and sugar contents (mg/g of fresh weight) in seedlings.

| Total soluble sugars | *Fusarium verticillioides* | *Macrophomina phaseolina* | Total soluble phenols | *Fusarium verticillioides* | *Macrophomina phaseolina* |
|---|---|---|---|---|---|
| Symptomatic | 5.33[a] | 3.48[a] | Symptomatic | 9.39[a] | 4.68[a] |
| Asymptomatic | 4.28[b] | 2.90[b] | Asymptomatic | 5.24[b] | 2.19[b] |
| Control | 2.84[c] | 2.84[b] | Control | 2.18[c] | 2.18[b] |
| CD (%) | 0.29 | 0.01 | | 0.61 | 0.04 |

Values followed by different letters are significantly different in a column.

soluble phenols are significantly different from each other, in *F. verticillioides* infected seedlings for symptomatic, asymptomatic and control seedlings. In seedlings infected by *M. phaseolina*, the values of total soluble sugars and total soluble phenols are significantly different among symptomatic and asymptomatic ones. However, the values of asymptomatic and control seedlings are not significantly different.

## DISCUSSION

Stalk rot disease caused by *F. verticillioides* and *M. phaseolina* was studied at seed and seedling stages, and parameters associated with disease were marked, which led to a better understanding of host-pathogen interaction. This study aims to compare the symptom appearance at seed and seedlings stages inoculated by *F. verticillioides* and *M. phaseolina*. Both these pathogens are associated with stalk rot disease but both differ considerably in life cycle and environmental conditions required for their development. Though seeds were surface sterilized, there was an attack of pathogen in varied degree, ranging from high surface infection to low surface infection, and in some case no infection. Seed rotting, and ultimately no germination, was observed in few seeds in both *F. verticillioides* and *M. phaseolina* infections. However, the seed rotting percentage was higher in *F. verticillioides* (36.6%) as compared to *M. phaseolina* (10%) infection. There are various reasons for seed rotting; first one is kernel infection which occurs through silk channel where airborne spores of *F. verticillioides* are produced on corn residue, and can land on corn silks. Once *F. verticillioides* is in contact with kernels, it may enter them through the silk scars or through cracks and breaks in the seed coat. Secondly, kernels may also become infected through internal, systemic infection of the corn plant. Thirdly, due to shelling, seeds may have mechanical injury paving entry to pathogen spores in the developing kernels. These spores develop symptoms when the favourable conditions occur. In our study, maybe the presence of *F. verticillioides* spores inside kernel and heavy inoculums load provided externally led to seed rotting.

*M. phaseolina* is predominantly soil borne fungi. However, seed coat may also carry this fungus. Such seeds do not germinate or produce seedlings, and die soon after emergence. Pre-emergence mortality is directly related with the ability of the strain of *M. phaseolina* to infect the embryo (Burney et al., 1984; Dhingra and Sinclair, 1978; Francl et al., 1988). Strains of the fungus responsible for pre-emergence mortality can penetrate 5 to 10 μ intact thick membrane. This is also evident in our experiment, as *M. phaseolina* inoculated seed expressed higher percentage of germination and low seed rotting. This pathogen is effectively spread by sclerotia which can survive years together in the soil. Hence, the inoculation of seed with *M. phaseolina* in petriplates was less infectious, leading to better seed survival.

The second experiment included infection on seedlings by spore suspension spray of *F. verticillioides* and *M. phaseolina*. There were symptoms such as tip drying, necrotic lesions and yellowing, chlorotic bands and yellowing of margins in symptomatic seedlings. It is always difficult to discuss with certainty the role of *F. verticillioides* in the etiology of seedling diseases, as seedling infections and the disease development are produced by the seed affected by a disease or a contaminated soil, and depend on the temperature during the maize growing period (Komme-dahl and Windels, 1981). In the present study, *M. phaseolina* expressed less symptoms on seedlings, major symptom was pale green appearance of leaf (53.33%). *M. phaseolina* is a seed and soil borne pathogen with a wide distribution and a wide host range. The evidence suggests that it is primarily a root inhibiting fungus and produces tuber or cushion shaped 1 to 8 mm diameter black sclerotia. These sclerotia serve as a primary means of survival (Smith, 1969; Mirza, 1984; Kaisar and Das, 1988). Hence, root inoculation for disease screening at seedling stage is the best method in case of *M. phaseolina*.

As discussed earlier, *F. verticillioides* spores are widespread and move easily by air, resulting in increased seedling symptoms. Inoculation technique by spraying is effective in developing symptoms and screening for *F. verticillioides* infection at seedling stage unlike *M. phaseolina*, where spraying was not very effective. However, *M. phaseolina* appears to affect some seeds less severely than *F. verticillioides*, which suggests the existence of a potential genetic resistance to *M. phaseolina* in experimental line. This result was

confirmed by the biochemical study wherein the contents of total soluble sugars and total soluble phenols were estimated in seedling samples. Biochemical assays lay down an approach to investigate expression level of biochemical factors due to cell response that is, defence mechanism under stress conditions. Total phenolics and soluble sugar in the plants act as a biochemical markers to analyse disease severity which varies in host cell according to fungal attack. The phenols are a secondary metabolite secreted by plants in their defence mechanism against stress such as disease. In the present study, total soluble phenols (9.39 mg/g) and sugar content (5.33 mg/g) were high in symptomatic seedlings inoculated by *F. verticillioides* as compared to control (2.18 and 2.84 mg/g, respectively) indicating that total soluble phenols reach a level to inhibit the further advancement of fungal growth. This increase in phenolics is expressed as browning of leaf tissues, developing necrotic lesions. Soluble sugar content increased after pathogen treatment (about 21.50%). It has been proposed that plants switch off photosynthesis and other assimilatory metabolism to initiate respiration and other processes required for defence (Berger et al., 2007). The same trend was observed in *M. phaseolina* infection also.

Another significant result in our seedling inoculation experiment was the appearance of asymptomatic along with symptomatic ones. In *F. verticillioides* and *M. phaseolina*, the percentage of asymptomatic seedling observed was 13.3 and 23.3%, respectively. Fungus moves to above ground parts in early stages of host-pathogen interaction. Relatively small fungal biomass does not cause disease symptoms. The load of fungal inoculums influences growth enhancement and growth retardation in host. *F. verticillioides* are less aggressive and develops more slowly, giving the host more time to respond and restrict fungal growth by means of producing biochemical metabolites (Yates et al., 1997; Murillo et al., 1999). Fungus may be restricted to specific tissues within which it reproduces without damaging the surrounding cells. Similarly, in *M. phaseolina* infection, the biochemical estimation showed initiation of resistance without expression of symptoms, thereby confirming disease escape. The severity of this disease is directly related to the population of viable sclerotia in the soil. The activity of exudations from the sclerotia and utilization of soil nutrients also explain the pathogenic importance of a soil borne fungus (Filnow and Lockwood, 1983; Mazzola et al., 1996). This also showed that the method of suspension spray was ineffective to cause disease in *M. phaseolina*. However, the appearance of asymptomatic seedlings requires further study.

This study led down a pathway to select crop varieties more carefully, as results embarked no assurance of complete disease-free plants. Symptomless plants remained symptomless throughout the observation period; this indicates that the symptomless state persists beyond the seedling stage and could contribute, without visual signs, to the total mycotoxin contaminants of maize both before and during kernel development. To combat yield loss at maturity along with recommended cultural practices, breeding for resistant sources using conventional and molecular tools should be encouraged. The study also suggests the need to develop bioassays to estimate disease infection at early stage, so that economic and yield loss due to Stalk rot disease can be curtailed.

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