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Effects of Fungal Filtrates on Seed Germination and Leaf Anatomy of Maize Seedlings (*Zea mays* L., Poaceae)

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KEY WORDS: Anatomy, fungi, fungal filtrates, seed germination and *Zea mays*

ABSTRACT: This study was carried out to investigate the effects of 7-day-old fungal filtrates of *Aspergillus niger* and *Penicillium chrysogenum* isolated from maize seeds on percentage germination, morphological and anatomical structures of maize seedlings. The seeds were soaked in culture filtrate of each fungus for 12hrs before planting. Blotter method was used to observe seed germination. Results showed that the percentage germination of the seeds treated with culture filtrates of *A. niger* and *P. chrysogenum* (65.33% and 79.67% respectively) was lower than the control (100%) and significantly different from each other at significant level of P ≥0.05. The leaf area showed significant difference between the experimental and control plants but there was no significant difference in the leaf number. The tetracytic stomatal complex type and wavy anticlinal walls remained constant in all the treatments and control. The stomatal index of seed treated with *A. niger* on abaxial leaf surface (43.61%) showed significant difference with adaxial leaf surface (31.97%). The treatments had no significant difference on stomatal density at abaxial surfaces. Reduction in stomatal size and density suggests physiological implication.

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Introduction

Maize (*Zea mays*) is a cereal crop that is cultivated widely in a range of agro-ecological environment. It belongs to Poaceae (Graminae) Family along with wheat, millet and rice. Maize plant often attains 2.5m (8ft) in height and may grow up to 12m (40ft) in some natural strains. Corn seed is also called kernel and known botanically as caryopsis. The endosperm forms most of the volume and weight of the kernel.

Maize is one of the most important cereal crops in the world but its production does not meet the need. Shetty (1988) reported that fungi accounted for 75% of seed-borne pathogens which have been found to cause infectious diseases such as rot, discoloration, necrosis and blight. *Fusarium* spp. is reported to be the most important field fungi worldwide and produce over 100 secondary metabolites that are hazardous to the maize seed consumers (Owolade *et al.*, 2005). Maize seeds and seedlings are susceptible to soil and seed-borne diseases as many seeds may decay before or after germination. Also, affected plants may suffer from stunted growth, reduced ear size and even in severe condition, may die as a result of poor root system (Vincelli, 2008).

Fungi of the genera *Aspergillus, Fusarium, Penicillium* and *Rhizoctonia* are known to produce mycotoxins, toxic metabolites (Singh *et al*., 1991). These mycotoxins had been reported to degrade seed quality and reduce their viability (Caster and Frederiksen, 1980; Gopinath and Shetty, 1988). Culture filtrates of *Aspergillus* spp. caused reduction in seed germination and root-shoot elongation and the filtrate of *A. niger* was found to be inhibitorier (Jalander and Gachande, 2012). Seed-born diseases play a significant role in the quantity and quality of agricultural produce. Seed rot, seedling blight, *Bipolaris* leaf spot and *Cuvularia* leaf spot are etiologically caused by *Penicillium* spp. and *Fusarium oxysporium, Aspergillus* spp., *Bipolaris maydis* and *Curvularia lunata* respectively (Debnath, 2012). Symptoms expressions are affected by plant age, plant species and environmental factors.

The etiological agents of disease penetrate host plants by direct penetration using mechanical force or indirect penetration through wound and natural openings such as lenticels, hydathodes and stomata (Agrios, 2005). As alternative to direct penetration, fungi attack foliage and develop infectious structures that may consider stomata as their penetration route. The dysfunction of stomata affects host's

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on seedling physiological activities, include transpiration and respiration. The phyllosphere of terrestrial plants provides one of the most important niches for microbial inhabitation (Upper and Hirano, 1999; Lindow and Brandl, 2003). Meanwhile, plants rely on complex innate immune systems to make up for their lack of adaptive systems. They use complex signaling cascades that can detect and respond to bacterial and fungal pathogens both inside and outside their cells. One type of response is for the plant to close their stomata, the small openings on its leaves which deny the pathogen to gain entrance into the plant. Stomata play an important role in plant innate immunity by limiting pathogen entry into leaves (Gudesblat *et al*., 2009; Zeng *et al*., 2010).

The present study is to elucidate the effects of *Aspergillus niger* and *Penicillium chrysogenum* filtrates on seed germination and leaf anatomy of the maize seedling (*Zea mays*).

MATERIALS AND METHODS

Collection of seeds: The seeds of *Zea mays* (OBASUPER Variety) were procured from Kwara State Ministry of Agriculture, Ilorin. These were kept in a sterile polythene bag and moved to the Plant Biology Laboratory, University of Ilorin, for research analyses.

*Viability test of the seeds***:** The test was carried out using floatation method as described by Anoliefo (2006). Physical assessment of the seeds was done and cracked or broken seeds were discarded. The intact ones were put in a bowl containing water. The seeds with living embryo sank and were used for this research work while the floated ones were discarded.

Preparation of fungal filtrates **:** The pure cultures of *Aspergillus niger* and *Penicillium chrysogenum* were collected from Microbiology Department, University of Ilorin, Ilorin, Kwara State, Nigeria. Each fungus was grown in 250ml conical flask containing 100ml Potato Dextrose Broth and incubated at 25±2^{o}C for 14 days (Hajieghrari, 2010). After incubation, the culture filtrates were filtered into pre sterilized conical flasks using Whatman no. 1 filter paper. The filtrates were stored in a refrigerator at 4±2^{o}C (Jalander and Gachande, 2012).

*Determination of effects of fungal filtrates on seed germination***:** Two hundred (200) viable maize seeds were surface sterilized with 70% ethanol and then rinsed three consecutive times with sterile distilled water. Sixty (60) seeds were soaked in each fungal filtrate for 12hours. After the soaking period, the seeds were removed and rinsed with sterile distilled water separately. Twenty seeds were put in sterilized Petri dishes containing three layered wet blotter papers (Patil *et. al.,* 2012). Each treatment was done in three replicates and control experiment was treated and maintained with distilled water in the same number of replicates. After seven days of incubation, percentage of germination as well as plumule and radicle length of the seedlings were determined. The vigor index was calculated using the following mathematical representation as described by Khokhar *et al.* (2013):

Vigor Index (V.I) = {length of plumule (cm) + length of radicle (cm)} × percentage of germination

Determination of effects of fungal filtrates morphology: The soil used for the experiment was sieved to remove large particles, debris and stones. They were sterilized in autoclave at 121^{o}C for 1 hour as described by Devash *et al.* (1980). Four kilogram of sterilized soil was poured in each 7×7 pre-sterilized plastic pot. The treated seeds were planted in the pot (3 seeds per pot) and each treatment was done in three replicates (Hajieghrari, 2010). The pots were watered twice daily throughout the experimental period.

Isolation of leaf epidermal layers: Leaf segment of an area of 1 cm square from each specimen was cut and immersed in concentrated solution of nitric acid for maceration. The upper (adaxial) and lower (abaxial) layers were separated with dissecting needle and forceps. These samples were picked from acid and rinse with sterile distilled water (Alvin and Boulter, 1974).

Determination of Frequency of Stomatal Complex Types (SCT): Prepared slides of macerated cuticles from the leaves of the seedlings were observed. Different types of stomatal complex present in each specimen were also observed using 35 fields of view at x40 objectives as quadrats. The number of subsidiary cells per stoma was noted and recorded to determine the frequency of the different stomatal complex types present in each specimen. Frequency of each complex type was expressed as percentage occurrence of such complex type based on all occurrences (Obiremi and Oladele, 2001). The terminologies for naming *SCT* followed those of Dilcher (1974) and Metcalfe and Chalk (1988).

Determination of Stomatal Density (SD) and Stomatal Index (SI): The SD was determined as number of stomata per square millimeter (Stace, 1965). The SI was determined as follows: $SI = \frac{SD}{SD+E} \times 100$ .Where SD = numbers of stomatal per square millimeter E = number of ordinary epidermal cells per square millimeter

Determination of stomatal size (SS) and epidermal cell size (ECS): The mean stomatal size of the plant was determined as product of length and breadth of guard cells using eye piece micrometer. Epidermal cell size was determined as product of length and breadth of a cell based on a sample size of 35 (Abdulrahaman *et al*., 2013).

RESULTS AND DISCUSSION

The percentage of germination of maize seeds soaked in *A. niger* filtrate (65.33%) was significantly different from its counterpart seeds that soaked in *P. chrysogenum* filtrate (79.6%). The control experiment recorded 100% seed germination. The vigor indices of experimental plants were significantly different from each other at $p \leq 0.05$ as shown in Table 1.

The culture filtrates of *A. niger* and *P. chrysogenum* did not influence any significant difference in shoot length of the maize seedlings but had inhibitory impact on the leaf area when compared with the control (Table 2). The anatomical response of maize seedling to the fungal filtrates was noticeable as the experimental seedlings showed the same stomatal complex type (tetracytic) at both the abaxial and adaxial leaf surfaces. The culture filtrates of *A. niger* reduced the epidermal cell size of the seedlings to 325.64µm at adaxial surface and higher than the size value obtained from the seedling treated with *P. chrysogenum* filtrate (287.56µm) but both are significantly different from the value obtained from control (496.44µm) at adaxial surfaces of the seedlings as shown in Table 3. Both the stomata index and stomata density were affected by the fungal filtrates in this study. There was no clear alterations in the anticlinal cell wall patterns which were wavy in all the treatments and in the occurrence of tetracytic stomatal complex type on both leaf surfaces that is, an amphistomatic condition (Table 3; Figs. 1 – 6).

A. niger and *P. chrysogenum* were found to be seed-borne fungi. Debnath *et al*. (2012) reported that fungi associated with maize seeds were *A. niger, A. flavus, P. oxalicum and Rhizopus stolonifer*. Pathogenic fungi are known to produce mycotoxins. These mycotoxins are located in different parts like the seed coat, endosperm and/or cotylendon (Patil *et al*., 2012). The fungal filtrate of *A. niger* inhibited the seed germination to 65.33% and this result was supported by Ibraheem *et. al.* (1987) who observed that *A. niger, A. flavus* and *Alternaria alternata* had inhibitory power to reduce seed germination.

P. chrysogenum decreased percentage seed germination by 20.33%. Khokhar *et. al.* (2013) reported that the fungus had shown poisoning effect on the seedling of cereal as evidenced in inhibition of the seed germination percentage of wheat at higher percentage as 90%. The inhibition may be indication that the tested fungi produce toxic metabolites in the broth which they were cultured. *A. niger* and *P. chrysogenum* produced metabolites which are known to reduce germination and seedling development (Haikal, 2008). *A. niger* can produce mycotoxins such as oxalic acid crystals, kojic acid and malformins depending on the growth condition and the strain of the organism (TSCA, 2012).

Stomata are small pores located on the leaf surface that allow plants to exchange gases with the environment. They play an essential role in the intake of CO_2 for photosynthesis, moisture regulation and temperature control, but at the same time, they allow water loss by transpiration. Their position at the interface between internal plant tissues and the environment make them convenient gates for endophytic colonization by phytopathogens (Gudesblat *et al*., 2009). For this reason, plants have evolved the capacity to adjust stomatal apertures not only in response to hormones like abscisic acid (ABA) and to diverse environmental factors such as light, air humidity and carbon dioxide but also in response to pathogens. Past studies, conducted with fungal and bacterial pathogens that enter leaves through stomata, have shown that many of these organisms display tropic movements towards the stomata of the maize seedlings (Gudesblat *et al*., 2009).

The variations in other stomatal features namely density, index and size might be due to the response of the photosynthetic pathways to the presence of the pathogens. These features were in most cases higher or larger as the case may be on the abaxial surface (which is likely away from direct contact with the pathogens) than on the adaxial surfaces (which is likely in direct contact with the pathogens). In most cases, the adaxial surface is the first contact of the pathogens on the phyllosphere. Accordingly, plants regulate stomatal aperture in response to environmental conditions such as relative humidity, CO_2 concentration, and light intensity. Stomatal openings are also a major route of pathogen entry into the plant and plants have evolved mechanisms to regulate stomatal aperture as an immune response against bacterial invasion (Zeng *et al*., 2010; Lee *et al*., 1999; Mott *et al*., 1989; Turner *et al*., 1969).

Stomata are functional units of the epidermis responsible for the exchange of gases between the intercellular spaces of the plant and environment.

Stomata help in photosynthesis, moisture regulation and temperature control. The fungal cultures reduced both epidermal and stomatal sizes as well as stomatal density but the reduction was more pronounce in the seeds treated with *A. niger*.

Conclusion: The culture filtrates of both *A. niger* and *P. chrysogenum* affected not only percentage of seed germination but also the morphology and anatomy of maize seedlings. It adversely affected the epidermal cells and stomata. The impairment of the seedling anatomy features suggests physiological implications to the plants.

Table 1: Percentage of germination and vigor indices of maize seeds

	Seed germination (%)	Radicle length (cm)	Plumule length (cm)	Vigor index ×100
Control	100.00a	8.97a	11.47a	20.43a
Aspergillus niger	65.33b	5.33b	7.47c	8.36c
Penicillium chrysogenum	79.67c	6.80b	9.80b	13.22b
Mean	81.67	7.03	9.58	14.01
Standard Error	5.05	0.57	0.62	1.76
p-value	0.00	0.003	0.002	0.00

Mean with the same letter(s) down the column are not significantly different 0.05 α level

Table 2: Morphological response of maize seedling to fungal filtrates

	Shoot length (cm)	No of leaf (cm)	Length of leaf (cm)	Width of leaf (cm)	Area of leaf (cm^2)
Control	22.87a	7.75a	61.98a	3.43a	212.43a
Aspergillus niger	19.83a	6.75a	55.05b	2.53b	138.93b
Penicillium chrysogenum	21.85a	7.25a	58.13ab	2.97ab	173.41b
Mean	21.52	7.25	58.38	2.98	174.92
Standard Error	0.74	0.22	1.26	0.14	10.85
p-value	0.25	0.18	0.06	0.01	0.05

Mean with the same letter(s) down the column are not significantly different 0.05 α level

Table 3: Anatomical responses of maize seedlings to fungal filtrates

	Leaf surface	Stomatal complex type	Stomatal index (%)	Stomatal density (mm^{-2})	Stomatal size (µm)	Epidermal cell size (µm)
Control	Abaxial	Tetracytic	47.90a	14.20a	146.94a	486.17a
	Adaxial	Tetracytic	39.64ab	12.00ab	72.36c	496.44a
Aspergillus niger	Abaxial	Tetracytic	43.61ab	13.40a	97.49b	439.56ab
	Adaxial	Tetracytic	31.97c	10.20b	59.96c	325.64bc
Penicillium chrysogenum	Abaxial	Tetracytic	45.79ab	13.40a	103.33b	444.01ab
	Adaxial	Tetracytic	36.78bc	10.80b	69.68c	287.56c
Mean			40.95	12.33	91.62	413.23
Standard Error			0.39	1.47	5.81	20.68
p-value			0.006	0.005	0.003	0.00

Mean with the same letter(s) down the column are not significantly different at 0.05 α level

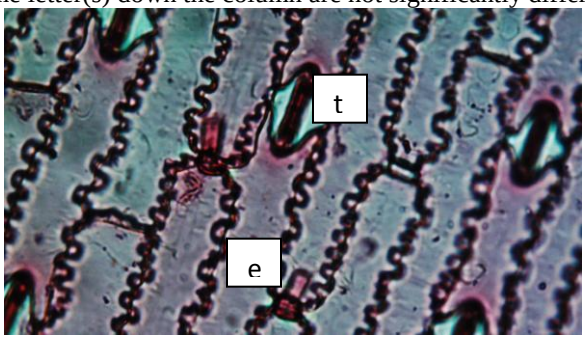

Fig. 1: Abaxial leaf epidermal surface of *Zea mays* (control) showing tetracytic stomata (t) and wavy epidermal cells (e)x400

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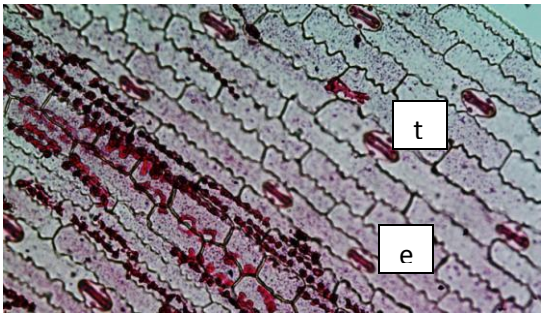

Fig. 2: Adaxial leaf epidermal surface of *Zea mays* (control) showing tetracytic stomata (t) and wavy epidermal cells (e)x400

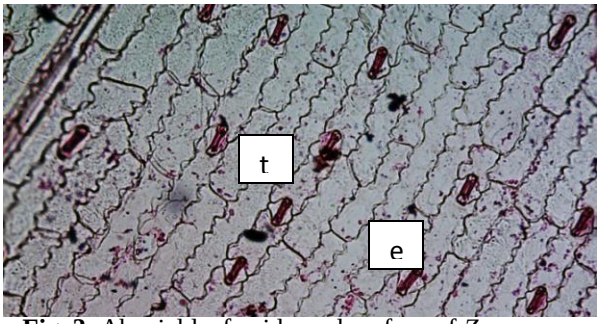

Fig. 3: Abaxial leaf epidermal surface of *Zea mays* (*Aspergilus niger*) showing tetracytic stomata (t) and wavy epidermal cells (e)x400

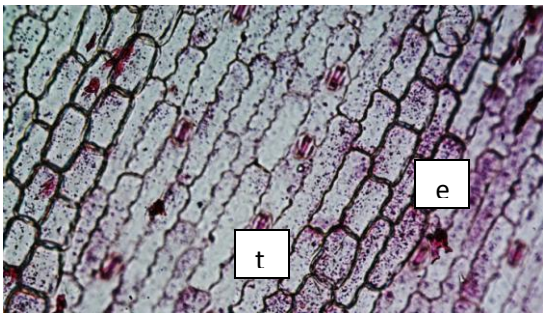

Fig. 4: Adaxial leaf epidermal surface of *Zea* (*Aspergilus niger*) showing tetracytic stomata (t) and wavy epidermal cells (e)x400

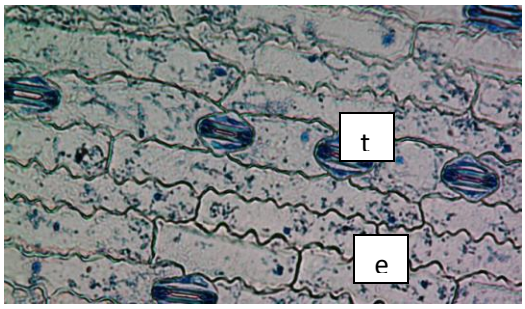

Fig. 5: Abaxial leaf epidermal surface of *Zea mays* (*Penicillium chrysogenum*) showing tetracytic stomata (t) and wavy epidermal cells (e)x400

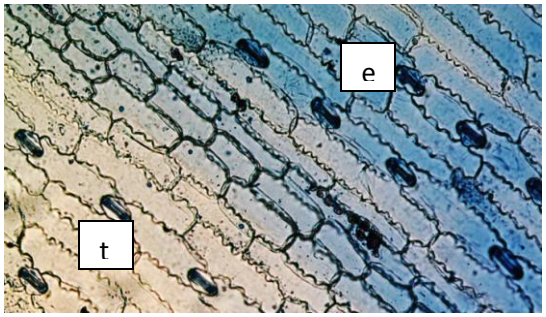

Fig. 6: Adaxial leaf epidermal surface of *Zea mays* (*Penicillium chrysogenum*) showing tetracytic stomata (t) and wavy epidermal cells (e) x400

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