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Ultrastructure of the Infection of *Sorghum bicolor* and *Zea mays* by *Pythium* species

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Key words: pathogenesis, light microscopy, electron microscopy, maize, sorghum, beans

Abstract (192 words)

The practice of cultivating mixed crops is common in Tropical Africa and elsewhere especially in areas of high population density. The practice of mixed cropping can promote disease spread especially in multi-host pathosystems. *Pythium* is a soil borne oomycete with a wide host range affecting both cereal and legumes. In this paper we examine pathogenesis by *Pythium* species in maize and sorghum, crops commonly included as intercrops in south western Uganda. In this study, both electron and light microscopy were used to study infection process using bean derived *Pythium* species- *Pythium ultimum* (MS 61) and *Pythium irregulare* (DFD 47) on beans, maize and sorghum. Electron microscopy revealed that on maize *P.irregulare* hyphae remained extracellular while *P. ultimum* hyphae in epidermis underwent necrosis after 9 days. In sorghum on the contrary, *P. ultimum and P. irregulare* extensively colonised both the epidermis and endodermis. In this study, *P. ultimum* also had two types of hyphae which mediated infection thus making it more virulent than *P. irregulare*. The results of this

study confirm that *Pythium spp.* are pathogenic on sorghum and therefore the role of sorghum in *Pythium* inoculum build-up in bean fields cannot be precluded.

Introduction

Pythium spp. are soil borne oomycetes of worldwide distribution. The ability of highly pathogenic *Pythium* species including *Pythium ultimum, Pythium aphanidermatum* and *Pythium irregulare* to induce plant wilting and root rotting has been abundantly documented (Blanchard *et al*.,1992).The colonisation of plant roots by *Pythium* species causes damping-off, severe necrosis and rots on the roots and stems of mature plants (Rey *et al.,* 2001). In general, *Pythium* infection of hosts causes visible symptoms on roots and shoots (Gichuru, 2008). However in some cases, *Pythium spp.* have been isolated from asymptomatic plants. Thus the occurrence of various *Pythium spp*. in necrotic as well as asymptomatic roots raises a question as to the extent to which such organisms are involved in disease epidemics (Hodges and Coleman, 1985). Elsewhere it has been shown that *Pythium* infections may compromise plant growth and yields without causing any visible symptoms on roots (Stanghellini and Rasmussen, 1994; Rey *et al.*, 1998). A study on the role of other crop species on *Pythium* induced root rots in south west Uganda was investigated. The study isolated *Pythium spp.* from both sorghum and maize. Sorghum plants had symptoms of stress, suggesting susceptibility whereas maize did not exhibit symptoms of susceptibility (Gichuru, 2008). Such a study will confirm the potential role of sorghum in the current *Pythium* root rot epidemics in south western Uganda as well as other agroecologies where mixed farming is common.

Materials and Methods

Experimental set up

Experiments to investigate infection of crops commonly found in bean-intercrops were studied using bean derived *Pythium* species (Mukalazi, 2004). The *Pythium* species were *Pythium ultimum* (MS 21) and *Pythium irregulare* (DFD 47).

Autoclaved millet (100g) was mixed with water (200 ml) and used to culture these *Pythium spp.* After two weeks, pre sterilised soil was mixed with infested millet in a ratio of 1:10 v/v in wooden trays of 42 cm x 72 cm. A non-inoculated control was included. The experiment was set up in a Completely Randomised Block Design (CRBD), with three replications.

Sorghum, maize, CAL 96 (susceptible bean variety) and AND 1062 (resistant bean variety) were planted in the wooden trays. The trays were maintained in a screen house. After germination, the seedlings were watered everyday to provide a favourable environment for the pathogen establishment and development. Every week until three weeks, the test crop species were harvested for light and electron microscopy. The experiment was repeated once.

Tissue processing for light microscopy

Samples from inoculated and non-inoculated crop species were collected 7, 8 and 9 days after germination of seed. This sampling strategy was used because in general, it marks

the earliest point of symptom development (Gichuru, 2008). The root samples were cut using a sharp blade and placed in 5 % acetic acid for 24 hours so as to clear and fix the tissue. The roots were subsequently stained in lactophenol-tryphan blue (25% w/v phenol crystals, 50% lactic acid, and 2.5 mg/ml tryphan blue) for 4 minutes, then mounted on a glass slide and fixed in 80% glycerol. The roots were viewed using a light microscope Laborlux D (Leitz Wetzlar, Germany). Pictures were taken with a digital camera at x 40 magnification.

Tissue processing for electron microscopy

Samples from inoculated and non-inoculated test species were similarly collected at 7, 8 and 9 days after germination of seed. Root samples were immediately fixed in 2.5 %(vol/vol) phosphate buffer-glutaraldehyde (0.1M, pH7.2) for 24 hours. All samples were post fixed for 1 hour in 1 % osmium tetroxide in water. The samples were dehydrated in graded series of ethanol i.e. 25%, 50%, 70%, 90% and three times in 100% for 20 minutes each time. Then the samples were dried at the critical point with carbon dioxide using a drier SAMDRI-780A (Rockville MD). Gold plating of the samples at 30 nanometers was done Hummer VII Sputtering System (Anatech, Alexandria, A).The samples were then observed in a Scanning electron microscope (Marca Jeol JSM-820 Cambridge Instruments).

Results and Discussion

Light microscopy of plants infected with *Pythium irregulare* revealed that in resistant bean variety (AND 1062), a steady process of infection starting with attachment to root

epidermis and eventual entry into the tissues occurred by 9 days after germination of seed (Plate 1). From scanning electron microscopy of AND 1062, 7 days after germination of seed, hyphae was observed to penetrate into the epidermis. Eight days after germination of seed however, few hyphae were observed to be growing intracelluarly in the epidermis. In addition, the epidermal cells appeared to be fortified. Electron microscopy confirmed light microscopy results revealing few oomycete hyphae in epidermis of root tissue by 9 days after germination of seed (Plate 1).Conversely, light microscopy of infected lesions on the resistant bean variety (AND 1062) infected with *Pythium ultimum* revealed similar results to those observed with *Pythium irregulare.* However scanning electron microscopy revealed more details (Plate 2). For instance, 7 days after germination of seed, hyphae were observed to be undergoing necrosis on the epidermal layer of the root tissue; 8 days after germination of seed, two types of hyphae were found attached on the external surface of the root tissue. One group of hyphae was short and thick while the second group was long and thin. The epidermal cells were also observed to be greatly enlargened. Finally 9 days after germination of seed, the thin oomycete hyphae had penetrated into the upper layer of the epidermis. The ultrastructural investigation of resistant bean variety (AND 1062) infection with

P. irregulare showed the epidermal cells were fortified. This could suggest that the crop is able to put up mechanical resistance therefore contributing to the resistance of AND 1062 to this pathogen. Also *P. ultimum* hyphae were necrotic on resistant bean variety (AND 1062). This necrosis could have been initiated by some biochemicals due to the crop's response to infection with the pathogen. In addition, *P. ultimum* infection on resistant bean variety (AND 1062) was mediated by two groups of hyphae: - one group

was short and thick and the other group was long and thin. The fact that *P. ultimum had* primary and secondary hyphae suggest that *P. ultimum* is more adapted than *P. irregulare* on that host. In other studies, *P. ultimum* was reportedly more virulent on spruce seedlings (*Picea abies* (L.) Karst) than *P. irregulare* .The difference in virulence could be due to the reaction of the latter towards the respective pathogen (Kozlowski and Metraux, 1997). In addition for the less virulent P. *irregulare,* lignification and subernisation of endodermis constitutes a barrier, since this pathogen can not grow through such cells (Kozlowski and Metraux, 1997).

Light microscopy of infected lesions on the susceptible bean variety (CAL 96) infected with *Pythium irregulare* revealed that, 7 days after germination of seed, hyphae made contact with the root surface and was penetrating the epidermis. Eight days after germination of seed, the hyphae were attached on the root surface and both the epidermal and endodermal tissue had stained blue (Plate 3). Scanning electron microscopy of infected lesions on susceptible bean variety (CAL 96) infected with *P. irregulare*, 7 days after germination of seed revealed appressorium bending towards the epidermis; 8 days after germination of seed, the hyphae had penetrated the endodermis but without appressoria (Plate 3). The ultrastructural investigation of susceptible bean variety (CAL 96), sorghum and maize infection with *P. irregulare* revealed the formation of appressoria on hyphae.This is a structure used for pathogen entry into plants. During infection, *Pythium spp.* are known to produce appressoria that penetrate the cuticle and epidermal cell walls mechanically (Adegbola and Hagedorn, 1969). Appressorium formation has been been observed in *Phomopsis helianthi* and *Asochyta pisi* (Heath and Wood, 1969; Mutanola, 1989). Different species of *Phoma* can infect plants with or

without appressorium formation. The formation of appressorium is known to depend on different factors such as epicuticular waxes, rigidity and surface hardness (Höhl *et al*., 1990).

Scanning electron micrographs of *Pythium irregulare* infection on susceptible bean variety (CAL 96) and sorghum showed extensive colonisation of epidermal tissue. This reaffirms the susceptibility reaction of susceptible bean variety (CAL 96) which is already known and confirms that sorghum is also susceptible to *Pythium* pathogens.

On the susceptible bean variety (CAL 96), light microscopy of *P. ultimum* induced lesions showed hyphae attached to the surface of the root tissue (Plate 4). Scanning electron microscopy, 7 days after germination of seed, revealed extensive colonisation of epidermal tissue; the hyphae were short and thick. Eight days after germination of seed, the thin sized hyphae were observed to penetrate the epidermal tissue (Plate 4).

On maize, light microscopy of *P. irregulare* induced lesions, 8 days after germination of seed showed enlargened epidermal cells which had stained blue (Plate 5). No dye was taken up by the endodermis. This suggests that the *P. irregulare* did not penetrate beyond the epidermis. Electron microscopy revealed 8 days after germination of seed; the *P. irregulare* hyphae were growing intracellularly in the epidermis and moving towards the endodermis. Hyphae were observed to be long and to bear an appressorium. Nine days after germination of seed, that the hyphae were observed to be shorter and some of them had a paddle-like structure.

Light microscopy of *P. ultimum* induced lesions in maize, 8 days after germination of seed indicated that the hyphae had attached to the root tissue (Plate 6). However with Scanning electron microscopy, the hyphae were attached to root surface and were also

found in the epidermis and endodermis. Nine days after germination of seed, the hyphae in the epidermis were observed to undergo necrosis. The Scanning electron micrograph of *Pythium ultimum* infection of maize showed necrosis of hyphae in the epidermis. This suggests that although maize is infected by *Pythium* (symptoms of infection were observed in studies by Gichuru, 2008); the *Pythium* pathogen did not survive therefore was not able to spread in the tissue.

On sorghum, light microscopy of *P. irregulare* induced lesions showed 8 days after germination of seed, hyphae were attached to the external surface of the root and was penetrating into the epidermis (Plate 7). Scanning electron microscopy, 7 days after germination of seed, the oomycete hyphae were observed to attach on the outer surface of the root tissue and had began to penetrate the epidermis. Eight days after germination of seed, there was presence of numerous hyphae bearing appressoria on the root surface. Nine days after germination of seed, the hyphae were observed to form an intertwining network within the epidermis (Plate 7).

On sorghum, light microscopy of *P. ultimum* induced lesions showed 8 days after germination of seed, the hyphae were attached to the root tissue (Plate 8). Scanning electron microscopy, 7 days after germination of seed the oomycete hyphae were observed to be intercellular; 8 days after germination of seed, the hyphae were intracellular and 9 days after germination of seed, the hyphae were distributed on the external surface of root tissue as well as in the endodermis and epidermis (Plate 8).

Taken together, this study therefore demonstrates that *Pythium spp.* pathogenesis is influenced by host genotype and physical attributes. In sorghum, pathogen growth was found to be extensive and similar to growth and spread in the susceptible bean variety

(CAL 96). In maize, whereas the pathogen successfully attached itself to the root surface, the hyphae experienced some biochemical changes which led to necrosis of the hyphae and prevented further spread of the pathogen. Similar observations were made in the resistant bean variety (AND 1062). This study therefore confirms that sorghum is an alternative host of *Pythium* species while maize is not. Given that both sorghum and beans are commonly included in bean intercrops, the use of sorghum will promote *Pythium* root rot epidemics whereas maize will reduce *Pythium* inoculum load.

Acknowledgments

This study was funded by DFID-CSL through a grant awarded to NARO-CIAT-Africa. We thank the farmers in Kabale district for enabling sample collection from their farms. Technical assistance at National Agricultural Laboratories Institute (NALI) Kawanda and CIAT- Colombia is acknowledged.

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Figures

Plate 1: The panels marked A-D are pictures of light micrographs of resistant bean variety (AND 1062) tissue inoculated with *Pythium irregulare.* A: non-inoculated sample. B: tissue collected 7 days after germination of seed; stained blue indicating presence of pathogen hyphae.C: tissue taken 8 days after germination of seed; similarly staining blue and hyphae observed to be attached to the root surface. D: tissue collected 9 days after germination of seed; epidermis stained blue indicating colonisation of the epidermis.

The panels marked E-H are Scanning electron micrographs of resistant bean variety (AND 1062) tissue inoculated with *P.irregulare*. E: a non-inoculated control. F: infected root tissue taken 7 days after germination of seed showing appressorium on hyphae as indicated by the arrow. G: infected root sample taken 8 days after germination of seed showing hyphae growing intracellularly. H: infected tissue taken 9 days after germination of seed showing hyphae restricted in growth within the bean tissue.

Plate 2: The panels marked A-D are pictures of light micrographs of resistant bean variety (AND 1062) tissue inoculated with *Pythium ultimum.* A: non-inoculated sample. B: tissue collected 7 days after germination of seed; hyphae were attached on root surface. C: tissue collected 8 days after germination of seed; epidermal surface stained blue indicating colonisation of surface. D: tissue collected 9 days after germination of seed; epidermis stained blue indicating colonisation of the epidermis.

The panels marked E-H are Scanning electron micrographs of resistant bean variety (AND 1062) tissue inoculated with *P.ultimum*. E: a non-inoculated control. F: infected root tissue taken 7 days after germination of seed showing hyphae undergoing necrosis in epidermal tissue. G: infected root sample taken 8 days after germination of seed showing two types of hyphae: - short and wide, long and thin hyphae attached on the root surface. H: infected tissue taken 9 days after germination of seed showing thin hyphae penetrating the epidermis.

Plate 3: The panels marked A-D are pictures of light micrographs of susceptible bean variety (CAL 96) tissue inoculated with *Pythium irregulare.* A: non-inoculated sample. B: tissue collected 7 days after germination of seed hyphae attached on root surface. C: tissue collected 8 days after germination of seed; only a small area of the endodermis did not stain blue indicating that it was not colonised by oomycete. D: tissue collected 9 days after germination of seed; epidermis and endodermis stained blue indicating colonisation by oomycete.

The panels marked E-H are Scanning electron micrographs of susceptible bean variety (CAL 96) tissue inoculated with *P. irregulare.* E: a non-inoculated control. F: infected root tissue taken 7 days after germination of seed showing hyphae attached on root surface. The arrows indicate the hyphae. G: infected root

sample taken 8 days after germination of seed showing hyphae without appressorium in the endodermis. H: infected tissue taken 9 days after germination of seed showing hyphae in epidermis and endodermis.

Plate 4: The panels marked A-D are pictures of light micrographs of susceptible bean variety (CAL 96) tissue inoculated with *Pythium ultimum*. A: non-inoculated sample. B: tissue collected 7 days after germination of seed, stained blue indicating colonisation by oomycete. C: tissue collected 8 days after germination of seed; hyphae attached on root surface. D: tissue collected 9 days after germination of seed, epidermal tissue was stained blue indicating colonisation by oomycete.

The panels marked E-H are Scanning electron micrographs of susceptible bean variety (CAL 96) tissue inoculated with *P. ultimum.* E: a non-inoculated control. F: infected root tissue taken 7 days after germination of seed showing extensive colonisation of epidermal tissue by thin oomycete hyphae. G: infected root sample taken 8 days after germination of seed showing thin hyphae penetrating the epidermis. H: infected tissue taken 9 days after germination of seed indicating short, thick hyphae attached on the root surface.

Plate 5: The panels marked A-D are pictures of light micrographs of maizetissue inoculated with *Pythium irregulare*. A: non-inoculated sample. B: tissue collected 7 days after germination of seed, epidermis stained blue indicating colonisation of epidermis by oomycete. C: tissue collected 8 days after germination of seed showed epidermal cells stained blue and enlargened. D: tissue collected 9 days after germination of seed showed epidermis stained blue indicating colonisation by oomycete.

The panels marked E-H are Scanning electron micrographs of maize tissue inoculated with *P. irregulare*. E: a non-inoculated control. F: infected tissue taken 7 days after germination of seed showed hyphae growing intracellularly. The arrows in the diagram indicate hyphae. G: infected root sample taken 8 days after germination of seed showing hyphae which is long and bearing appressoria (bulbous-like structures). H: infected tissue taken 9 days after germination of seed showed shorter hyphae bearing paddle-like structures.

Plate 6: The panels marked A-D are pictures of light micrographs of maize tissue inoculated with *Pythium ultimum*. A: non-inoculated sample. B: tissue collected 7 days after germination of seed showed hyphae attached to the root surface. C: tissue collected 8 days after germination of seed showed epidermal cells stained blue indicating presence of oomycete. D: tissue collected 9 days after germination of seed showed epidermal cells stained blue.

The panels marked E-H are Scanning electron micrographs of maize tissue inoculated with *P. ultimum*. E: a non-inoculated control. F: infected tissue taken 7 days after germination of seed showed hyphae on root surface. The arrows

indicate the hyphae. G: infected root sample taken 8 days after germination of seed showing few hyphae on root surface. H: infected tissue taken 9 days after germination of seed showed hyphae undergoing necrosis.

Plate 7: The panels marked A-D are pictures of light micrographs of sorghum tissue inoculated with *Pythium irregulare*. A: non-inoculated sample. B: tissue collected 7 days after germination of seed showed epidermis stained blue indicating colonisation by hyphae. C: tissue collected 8 days after germination of seed showed hyphae attached to root surface and penetrating epidermis. D: tissue collected 9 days after germination of seed showed epidermis and endodermis stained blue indicating colonisation by hyphae.

The panels marked E-H are Scanning electron micrographs of sorghum tissue inoculated with *P. irregulare*. E: a non-inoculated control. F: infected tissue taken 7 days after germination of seed showed hypha bearing appressoria and the hyphae were penetrating the epidermis. The arrows indicate the hyphae. G: infected root sample taken 8 days after germination of seed showing numerous hyphae on root surface. H: infected root tissue taken 9 days after germination of seed showed hyphae forming an extensive network in the epidermis.

Plate 8: The panels marked A-D are pictures of light micrographs of sorghum tissue inoculated with *Pythium ultimum*. A: non-inoculated sample. B: tissue collected 7 days after germination of seed, epidermis stained blue indicating colonisation by hyphae. C: tissue collected 8 days after germination of seed hyphae was attached to root surface. D: tissue collected 9 days after germination of seed, epidermis stained blue indicating colonisation by hyphae.

The panels marked E-H are Scanning electron micrographs of sorghum tissue inoculated with *P. ultimum.* E: a non-inoculated control. F: infected tissue taken 7 days after germination of seed showed hyphae growing intercellularly.The arrows are pointing to hyphae. G: infected root sample taken 8 days after germination of seed showed that hyphae were intracellular. H: infected tissue taken 9 days after germination of seed had numerous hyphae on root surface.