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Full Length Research Paper

Bioassay of *Geniculosporium* species for *Phytophthora megakarya* biological control on cacao pod husk pieces

P. R. TONDJE[1]*, K. P. HEBBAR[2], G. SAMUELS[4], J. H. BOWERS[3], S. WEISE[5], E. NYEMB[1], D. BEGOUDE[1], J. FOKO[+] and D. FONTEM[6]

[1]Regional Biological control and Applied Microbiology Laboratory, Institute of Agricultural Research for Development (IRAD), P.O. Box 2123 Yaoundé, Cameroon.

[2]MARS Inc./US Deparment of Agriculture, Agricultural Research Service, Plant Science Institute, Alternate Crops Systems Lab, Beltsville, MD 20705, USA.

[3]US. Deparment of Agriculture (USDA), Agricultural Research Service (ARS), Plant Science Institute (PSI), Alternate Crops Systems Lab. Beltsville, MD,20705 USA.

[4]US. Deparment of Agriculture (USDA), Agricultural Research Service (ARS), Plant Science Institute (PSI), Systematic and Mycology Lab. Beltsville, MD,20705 USA.

[5]International Institute of Tropical Agriculture (IITA), HFS- P.O.Box 2008 Yaounde, Cameroon.

[6]University of Dschang, Faculty of Agronomy and Agricultural Science, P.O.Box 222, Dschang - Cameroon.

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Fungal endophytes (*Geniculosporium* sp.) isolated from cacao leaves were screened for biological control of *Phytophthora megakarya* the cacao black pod disease pathogen, using Cacao Pod Husk Pieces (CPHP). CPHP were pre-treated with spore suspensions of *Geniculosporium* sp. [BC13 (GJS 01-196), BC108 (GJS 01-192), BC118 (GJS 01-197), BC177 (GJS 01-198)], and were infected with *P. megakarya* zoospore suspensions (10^5 zoospores/ml) . Effects on *P. megakarya* were noticed at pod infection, mycelia growth inside infected tissues, and fungal sporulation, that are major stages of the black pod disease cycle on cacao pods. CPHP pre-treated with BC108 expressed the lowest early Infection Index (EII), but could not control disease progress into infected cacao pod husk tissues. On CPHP pre- treated with BC13, average growth rate of the necrosis was significantly reduced, but no control on *P. megakarya* sporulation was observed, while CPHP pre-treated with BC177 significantly reduced *P. megakarya* sporulation.

Key words: Cacao, biological control, *Geniculosporium sp,* endophytes, *Phytophthora megakarya,* pod husks pieces.

INTRODUCTION

Phytophthora megakarya the causative agent of cacao black pod disease is present only in Central and West African cocoa producing countries, and is the most damaging of the cocoa diseases in this region. Annual losses due to this disease range from 30 to 90% of total production (Dakwa, 1987; Djiekpor et al., 1981). To combat this disease, copper and metalaxyl-based fungicides are applied (Matthews et al., 2003). Apart from being very expensive for many African farmers, heavy reliance on such chemicals can be associated with non-target effects (WHO, 1986; Mwanti and Kimani, 1993), loss of biodiversity and spoilage of land and water and may also lead to the development of resistance by the pathogen (Davidse et al., 1981; Deahl et al., 1992; Fontem et al., 2005; Giller et al., 1998; He et al., 2005). As an alternative to chemical fungicides application, agronomic measures such as the removal of diseased pods and shade management have not proven effective enough (Tondje et al., 1993; Ndoumbe-Nkeng et al., 2004). Moreover, a cacao variety resistant to black pod disease caused by *P. megakarya* is yet to be made

*Corresponding author: Email: prtondje@yahoo.com.

available. In cacao, potential biological control agents for the control of the most commonly known causative agent of cacao black pod disease, *Phytophthora palmivora*, have been reported (Figueiro and Medeiros, 1977; Gallindo, 1992; Odamtten and Clerk, 1984; Odigie and Ikotun, 1982). However, these results were not applied in natural conditions. Yet, lack of consistency was reported as one draw back of the biological control (Chalutz and Droby, 1997).

The microorganisms in the phyllosphere are frequently affected by many factors: low nutrient availability, extreme temperatures, drought, and intense radiation. However, the endophytes which live in an environment protected against sudden weather changes and radiations are more stable (Burrage, 1971). Endophytes are microorganisms that colonize the tissues of living plants and grow within healthy tissues without inducing disease symptoms in the plant (Carroll, 1998, Arnold et al. 2003). Several endophytes have been reported to support growth and improve the health of plants (Hallmann, et al. 1997; Kirchhof et al. 2001; Nowak, 1998; Sharma and Novak, 1998; Stoltzfus et al. 1998). Endophytes may be important sources of biological control agents in cacao (Arnold et al., 2003, Rubini et al., 2005). *Geniculosporium* species are commonly found as endophytes of tree species (Petrini and Petrini, 1985). *Geniculosporium* is the anamorph (asexual) form of several species of *Hypoxylon* (Ascomycetes, Xylariales); it is not a name previously found in the biological control literature. However, *Hypoxlon* species are common in natural forests as well as agricultural settings in all temperate and tropical regions (Ju and Rogers, 1996). *Geniculosporium* species produce dry conidia profusely in agar culture, thus providing an easy means to mass produce inoculum for biological control.

The initial step of screening for microbes with potential for disease control is the most critical in biological control of plant pathogens (Fravel, 1999). The validity of simple agar plates screening tests in selecting for antagonists is questionable; the difference between the ability of an antagonist under laboratory and field situations is frequently experienced in biological control research (Tronsmo, 1992; Huber and Watson, 1966).

The aims of this study were to evaluate (i) the antagonistic potential of the endophytic, *Geniculosporium* spp. [BC13 (GJS 01-196), BC108 (GJS 01-192), BC118 (GJS 01-197), BC177 (GJS 01-198)] against *P. megakarya*, and (ii) the use of cacao pod husk pieces for screening microbial candidates for *P. megakarya* biological control.

MATERIAL AND METHODS

Isolation of the fungal endophytes from cacao leaves

Twenty seven healthy cacao leaves from a farmer's field at Nkometou lll (Cameroon) were randomly collected and kept individually in polyethylene plastic bags. Collected samples were stored in a freeze container at 4°C temperature before processing in the laboratory two hours later (Bills, 1996). From each leaf, 20 healthy leaf disks (5 mm diameter) were carefully cut using a sterile cork-borer. These leaf disks were then surface- sterilized with sequential washes in tap water, 95% ethanol (30 s), 10% (v/v) commercial sodium hypochlorite (10 min), 75% ethanol (2 min), and sterile distilled water (3 rinses) (Arnold, 1999). The sterile leaf disks were then serially placed on water agar plates and incubated in the laboratory at room temperature. Emerging hyphae were plated on Potato Dextrose Agar (PDA) medium (per litre: 200 g pealed and sliced potatoes, 20 g dextrose, 15 g agar) and purified.

Several fungi were isolated from symptomless cacao leaves from a farmer's field at Nkometoull (Cameroon). Among these, *Geniculosporium* isolates (GJS 01-192), BC118 (GJS 01-197) and BC177 (GJS 01-198) expressed some antagonism to *P. megakarya* in preliminary experiments (Mbenoun, 2001).

Preparation of cacao pod husk pieces (CPHP)

About 18 to 24 healthy pieces (4 cm x 4 cm x 0.5 cm) of cacao pod husk were carefully cut from different mature, green, healthy cacao pod of the (*Theobroma cacao* L) cacao variety (SNK10) that was reported to be very sensitive to black pod disease in Cameroon (Blaha and Lotodé, 1976). To minimize pod origin and pod age effects (Turner, 1965) three randomly selected pieces of cacao pod husks were placed on moist filter paper in each Petri dish (90 mm diameter). Sterile distilled water was added when necessary to keep the filter paper continuously moist.

Biological control candidates and the pathogen

The endophytic *Geniculosporium* isolates were stored in sterile distilled water (Jones et al., 1991) on small plugs of potato dextrose agar (PDA). Conidia from 10-day-old PDA agar cultures were aseptically harvested and suspended in sterile distilled water at a concentration of $1x10^6$ sporangia ml^{-1}, using a MALASSEZ hemacytometer (SOVIREL, France).

P. megakarya strain NKOM3-00 was isolated from a naturally infected cacao pod in a farmer's field at Nkometou III and stored in sterile distilled water on small pieces of V8 agar (200 g V8 juice, 3 g $CaCO_3$, 15 g agar and 1000 ml distilled water). Zoospores were obtained from sporangia of artificially infected cacao pods maintained in humid plastic boxes at 28°C (room temperature) for 10 days. Sporangia were scraped from the surface of the infected cacao pod and placed in sterile distilled water. The suspension of sporangia was placed in a refrigerator at 4°C for 3 min, and immediately transferred to room temperature for 15 min. The concentration of zoospores was adjusted to $1x10^5$ zoospores ml^{-1}, using the hemacytometer.

Pretreatment of cacao pod husk pieces (CPHP)

For each of the five biological control candidate to be tested, six CPHP, distributed in two Petri dishes (90 mm diameter), were pre-treated by spreading a 50 µl drop of conidial suspension of the biological control candidate at a concentration of $1x10^6$ conidia ml^{-1} onto the upper surface of the CPHP to facilitate the pre-colonization process. For the control treatments, 50 µl of sterile distilled water was spread on the upper surface of the CPHP. Plates were kept open for about 5 h at room temperature to speed up the drying process of the upper surface of the CPHP. Closed plates were then incubated at room temperature (~28°C) for 4 days. During incubation the filter paper was kept moist inside the Petri dishes using sterile distilled water to allow for the germination

Table 1. Early Infection Index (EEI), Production of Sporangia (PS) /cm^2 and Radial Growth of the necrosis on pre-treated cacao pod husk pieces with some selected biocontrol candidates of *P.megakarya*.

Treatment	$^{\alpha}$Early Infection Index (EII) (%)	$^{\beta}$Production of Sporangia (PS)	$^{\delta}$Radial progress of the necrosis
Control	100	5.201a	30.167a
BC13	46.15	5.000ab	12.717d
BC181	51.28	4.934b	22.983c
BC118	73.07	4.646b	29.700a
BC108	39.74	4.739b	26.000b
BC177	47.43	3.985c	22.250c
	F	7.619***	43.18***
	P	0.005	0.001
	CV	3.92	9.97

$^{\alpha}$values are expressed in % relative to control.
$^{\beta}$Expressed as $\log_{10}$[(PS)/cm^2] 10 days after inoculation with *P. megakarya* .
$^{\delta}$Expressed as *P. megakarya* necrosis (mm/24 h) during the first 4 days after a successful infection by *P. megakarya*, on pre-treated CPHP with *Geniculosporium* spp. [BC13 (GJS 01-196), BC108 (GJS 01-192), BC118 (GJS 01-197), BC177 (GJS 01-198)].
Values in a column followed by the same letters are not significantly different.

and growth on CPHP surfaces of sporangia of the biocontrol candidates.

Inoculation of pre-treated cacao pod husk pieces

A 15-µl drop of zoospore suspension of *P. megakarya* at a concentration of 1x10^5 zoospores ml^{-1} was inoculated to the center of the completely dried surface of each CPHP. First observations were made two days after inoculation, and then every day for two weeks.

Early Infectivity of *P. megakarya* on pre-treated CPHP

An index for early infectivity (EII) on CPHP was calculated using the following formula adapted from Soria and Esquivel (1970)

$EII = 6X_1 + 4X_2 + 2X_3 + X_4$

In this formula, X_i (where *i* =1, 2, 3, and 4), for each treatment (EII) represents the sum of CPHP on which *P. megakarya* infection was observed from the point of inoculation on the 1st, 2nd, 3rd, and 4th day of observation.

Radial growth of the necrosis (RGN)

Radial growth of the necrosis on the CPHP was measured each 24 h after successful infection; for the first 4 days after inoculation with *P. megakarya*. The CPHP were incubated for one more week for observations on sporangia production potentials on pre-treated CPHP.

Production of sporangia

Sporangia were harvested 4 days after inoculation with *P. megakarya* by scraping a 1 cm2 area of the surface of each infected CPHP with a glass slide and flushed with 1 ml of distilled water into a 1.5 ml polypropylene micro tube. The suspension was mixed with a vortex mixer for 15 s, and sporangia were counted in a hemacytometer. Data was recorded as production of sporangia (PS) per square centimeter, PS/cm^2.

Data analysis

All experiments were performed twice. A completely randomized design was used in the experiments. The results for the two experiments were pooled and the homogeneity of variances confirmed by Bartlett's test. Results of radial growth of the necrosis and production of sporangia were analyzed by ANOVA followed by the Student, Newman, Keuls test to differentiate treatments using SAS (Statistical Analysis System, SAS Institute, Cary, NC).

RESULTS

All the strains of *Geniculosporium* [BC13 (GJS 01-196), BC108 (GJS 01-192), BC118 (GJS 01-197), and BC177 (GJS 01-198)] tested expressed some degree of antagonism to *P. megakarya.* The three parameters used were: Early infection Index (EII), radial growth rate of the necrosis (RGN) and production of sporangia (PS) (Table 1)

Early infection index (EII) of pre-treated CPHP

In this study, of all the strains screened, the lowest EII of pre-treated CPHP was observed for CPHP treated with strain BC108 (Table 1). We did not observe any strain of the *Geniculosporium* spp. screened so far that could completely prevent cacao pod husks pieces from being infected by *P. megakarya.*

Radial growth rate of the necrosis (RGN)

Pre-treated CPHP with strain BC13 showed a particular capacity to slow the growth of the necrosis (12.717 mm/24 h) relative to control (30.167 mm/24 h), after a successful infection by *P. megakarya.* Significant differences (*P*<0.001) were observed between isolates of

biocontrol candidates, but none were able to completely inhibit the radial growth of the necrosis caused by *P. megakarya* on cacao pods (Table1).

Production of sporangia (PS) of *P. megakarya* on CPHP

Significant differences were found among biological control candidates on pre-treated CPHP in regard to the production of sporangia by *P. megakarya*. Isolate BC13 was able to reduce the radial growth of the necrosis but showed no capacity to inhibit the production of sporangia 10 days after a successful infection by *P. megakarya*. However, an interesting reduction by BC177 on the number of sporangia produced on CPHP by *P. megakarya* was observed (Table1).

DISCUSSION

In this study, differences were sought according to disease assessment parameters for black pod disease incidence on pre-treated CPHP with microbial biological control candidates. Relative to control, the lowest early Infection Index (EII) was observed for pre-treated CPHP with BC108 (39.74%). However, the average production of sporangia was high (Table 1). This result reveal more specific responses of these endophytic biological control candidates on particular points of *P. megakarya* cycle on cacao pods: pod infection, mycelia growth inside infected tissues, and fungal sporulation on infected tissues.

One major difference between *P. palmivora* and *P. megakarya,* the most damaging species on cacao, is that the production, maturation, and liberation of sporangia are grouped in a short period of time for *P. palmivora*, and in an extended period for *P. megakarya* (Blaha, 1984). On a single cacao pod, zoospores can be released from sporangia for over 30 days when infected by *P. megakarya* (Depreaux et al., 1987). Based on this basic difference between the two species, we strongly believe that an effective screening strategy for biocontrol candidates of *P. megakarya* needs to lay emphasis on endophytic strains that could control the production and maturation of sporangia on cacao pods since sporangia and, in turn, zoospores are the major propagules for the dissemination of this fungal disease. The cacao pod is the most important site of the infection cycle of cacao black pod disease in cacao farms. Green cacao pod husk pieces prove to be usable for bio-tests in laboratory conditions. This material presents the advantage of making possible the screening procedure for endophytic biological control candidates of *P. megakarya* at infection of cacao pods, mycelia growth inside infected tissues, and fungal sporulation on infected cacao pods tissues, under laboratory conditions. However, as cacao pods are not available throughout the year, the possibility of using alternative organs as cacao leaves, needs to be investigated more, and could help for speeding the screening process for endophytic biological control candidates.

T. cacao is known to host many endophytic fungi, but work done to date has emphasized ecological aspects of the phenomenon of endophytism (Arnold and Herre, 2003) while suggesting economic potential (Rubini et al., 2005). In the present work we demonstrate biological control potential for cacao black pod disease with *Geniculosporium* species isolated from healthy leaves of cacao in Cameroon. The next steps will focus on on-farm evaluation tests.

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