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Comparing Oomycete species detection methods from soils using Oak (*Quercus* species) and Rhododendron (*Rhododendron* species) leaves as baits

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Abstract

Isolation of oomycete species from soils of cocoa fields, using young oak (Quercus species) and (Rododendron species) leaves as baits where carried out in a previous diversity study by the authour. In the study, isolation of oomycetes was done by baiting with young oak and rhododendron leaves and plating on PARPH-V8 selective medium. The identification of oomycete species was based on PCR analysis of the internal transcribed spacer region of rDNA and confirmed by morphological characteristics. According to the previous study, the oomycetes isolated and identified were Phytopythium vexans, Pythium cucurbitacearum, P. deliense, P. acanthicum, P. oligandrum and P. acanthophoron. Comparing the respective percentage isolation on each of the two baits in this present study showed that the use of more than one bait is advantageous when samples sources are multi-locational, with different environmental conditions and also when many species are the target of isolation. The result from this study generally showed that the percentages of isolation of the oomycetes were highest on oak leaves in the Humid Forest ecology while it was highest on rhododendron leaves in the Moist Derived Savanna Ecology; the only exception to this was Phytopythium vexans, whose percentages of isolation was location specific rather than the ecology. The use of the two baits was thus useful as a preliminary study to identify the most suitable bait for each of the identified species, for each respective location and ecology, for further studies.

Keywords: Oomycete species, cocoa, oak, rhododendron, Nigeria, PCR

Introduction

Because oomycete species like *Pythium* and *Phytophthora* can be difficult to isolate from diseased plants, plant pathologists often use baits, to detect them. Different baits vary in their susceptibility to the various oomycete species, and no single bait can detect all species (Swiecki and Bernhardt, 2015). Among the baits used, oak and rhododendron are readily available and are susceptible to many common oomycete species, and are relatively easy to interpret. The United Kingdom Plant Health Inspectors use Rhododendron and Pieris

leaves as baits in rainfall traps for the detection of some airborne oomycete species (Wedgwood *et al*., 2014).

Phytophthora spp. fungus-like organisms are reported among the most dangerous pathogens that worsen the health of rhododendrons in nurseries (Orlikowski and Szkuta, 2003).

Oomycete pathogens causes important diseases of environmentally and economically important tree species such as oaks, tanoaks, rhododendron, European beech, Japanese larch and many woody ornamental plants (Grünwald *et al*., 2008; Brasier and Webber, 2010; Grünwald *et al*., 2012). Disease symptoms are host specific, but can vary from necrotic lesions in leaves, to shoot dieback and bleeding cankers on the stem (Rizzo *et al*., 2005; Hansen *et al*., 2005). For effective control measures to be put in place, it is essential that rapid and reliable detection systems are available. Traditionally, standard detection methods have employed baiting, culture plating, or a combination of both (Pittis and Colhoun, 1984). These techniques take several days, require taxonomic expertise, and are often too slow to assist growers in disease management decisions. Technological advances in antibody-based assays have enabled rapid detection of *Pythium* and *Phytophthora* species in plant tissues (Rittenburg et al., 1988; Harrison et al., 1990; Lyons & White, 1992; Beckman et al., 1994); in soil (Miller et al., 1992; White et al., 1996; Miller et al., 1997); and in water samples (Ali-Shtayeh et al., 1991; Cahill & Hardham, 1994; Themann & Werres, 1996; Wakeham et al., 1997). Test formats vary, and employ either enzyme-linked immunosorbent assay (ELISA), membrane trapping assay, or a dipstick format. A number of these tests are commercially available as kits for on-site use (e.g. Adgen Ltd, Auchincruive, Ayr, KA6 5HW, UK), and enable growers to obtain results within 10 min of sample collection. However, most of these tests are limited by their inability to differentiate between live and dead propagules of target pathogens. According to Kowalik (2008), infected Rhododendrons plants develop discolouration, brown spots and necroses, affecting their aesthetic value. The symptoms of infection, observable from spring to autumn, increase when the plants are in bloom resulting in dieback and leaf drop. The damage is caused by fungi-like Oomycetes of genera *Pythium* and *Phytophthora* and fungi of genera: *Botrytis, Cercospora, Colletotrichum, Cylindrocladium, Exobasidium, Microsphaera, Ovulinia, Pestalotia, Phomopsis, Phyllosticta, Pycnostysanus, Ramularia, Rhizoctonia* and *Septoria* (Farr *et al*., 1996; Werner, 1998; Werres, 2000; Orlikowski, 1999; Labanowski *et al*. 2001; Kita, 2003; Kowalik *et al*. 2006; Kowalik, 2007). Recent works also confirm occurrence of symptoms of discolouration, leaf spot and necroses resulting in dieback and leaf fall in rhododendron (Kowalik *et al*., 2010a; Kowalik *et al*., 2012). Fungi and fungus-like organisms as being the agents of rhododendron leaf infection have also been recently confirmed (Kowalik, 2009; Kowalik *et al*., 2011; Kowalik *et al*., 2010b).

Research carried out to compare different diagnostic technique for *Phytophthora* and *Pythium* species in water samples, Pettitt *et al*. (2002) showed the higher the zoospore concentration in water the greater proportion of rhododendron leaf discs baits became infested; with 6000 zoospores/L giving 100% infestation. Aigbe and Woodward (2018), recently for the first time, isolated and identified species of *Pythium* and *Phytopythium* in soils from cocoa fields, using oak and rhododendron leaves as baits. Three of these species that have been reported previously as damping-off and rot pathogens of many economically important crops in other countries. Aigbe and Woodward (2018), also recently and for the

first time, isolated and identified another 3 species of *Pythium* that have been reported elsewhere to be mycoparasites of important crop pathogens, including *Phytophthora* spp. *Phytophthora megakarya* and *P. palmivora* are the predominant oomycete pathogens causing black pod of cocoa (*Theobroma cacao* L.) in Nigeria, resulting in yield losses of 10-80% (Zentmeyer, 1987). There is currently paucity of information concerning oomycete species diversity and their impact on Nigerian economic crops. In bid to reducing this paucity of information gap, there is need therefore to identify the most suitable bait for the rapid isolation, detection of oomycete species in Nigerian crop ecologies for the much needed study and management of these species. The purpose of this study therefore is to compare oomycete species detection methods from soils using oak and rhododendron leaves for baiting.

Materials and methods

The isolates compared in this study were obtained in soils samples collected in an oomycete survey experiment of cocoa fields in Southern Nigeria (Aigbe and Woodward, 2018). In the experiment, soil samples were collected from soils of cocoa fields in the major cocoa growing states in Southern Nigeria. Eighteen different Local Governments Areas, from 8 states (Cross Rivers, Abia state, Edo, Ondo, Ekiti, Osun, Oyo and Ogun states) were visited for soil samples collection, in Southern Nigeria. The fungus was isolated by means of baiting methods with young oak and *Rhododendron* leaves following incubation on PARPH-V8 selective medium. Genomic DNA of isolate was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The internal transcribed spacer (ITS) regions of rDNA were amplified using the primers ITS6 and ITS4 and the amplified PCR product was purified using a QIAquick PCR purification Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The PCR products were sequenced by 'Source BioScience' company, Scotland and analysed with the CLC Main Workbench software. DNA sequences were blasted at NCBI database using BlastN to identify the sequences. The molecular results were confirmed by morphological studies of the isolates by comparison with previous descriptions by other workers (Middleton,1943; Waterhouse, 1968; Van der Plaats-Niterink, 1981; Dick, 1990). Several isolates of the fungus were grown on Five percent CV8A, Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) for morphological studies.

Results and discussion

Soil Sample collection from Nigerian cocoa farms

The soil samples were collected from black pod infested cocoa fields in eight different states: Cross Rivers, Abia state, Edo, Ondo, Ekiti, Osun, Oyo and Ogun states consisting of 18 different Local Governments Areas of Southern Nigeria, where cocoa is predominantly grown (Aigbe and Woodward, 2018). Based on the study, the identifications of the 721 isolates of *Pythium* and *Phytopythium* were based on morphological characteristics and result of a BLAST search with ITS 4 sequences of isolates of *Pythium* and *Phytopythium* species from GenBank. The species identified include *Phytopythium vexans*, *Pythium cucurbitacearum*, *P. deliense*, *P. acanthicum*, *P. oligandrum* and *P. acanthophoron*. Their occurrence and distribution in Southern Nigeria are summarized in Tables 1 and the sampling sites are represented in Figure 1.

The percentage of isolation of *Pythium cucurbitacearum* was higher from oak in Cross Rivers Local Government areas (LGA); with the exception of Boki that was slightly higher for rhododendron. In Abia,

Edo and Ogun states, oak leaves similarly produced the highest percentages of isolation for *P. cucurbitacearum*; again, with the exception of Ogbere LGA in Ogun State. In contrast to this, rhododendron had the highest percentages of isolation for *P. cucurbitacearum* in Ondo, Osun and Oyo staes, in addition to Ogbere in Ogun state; interestingly, all these locations are in the Moist Derived Savanna. The locations were the percentages of isolation for *P. cucurbitacearum* were generally higher on oak were in the Humid forest (Table 1).

Table 1. Percentage Isolation of oomycetes from soils of cocoa fields in Nigeria on young Oak and Rhododendron leaves.

States	Local Government Areas (LGA)	Percentage Isolation Oak leaves	Rhododendron leaves	Agro ecology	Preference
Pythium cucurbitacearum					
Cross Rivers	Etung	21.8	4.4	Humid Forest	Oak
Cross Rivers	Ikom	23.3	6.7	Humid Forest	Oak
Cross Rivers	Boki	20.0	22.9	Humid Forest	Rhododendron
Abia state	Bende	84.0	56.7	Humid Forest	Oak
Edo	Uhommora	24.0	0.0	Moist Derived Savanna	Oak
Ondo	Idanre	10.0	16.7	Moist Derived Savanna	Rhododendron
Osun	Oriade	13.3	16.7	Moist Derived Savanna	Rhododendron
Oyo	Akinyele	3.3	10.0	Moist Derived Savanna	Rhododendron
Ogun	Ijebu Ode	16.7	0.0	Humid Forest	Oak
Ogun	Ogbere	0.0	25.0	Humid Forest	Rhododendron
Pythium acanthicum					
Cross Rivers	Etung	1.8	2.2	Humid Forest	Rhododendron
Edo	Owan East	25.0	60.0	Moist Derived Savanna	Rhododendron
Edo	Uhommora	13.3	10	Moist Derived Savanna	Oak
Edo	Esan West	13.4	30.0	Moist Derived Savanna	Rhododendron
Ondo	Owo	40.0	15.0	Moist Derived Savanna	Oak
Ekiti	Ikere	16.6	10.0	Moist Derived Savanna	Oak
Oyo	Akinyele	46.6	73.4	Moist Derived Savanna	Rhododendron
Oyo	Ibarapa	83.2	20.0	Moist Derived Savanna	Oak
Ogun	Ijebu Ode	56.6	3.4	Humid Forest	Oak
Ogun	Ogbere	35.0	5.0	Humid Forest	Oak
Pythium oligandrum					
Cross Rivers	Etung	1.8	2.2	Humid Forest	Rhododendron
Edo	Owan East	25.0	60.0	Moist Derived Savanna	Rhododendron
Edo	Uhommora	13.3	10	Moist Derived Savanna	Oak
Edo	Esan West	13.4	30.0	Moist Derived Savanna	Rhododendron
Ondo	Owo	40.0	15.0	Moist Derived Savanna	Oak
Ekiti	Ikere	16.6	10.0	Moist Derived Savanna	Oak
Oyo	Akinyele	46.6	73.4	Moist Derived Savanna	Rhododendron
Oyo	Ibarapa	83.2	20.0	Moist Derived Savanna	Oak
Ogun	Ijebu Ode	56.6	3.4	Humid Forest	Oak

(Table 1. Continued)

Ogun	Ogbere	35.0	5.0	Humid Forest	Oak
Pythium deliense					
Abia state	Bende	40	20	Humid Forest	Oak
Oyo	Ibarapa	20.0	0.0	Moist Derived Savanna	Oak
Phytopythium vexans					
Cross Rivers	Etung	12.7	2.2	Humid Forest	Oak
Abia state	Bende	0.0	16.7	Humid Forest	Rhododendron
Edo	Uhommora	24.0	0.0	Moist Derived Savanna	Oak
Ondo	Idanre	26.6	43.4	Moist Derived Savanna	Rhododendron
Ekiti	Ekiti South West	6.6	10.0	Moist Derived Savanna	Rhododendron
Osun	Oriade	26.6	10.0	Moist Derived Savanna	Oak
Ogun	Ijebu Ode	0.0	10.0	Humid Forest	Rhododendron
Pythium acanthophoron					
Ogun	Ijebu Ode	16.6	3.3	Humid Forest	Oak

For *Pythium acanthicum, P. oligandrum,* and *P. acanthophoron*, the percentages of isolations were generally higher on oak in the Humid Forest locations and on rhododendron in the Moist Derived Savanna, especially when inoculum load is high. There where however exceptions to this in Owo LGA of Ondo state in the Moist Derived Savanna, where the percentages of isolation was higher on oak and in Ibarapa LGA of Oyo state, still in the Moist Derived Savanna and where percentages of isolation was also highest on oak (Table 1). In a similar trend to *P. cucurbitacearum, Pythium acanthicum, P. oligandrum,* and *P. acanthophoron,* the percentage of isolation for *Pythium deliense* was highest on oak leaves, in the Humid Forest of Bende LGA of Abia State. This however contrasted to Ibarapa LGA of Oyo state, in the Moist Derived Savanna, where it was isolated only from oak leaves (Table1). For *Phytopythium vexans*, the percentages of isolation did not follow the previously observed trend for *P. cucurbitacearum, Pythium acanthicum, P. oligandrum* and *Pythium deliense*; bait preference was location specific, rather than ecology (Table 1).

Results from this study shows that *P. cucurbitacearum*, *Pythium acanthicum, P. oligandrum,* and *P. acanthophoron* generally have the highest percentages of isolation on oak leaves in the Humid forest and on rhododendron in the Moist Derived Savanna. It was only *Phytopythium vexans* that did not follow this trend. The results from this study therefore confirmed the report by Swiecki and Bernhardt (2015), that different baits vary in their susceptibility to the various oomycete species, and that no single bait can detect all species. When general isolation of oomycetes is to be carried out, it is therefore recommended that more than one bait should be used for the preliminary isolation study to identify preferred bait for each species in each ecology and location. The preferred bait can thereafter ward be used for further and more detail studies for the respective species.

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