Journal of Microbiology and Antimicrobials Vol. 5(3), pp. 25-28, March 2013
Available online http://www.academicjournals.org/JMA
DOI:10.5897/JMA12.003
ISSN 2141-2308 ©2013 Academic Journals

Full Length Research Paper

Ridding *Phytophthora palmivora* cultures of contamination by *Colletotrichum gloeosporioides*

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Accepted 31 January, 2013

0, 75, 100, 125 and 150 ppm a.i. concentrations of benomyl in Green Cocoa Mucilage Agar (GCMA) plates were evaluated for suppression of *Colletotrichum gloeosporioides* in *Phytophthora palmivora* plates contaminated by *C. gloeosporioides.* To do this, GCMA plates amended with the above concentrations of benomyl were seeded with mycelia plugs from a contaminated *P. palmivora* culture and radial growth rates of the resulting *P. palmivora* colonies were recorded. Mycelial bits from the centres, middle and peripheries of such colonies were examined at 7 days interval for *P. palmivora* and the contaminating *C. gloeosporioides*. Growth rates of pure *C. gloeosporioides* cultures were also studied on benomyl plates. Growth rates of 7.15, 3.10, 2.30, 2.05 and 1.60 mm/day were obtained for *P. palmivora* on plates, respectively, containing 0, 75, 100, 125 and 150 ppm benomyl. Growth rates of 2.95, 1.65, 1.60, 1.60 and 1.55 mm/day were respectively, obtained for the pure cultures of *C. gloeosporioides* indicating that on contaminated plates, growth of *C. gloeosporioides* will lag behind that of *P. palmivora.* On plates with 0 to 125 ppm benomyl, *P. palmivora* was detected at the centres, middle and peripheries of all colonies within the first week but *C. gloeosporioides* was restricted to the centres and middle portions of such colonies due to its slower growth rate. When subcultures were made at day 7 from the peripheries of colonies on 0 to 125 ppm benomyl plates onto benomyl-free plates, *C. gloesporioides* was not detected from the resulting cultures which were clean.

Key words: *Collectotrichum gloeosporioides*, contamination, selective culturing, *Phytophthora palmivora*.

INTRODUCTION

The causal agents of black pod disease of cocoa in Ghana are *Phytophthora palmivora*, (Brassier and Griffin, 1979) and *Phytophthora megakarya* (Dakwa, 1987). The disease is a major constraint to cocoa production and causes severe economic losses worldwide. Van der Vossen (1999) reported that black pod disease causes about 44% of crop loss globally. Total yield losses in Ghana due to *P. palmivora* and *P. megakarya* are 4.9 to 19 % (Dakwa, 1984) and 60 to 100% (Dakwa, 1987), respectively.

Contamination of *P. palmivora* cultures by other fungi, notably *Colletotrichum gloeosporioides* is problematic. *C. glooesporioides* is a cosmopolitan saprophyte which invades tissues (Uchida and Kodaaka, 1997) and has been reported on diseased cocoa pods (Lass, 1985). Akrofi (personal communication, 2005) at the Cocoa Research Institute of Ghana (CRIG), observed that *C. gloeosporioides* was involved in severe chellere and pod rot disease of cocoa in Ghana. Thus, in culturing *P. palmivora,* the possibility of contamination by *C. gloeosporioides* is high. Such contaminated cultures are unsuitable for pathological and mycological studies on *P. palmivora.* Means of ridding such cultures of the contaminating *C. gloeosporioides* based on the use of a selective medium which selects for *P. palmivora* must be developed.

Selective isolation of *Phytophthora* spp. by incurporating chemicals into the culture medium is well known.

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Table 1. Radial growth rate of *P. palmivora* and *C. gloeosporioides* on GCMA plates at different concentrations of benomyl[1].

Benomyl (ppm)	Radial growth rate (mm/day)	
	P. palmivora	***C. gloeosporioides***
0	7.15	2.95
75	3.60	1.65
100	2.30	1.60
125	2.05	1.60
150	1.60	1.55
Cv (%)	2.77	0.46
LSD (0.05)	0.16	0.01

[1]The *P. palmivora* culture was contaminated by *C. gloeosporioides.*

Ritchie (2002) indicated that benomyl can be used in a medium to culture fungi in the Phylum *Oomycota*, which includes *Phytophthora palmivora* (Alexopolous et al., 1996). Benomyl (50 % WP), a benzimidazole fungicide, is reported to suppress growth of a large number of fungi including many *Deuteromycetes* (Agrios, 1997) to which *C. gloeosporioides* belongs (Alexopolous et al., 1996). One possible means we thought could be effective in cleaning *P. palmivora* cultures contaminated by *C. gloeosporioides* was by incorporating benomyl in the culture medium to selectively inhibit *C. gloeosporioides*. There is, however, no report on the concentration of benomyl that will effectively inhibit *C. gloeosporioides* while enabling growth of *P. palmivora*.

The main objective of the present study was to find means of cleaning *P. palmivora* cultures contaminated by *C. gloeosporioides*. Specific objectives, therefore, were to: 1. Determine the effect of different concentrations of benomyl on the growth of *C. gloeosporioides* and *P. palmivora* and 2. Determine the threshold period for subculturing from different portions of a contaminated culture of *P. palmivora.*

MATERIALS AND METHODS

Preparation of selective medium for *P. palmivora*

Green Cocoa Mucilage Agar (GCMA) was prepared from the mucilage of cocoa beans according to the method of Awuah and Frimpong (2002). Fresh beans (250 g) from matured green cocoa pods was stirred vigorously with an electric stirrer for 10 min in 500 ml sterile distilled water (SDW) and the suspension was filtered through a double layer cheesecloth. Sodium hydroxide solution (0.1 M) was added to the filtrate (10% v/v) and agar added at 2% (w/v). The mixture was dispensed into 200 ml Erlmeryer flask (100 ml per flask). Benomyl (benlate 50% WP) was added to the content of the flasks to establish concentrations of 75, 100, 125 and 150 ppm a.i. (corresponding to 150, 200, 250 and 300 mg, respectively, of the benomyl powder). The media were autoclaved, dispensed into 9 cm diameter Petri dishes and used after 3 days. Medium without benomyl served as control.

Assay of media for selectivity against *C. gloeosporioides*

A 2-week-old culture of *P. palmivora* contaminated by *C. gloeosporioides* and growing on Oat Meal Agar (OMA) was the source of the two fungi. The original culture was obtained from the Cocoa Research Institute of Ghana (CRIG), Akim Tafo and named in the present study as CRIG-2. Subcultures of CRIG-2 (also contaminated by *C. gloeosporioides*) were maintained on GCMA before use for an experiment. Three replicate plates containing a particular concentration of benomyl were centrally seeded with 7 mm diameter mycelial plugs of CRIG-2 (one plug, top down, per plate) and incubated in the dark at 27 ± 2°C. Diameters of the resulting contaminated *P. palmivora* colonies were measured at 24 h interval for 5 days along two perpendicular planes and the mean diameter for each plate was calculated. From these, the average radial growth rate per day was determined. Additionally, mycelia bits were taken at 7, 14 and 21 days after incubation from the centre (0 to 15 mm), middle (15 to 30 mm) and periphery (outer 2 mm) of the contaminated *P. palmivora* cultures and were examined with a microscope for the presence or absence of *P. palmivora* and *C. gloeosporioides.* Because *C. gloeosporioides* was not usually detected at the peripheries of colonies, mycelia bits from the peripheries of all plates were plated on benomyl-free GCMA plates, grown in the dark and examined weekly (for 3 wks) as before to confirm absence of *C. gloeosporioides*. A set-up using a pure culture of *C. gloeosporioides* was maintained and radial growth rate of the fungus was determined as with the *Phytophthora* colonies.

Data analysis

Radial growth rate data were analysed with the analysis of variance (ANOVA) to assess the effect of benomyl concentrations on the growth of *P. palmivora* and *C. gloeosporioides*. These were compared with the least significant different (LSD) test at 5% probability level.

RESULTS

Radial growth rates of *P. palmivora* cultures (in contaminated states) and those of pure cultures of *C. gloeosporioides* on plates amended with different concentrations of benomyl are presented in Table 1. When no benomyl was added to the medium, the highest radial growth rate of 7.15 mm/day was recorded for *P. palmivora* and this was significantly different ($P< 0.05$) from values obtained when benomyl was incorporated into the medium. Growth rates of *P. palmivora* were significantly reduced with increasing concentrations of benomyl with the lowest value (1.60 mm/day) being recorded when the fungicide was used at 150 ppm. With *C. gloeosporioides*, the highest growth rate of 2.95 mm/day was obtained when the medium was devoid of benomyl. In general, increasing concentrations of benomyl significantly reduced the growth rate of the fungus and the lowest growth rate of 1.55 mm/day was recorded on medium with 150 ppm benomyl. This value was similar to the growth rate of *P. palmivora* at the same concentration of benomyl (Table 1).

At 7 days, *C. gloeosporioides* was present at the centre and the middle but generally not at the peripheries of colonies growing on plates containing 0 to 125 ppm benomyl (Table 2). By 14 days, the fungus had advanced

Table 2. Presence (+) or absence (-) of *P. palmivora* and *C. gloeosporioides* at different locations on colonies grown on media with different concentrations of benomyl[1].

Benomyl (ppm)[2]	Presence (+) or absence (-) of *Phytophthora* and *Colletotrichum*[2]																	
	7 day						14 day						21 day					
	P. palmivora			*C. gloeosporioides*			*P. palmivora*			*C. gloeosporioides*			*P. palmivora*			*C. gloeosporioides*		
	Cen	Med	Peri	Cen	Med	Peri	Cen	Med	Peri	Cen	Med	Peri	Cen	Med	Peri	Cen	Med	Peri
0	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
75	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+
100	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-
125	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+
150	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[1]The colonies were established from a *P. palmivora* culture contaminated by *C. gloeosporioides*. [2]Cen = centre, Mid = middle, Peri = periphery of colonies.

Table 3. Detection of *P. palmivora* and *C. gloeosporioides* on cultures established from peripheral transfers from mixed cultures of *P. palmivora* and *C. gloeosporioides*[1].

Benomyl (ppm)[2]	7 day		14 day		21 day	
	P. palmivora	*C. gloeosporioides*	*P. palmivora*	*C. gloeosporioides*	*P. palmivora*	*C. gloeosporioides*
0	+	-	+	-	+	-
75	+	-	+	-	+	-
100	+	-	+	-	+	-
125	+	-	+	-	+	-
150	+	+	+	+	+	+

[1] + = Present, - = absent. [2]Benomyl concentrations of the original GCMA plates from which subcultures were made.

to the periphery of colonies on the benomyl-free plates but was absent from the periphery of colonies on plates containing 75 to 125 ppm benomyl. It was also present at the peripheries of colonies on plates containing 150 ppm benomyl and was constantly detected at that location as the colonies grew. In general, *C. gloeosporioides* was detected at the peripheries of all plates by 21 days (Table 2). On the other hand, *P. palmivora* was detected at the centre, middle and peripheries of colonies grown on all plates, irrespective of the concentration of benomyl used and colony age (Table 2).

When sub-culturing was done at day 7 from the peripheries of colonies on 0 to 125 ppm benomyl plates onto benomyl-free media, *C. gloeosporioides* could not be detected on any of the resulting cultures even after 3 weeks. It was, however, present on cultures originating from colony peripheries on plates containing 150 ppm benomyl (Table 3).

DISCUSSION

In the present study, it was thought that the principle of selective isolation (Tsao, 1987) could be used to clean up *P. palmivora* cultures contaminated by *C. gloeosporioides*. Selective isolation involves prevention or slowing the growth of the undesirable organism (in this case *C. gloeosporioides*) so as to promote growth of the desirable one (*P. palmivora* in the present study) and permit the desirable fungus to be isolated and cultured in a pure state. Because selective isolation is usually achieved by incorporating fungicides into the culture medium, it was assumed that use of the fungicide benomyl would completely suppress *C. gloeosporioides* on the culture medium, allowing *P. pal-*

mivora to grow and be isolated in a pure state. This was thought to be so because benomyl is known to be effective against *Deuteromycetes* (Agrios, 1997) to which *C. gloeosporioides* belongs (Alexopolous et al., 1996). Use of benomyl in the present study did not completely inhibit the *C. gloeosporioides* at the rate used (0 to 125 ppm). It rather slowed down the growth of the fungus relative to that of *P. palmivora*. The isolation of *P. palmivora* in a pure form from culture peripheries achieved in the current study, was therefore, not due to complete inhibition of *C. gloeosporioides* but rather due to the inherently faster growth rate of *P. palmivora* on the medium even though benomyl also slowed down *P. palmivora*. This enabled *P. palmivora* to grow out of the contaminated zone, permitting its isolation in a pure form when transfers were made from colony peripheries. Sub-culturing from the centre and middle of colonies did not result in pure cultures of *P. palmivora*, indicating that these areas were already contaminated by *C. gloeosporioides*. Thus, this procedure can be used to obtain pure cultures of *P. palmivora* contaminated by any fungus with a slower growth rate.

In this study, we have demonstrated that pure cultures of *P. palmivora* can be obtained even from culture plates devoid of benomyl. The basis of this is again the differential growth rate of the two fungi indicated above. However, on plates devoid of benomyl, sub-culturing from the colony peripheries ideally should be within 7 days when *C. gloeosporioides* was restricted to the centre and middle portions of colonies. To prolong the period of sub-culturing from colony peripheries, amending the medium with benomyl at a 75 to 125 ppm a.i. to further slow down *C. gloeosporioides* is recommended. At these benomyl concentrations, the growth of *C. gloeosporioides* is highly reduced, further delaying its growth into the *P. palmivora* portion of the colony. In all cases, however, the interval of sub-culturing from colony peripheries should not exceed 14 days. If plates are kept for 3 weeks before sub-culturing, the possibility that *C. gloeosporioides* would have completely contaminated plates would be high and obtaining pure cultures of *P. palmivora,* even from peripheries of such cultures, would be problematic. This explains why in the past it has been impossible to obtain pure cultures of *P. palmivora* from any portion of over-aged cultures.

At 150 ppm benomyl concentration, we were unable to get a pure culture of *P. palmivora* even from the peripheries of mixed cultures because at this concentration, the growth rates of both *P. palmivora* and *C. gloeosporioides* were not significantly different (1.60 mm/day for *P. palmivora* and 1.55 mm/day for *C. gloeosporioides*). Thus, contrary to Ritchie (2002), benomyl cannot be used under all conditions to selectively isolate fungi in the phylum *Oomycota.*

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