Formulation of dehydrated media from the bean mucilage and placenta of mature unripe cocoa pods for culturing *Phytophthora palmivora* (Butl.) Butl.

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ABSTRACT

Because of difficulty in obtaining cocoa pods during the off-cocoa growing season to prepare media from scratch, formulation of dehydrated media from the placenta and seed mucilage of green mature cocoa pods was attempted for use in culturing *Phytophthora palmivora*. Pieces of mucilage and placenta were oven-dried (70 °C, 6 h), milled and the resulting flakes sieved to obtain a fine powder. Four powder concentrations 0, 1, 2 and 3 per cent in water (w/v) were prepared, and agar and calcium carbonate added at 2 per cent (w/v) and 2.5 per cent (w/w), respectively, to each powder concentration. The media were autoclaved and evaluated in Petri plates for clarity and for capacity to support growth and sporulation of *Phytophthora palmivora*. Powder yield was higher for the placenta than for the mucilage-based media. The source of powder, powder concentration, and their interaction all had significant effects on radial mycelial growth of *P. palmivora*. At the same powder concentration, media prepared from the placenta supported significantly higher ($P \leq 0.05$) radial growth of the test fungus (5.59 – 7.10 mm/day) than media prepared from the mucilage powder (4.54 – 6.18 mm/day). Aerial mycelial growth improved slightly as powder concentration increased but media clarity decreased. Sporulation of *P. palmivora* was good on all the media. Powder source, powder concentration, and method of media preparation before autoclaving all had significant effects on radial fungal growth. At each concentration and for each type of powder, growth was faster when the medium was boiled before autoclaving. Media incorporating 2 per cent placenta powder, 2.5 per cent calcium carbonate, and 2 per cent agar prepared by boiling and filtering with cheese cloth before autoclaving had the best result and are recommended for culturing *P. palmivora*. This dehydrated medium is easy to prepare, has a *p*H of about 7, and can be stored for a long time without caking.

RÉSUMÉ

FRIMPONG, M. & AWUAH, R. T.: *Formulation de milieu déshydraté du mucilage de grain et du placenta des cosses de cacao mûr vert pour la production de* Phytophthora palmivora *(Butl.) Butl.* A cause de la difficulté d'avoir les cosses de cacao pendant la morte-saison de la production de cacao, pour préparer le milieu de griffure, formulation de milieu déshydraté du placenta et de mucilage de graine des cosses de cacao mûr vert était essayée pour utiliser à la production de *Phytophthora palmivora*. Les morceaux de mucilage et de placenta étaient séchés au four (70 °C, 6 h), moulus et les flocons résultants étaient tamisés pour avoir une poudre fine. Quatre concentrations de poudre 0, 1, 2 et 3 % dans l'eau (w/w) étaient préparées et gélose et carbonate de calcium étaient ajoutés respectivement à 2 % (w/v), et 2.5 % (w/w) à chaque concentration de poudre. Les milieux étaient stérilisé à l'autoclave et évalués dans les boîtes de Petri pour la clarté et la capacité de soutenir la croissance et la sporulation de *Phytophthora palmivora*. Le rendement de poudre était plus élevé pour le placenta que pour le milieu basé sur le mucilage. La source de poudre, la concentration de poudre er leur interaction avaient tous eu des effets considérables sur la croissance radiale et mycélienne de *P. palmivora*. A la même concentration de poudre, le milieu préparé de placenta soutenait la croissance radiale considérablement plus élevée ($P \leq 0.05$) de l'essai de fongus (5.59 - 7.10 mm/jour) que le milieu préparé de poudre de mucilage (4.54 - 6.18 mm/jour). La croissance mycélienne aérienne améliorait légèrement comme la concentration de poudre augmentait mais la clarté de milieu diminuait. Sporulation de *P. palmivora* était bonne sur tous les milieux. Source de poudre, concentration de poudre, méthode de préparation de milieu avant la stérilisation à l'autoclave tous avaient eu les effets considérables sur la croissance radiale fongique. A chaque concentration et pour chaque type de poudre, la croissance était plus vite lorsque le milieu était bouilli avant l'autoclave. Le milieu

Original scientific paper. Received 06 Sep 02; revised 29 May 03.

incorporant 2% poudre de placenta, 2.5 % carbonate de calcium et 2 % gélose préparé par le bouillonnement et la filtration avec mousseline à fromage avant l'autoclave donnait le meilleur résultat donc il est recommandé pour la production de *P. palmivora*. Ce milieu déshydraté est facile à préparer, ayant un *p*H d'environ 7 et peut être stocké pour longtemps sans durcissement.

Introduction

Black pod is a major fungal disease of cocoa in Ghana. The disease has long been known to be caused *by Phytophthora palmivora* (Butl.) Butl. (Blencove & Wharton, 1961), but a new and more virulent species, *P. megakarya* Brassier and Griffin, has been reported in Ghana (Dakwa, 1987). The less virulent, *P. palmivora,* is by far the most widespread, being present in almost every cocoa plantation and causing yield loss between 4.9 and 19 per cent (Dakwa, 1984). On the other hand, black pod due to *P. megakarya* can result in yield loss ranging from 60 to 100 per cent (Dakwa, 1987).

Available media for culturing *P. palmivora* in Ghana have not always produced the desired results. Therefore, an attempt was made to prepare media from various cocoa fruit parts for use in culturing the fungus. Media from the seed mucilage, especially the mucilage of mature green cocoa pods, were found to be ideal and recommended for use in growing *P. palmivora* (Awuah & Frimpong, 2002). The disadvantage of using this mucilage-based medium is that in the off-cocoa season, cocoa pods are difficult to obtain, and media preparation becomes problematic. The utility of the mucilage-based medium would be enhanced if a dehydrated, readily usable form is available. Dehydrated media have the advantage of being convenient to use.

In this study, the preparation of dehydrated media from the mucilage and placenta of mature green cocoa pods for laboratory culturing of *P. palmivora* was investigated.

Materials and methods

Powder preparation and initial evaluation of media

Seeds were extracted from mature green cocoa (hybrid of Amelonado and Amazon cross) pods collected from a farm belonging to the Department of Crop Science, KNUST, and the mucilage surrounding the seeds was carefully removed. The pod placenta was also removed and cut into pieces. The mucilage (360 g) and placenta (100 g) pieces were separately oven-dried into flakes in aluminium baking pans at 70 °C for 6 h. For each of them, the flakes were weighed, pulverised with a Waring blender (low speed, 2 min), and the resulting pulverized material sieved through a 75-mm sieve (ELE International Ltd, Hertfortshire, England). The fine powder collected after sieving was weighed. The remaining coarse powder was again milled, sieved as before, and the total weight of fine powder collected after the two cycles of milling determined. The powder was stored in a refrigerator until needed.

Media were prepared from the powders and tested in a completely randomised design (CRD) in a factorial manner with two levels of powder source (mucilage and placenta) and four levels of powder concentration (0, 1, 2 and 3 %). For each powder, 1, 2 and 3 g were separately mixed with 100 ml distilled water in 250-ml Erlenmeyer flasks. Agar was added at 2 per cent (w/v) and calcium carbonate at 2.5 per cent (w/w) to each medium. Media without powder but with only calcium carbonate and agar were also prepared to obtain media with 0, 1, 2 and 3 per cent powder concentrations. Media *p*H was measured with a Corning *p*H meter (Model 430 : Corning Inc. Science Product Division, Corning, NY). After autoclaving, the media were dispersed into 9-mm diameter Petri plates (four replicate plates per concentration, about 20 ml per plate).

An isolate of *P. palmivora* (Tafo-3) growing on green cocoa mucilage agar (GCMA) (Awuah &

Frimpong, 2002) was used to test the mycological utility of each medium as previously described (Awuah & Frimpong, in press). Briefly, mycelial plugs (7-mm diameter) of a 5-day-old culture of Tafo-3 were centrally placed (top down) on plates of each medium and incubated in the dark at 29 °C (± 2 °C). Colony diameters were measured at 1, 2 and 3 days and radial growth rate in mm/day was calculated for each assessment period and averaged. Media clarity, aerial mycelial production, and sporulation intensity of the test fungus were qualitatively assessed after 7 days (Awuah & Frimpong, 2002). The test was repeated.

Effect of powder source, powder concentration, and method of media preparation

The effect of the above on media clarity and on growth and sporulation of *P. palmivora* was studied in a CRD factorial experiment involving the following factors and levels: powder source (mucilage and placenta); powder concentration (2 and 3 %); method of media preparation before autoclaving (boiled before filteration and filteration without boiling). The requisite amounts of powder (2 and 3 g) were suspended in 100-ml distilled water in 250-ml Erlenmeyer flasks. Agar and calcium carbonate were added to media at 2 per cent (w/v) and 2.5 per cent (w/w), respectively. A medium was brought to boil on a Bunsen burner and filtered through eight layers of cheese cloth. A non-boiled medium was mixed by manual shaking for 10 min before filteration.

The sterilized media were dispensed into 9-cm diameter glass Petri dishes and evaluated for clarity and growth characteristics of *P. palmivora*.

Data analysis

The Chi-square test (Bartlett's test) of homogeneity of error variance (Gomez & Gomez, 1984) for radial growth data for repeat experiments indicated a significant homogeneity of the error variances; so the data for both experiments were combined before analysis of variance (ANOVA) was applied. Treatment means were compared with the least significance difference (LSD) test ($P < 0.05$).

Results

Drying 360 g mucilage and 100 g placenta at 70 °C for 6 h yielded 54.88 and 17.51 g of crispy flakes, respectively (Table 1). When these were milled and sieved twice successively, 20.71 and 6.78 g fine powder were, respectively, recovered from the mucilage and placenta flakes (Table 1).

Radial growth was significantly higher for the placenta-based media (5.59 – 7.10 mm/day) than for the mucilage-based ones (4.54 – 6.18 mm/day) (Table 2). As the powder concentration increased from 1 to 3 per cent, radial growth on the mucilage media significantly decreased from 6.18 to 4.54 mm/day. The same trend was observed for the placenta-based media for which radial growth decreased from 7.10 to 6.33 mm/day as the powder concentration was increased from 1 to 3 per cent. Acidity of the media also increased with increasing powder concentration, but the mucilage-based media were more acidic (Table 2). The source of powder, powder concentration, and their

TABLE 1

Comparative Recovery of Powders from the Mucilage and Placenta of Green Cocoa Pods

Preparation and powder recovery	*Amount of material (g)*	
	Placenta	*Mucilage*
Fresh pieces	360.00	100.00
Dried flakes	54.88	17.51
Pulverized flakes	54.76	16.16
Fine powder after sieving*	20.71	6.78
Coarse powder after sieving**	31.90	7.68
% recovery of fine powder from fresh material	5.75	6.78
% recovery of fine powder from dried material	37.74	38.72

*The flakes were pulverised in a Waring blender and sieved to obtain fine powder. The remaining coarse powder was again milled and sieved as before and the fine powders bulked.

**Coarse powder after two successive milling and sieving.

TABLE 2

Growth of P. palmivora *and Media Characteristics as Affected by Powder Source and its Concentration in the Medium*

Powder source and concentration (percent)	*Radial growth (mm/day)**	*Aerial mycelium***	*Sporulation intensity***	*Clarity****	p*H*
Mucilage 0	2.83	-	+(+)	++	9.41
" 1	6.18	+(+)	++(+)	+(+)	6.69
" 2	5.48	++	+++	+(+)	5.56
" 3	4.54	+++	+++	+	5.28
Placenta 0	2.83	-	+(+)	++	9.41
" 1	7.10	+(+)	+++	+(+)	7.09
" 2	6.33	++(+)	+++	+	6.76
" 3	5.59	+++	+++	(+)	6.44
LSD (≤0.05)	0.46				
CV (%)	6.32				

*Data represent the mean growth rates from two separate experiments (four replicate plates per treatment).
**Determined qualitatively by visual assessment at day 7: - = none; + = sparse; ++ = moderate; +++ = abundant.
*** + = fairly clear; ++ = very clear; (+) = rating approaching +.

interaction had significant effects on radial growth of *P. palmivora* (Table 3).

At the same powder concentration, boiling a medium and filtering before autoclaving significantly increased radial growth rate of the test fungus. For the placenta-based media, fungal growth rate increased from 6.84 to 7.58 mm/day when a medium with 2 % powder was boiled. At 3 per cent powder concentration, growth rate increased from 6.22 to 6.55 mm/day when the medium was boiled (Table 4). A similar trend was observed for the mucilage media. Powder source, powder concentration, and method of media preparation before autoclaving and most interactions of these factors also had significant effect on radial growth of *P. palmivora* (Table 5).

Media with 1 and 2 per cent powder concentrations were fairly clear (Tables 2 and 4), enabling partial visualization of fungal colonies from the underside of plates. Considerable aerial mycelial growth was also observed on media prepared from 2 and 3 per cent powder. Sporangia and chlamydospore production was good regardless of powder source, concentration, and method of preparation before autoclaving (Tables 2 and 4).

TABLE 3

Summary ANOVA for the Effect of Powder Source (Mucilage and Placenta) and Powder Concentration (0, 1, 2 and 3 %) on Radial Growth of P. palmivora

Source	*Degree of freedom*	*Mean square of errors*	*Probability values*
Powder source (A)	1	3.990	0.000
Concentration (B)	3	21.815	0.000
AB	3	0.457	0.003
Error	24	0.104	

Discussion

Previous efforts at preparing dehydrated media from local raw materials to cultivate a wide range of fungi in Ghana have been successful (Awuah, 1989, 1992, 1994). Even though the GCMA (Awuah

TABLE 4

Effect of Media Preparation Method, Powder Source, and its Concentration on Radial Growth of P. palmivora

Treatment	*Radial growth (mm/day)**	*Aerial mycelium***	*Sporulation intensity***	*Clarity****	*pH*
Mucilage 2 %; boiled	5.82	++	+++	+(+)	5.95
" 2 %; non-boiled	5.85	++(+)	+++	+(+)	6.21
" 3 %; boiled	5.57	++(+)	+++	+	5.76
" 3 %; non-boiled	5.11	++	+++	+	5.27
Placenta 2 %; boiled	7.58	+++	+++	+(+)	6.98
" 2 %; non-boiled	6.84	++(+)	+++	+(+)	7.16
" 3 %; boiled	6.55	++(+)	+++	+	6.33
" 3 %; non-boiled	6.22	++(+)	+++	(+)	6.74
LSD (≤0.05)	0.25				
CV (%)	2.87				

*Data represents the mean growth rates from two separate experiments (four replicate plates per treatment).
**Determined qualitatively by visual assessment at day 7: + = sparse; ++ = moderate; +++ = abundant; (+) = rating approaching +.
*** = fairly clear; ++ = very clear; (+) = rating approaching +.

TABLE 5

Summary ANOVA for the Effect of Powder Source (Mucilage and Placenta), Powder Concentration (2 and 3 %), and Method of Media Preparation Before Autoclaving (Boiled and Non-boiled) on Radial Growth of P. palmivora

Source	*Degree of freedom*	*Mean square of errors*	*Probability values*
Powder source (A)	1	11.725	0.000
Concentration (B)	1	3.465	0.000
AB	1	0.210	0.016
Method of prep. (C)	1	1.121	0.000
AC	1	0.200	0.018
BC	1	0.002	
ABC	1	0.412	0.001
Error	24	0.032	

Blank probability value for BC indicates a very high value.

& Frimpong, 2002) is useful for culturing *P. palmivora*, its utility is diminished because in the lean cocoa season, pods are not readily available, making media preparation from scratch almost impossible. This study has shown that dehydrated media can be formulated, not only from the seed mucilage, but also from the pod placenta of mature green cocoa pods. This is the first report of the preparation of dehydrated media from cocoa mucilage and placenta. The study, therefore, fills a critical gap in the culturing of *P. palmivora* in Ghana.

In general, powder preparation from the seed mucilage was more cumbersome than that from the pod placenta. Presumably, mucilage is of a higher sugar content and its flakes tend to be hygroscopic, making milling difficult. The powder particles were also sticky and sometimes clogged the pores of the sieve. These problems were not encountered during powder preparation from the placenta. Fine powder yield from the placenta was, therefore, comparatively higher than that from the mucilage. The removal of mucilage from the seed was also more laborious than extraction of the placenta from the pods. Therefore, media preparation from the placenta is preferred to that from the mucilage. The placenta powder, when

used at 2 % (w/v), had the best results.

Sodium hydroxide was previously used to increase *p*H of the mucilage-based media to 5 and above before autoclaving to enhance gelling (Awuah & Frimpong, 2002). This was necessary because media of high acidity such as the mucilage -based ones would not set after autoclaving (Johnston & Booth, 1983). In this study, sodium carbonate rather than sodium hydroxide was used because the latter tends to deliquesce, negating its usefulness in dehydrated media preparation.

Because of the generally low fine powder yield from the placenta, large quantities of mature green cocoa pods would have to be destroyed to obtain enough placenta for powder preparation. Economically, this will be unacceptable to growers. Thus, the placenta from ripe pods, which is a left-over product of seed extraction, should be studied for possible use in large-scale media preparation.

Media clarity, which is the least desirable characteristic of the placenta-based medium, can be improved by heating the recommended amount of powder and water, filtering the suspension before autoclaving. As with other previously formulated dehydrated media from local raw materials (Awuah, 1989, 1992, 1994), particles in the placenta-based medium cannot be avoided, but can be minimized if the medium is not overly agitated when dispensing into plates. This would leave out much of the particles which would have otherwise obscured media clarity.

For the same powder concentration, radial growth was faster on media that were boiled and filtered before autoclaving. This would be expected, since more nutrients would be released from the powder particles into solution during boiling. Consistency of the medium was also improved by boiling and filtering.

An inverse relationship was consistently observed between powder concentration and radial growth rate of *P. palmivora*. No specific reason was assigned to this, but the lower *p*H values of media with higher powder concentration could be a reason (Awuah & Frimpong, 2002).

Unlike commercial dehydrated mycological media, many of which tend to cake with time, possibly due to their high sugar content (Awuah, 1994), the dehydrated medium from the green cocoa pod, especially that from the placenta, does not readily cake. A sample of the placenta-based medium has maintained its powdery consistency for as long as 6 min without refrigerated storage. This is desirable.

Acknowledgement

This research was partially funded by the Ghana Cocoa Board.

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