ORIGINAL RESEARCH ARTICLE

Botryodiplodin (A Mycotoxin) detection in pathogenic *Botryodiplodia theobromae* isolated from diseased coconut fruits

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DOI: https://doi.org/10.57046/HPUP6912

Abstract

Botryodiplodia theobromae is a threat to crops because it produces botryodiplodin, that plays a role in the initial stages of plant infection, creating necrotic areas through which it can easily penetrate. In addition, the botryodiplodin produced is not easily detected during quarantine, and other techniques developed to detect botryodiplodin are not easily practicable for screening numerous samples. Hence, the need to develop an in-culture pigment formation method to identify and differentiate toxigenic and non-toxigenic pathogenic isolates of *B. theobromae*. In this study, to detect botryodiplodin produced by isolates of *B. theobromae*, PDA, CDA and modified CDA media were used. Only the modified CDA medium enhanced the detection of botryodiplodin produced by *B. theobromae* isolates due to the addition of glycine into the medium. The effect of modified CDA composition or formulation, sucrose, and glycine concentrations on botryodiplodin detection were also evaluated. Study on the effect of the modified CDA composition on the detection of botryodiplodin produced by isolates of *B. theobromae* revealed that only sucrose stimulated the detection of botryodiplodin in comparison with other ingredients in the modified medium. In addition, the results from the study also reveals that increasing sucrose and glycine concentrations directly enhanced botryodiplodin detection, with optimum concentration of sucrose and glycine for detecting botryodiplodin by isolates of *B. theobromae* established at 15 and 10 g/l respectively**.** Hence, there is no need to increase the concentration of both sucrose and glycine above these established concentrations when preparing an in-culture medium for screening *B. theobromae* isolates capable of producing botryodiplodin.

Key words: *Botryodiplodia theobromae, coconut fruit, pathogenic, in-culture, botryodiplodin*.

Introduction

Botryodiplodia theobromae (synonym *Lasiodiplodia theobromae* Pat.), is a cosmopolitan soil-borne fungus that causes field and storage diseases of major economic important crops, resulting in consequential yield loss of over 60% (Ekhorutomwen *et al*., 2019; Marques *et al*., 2013; Viana *et al*., 2007; Punithalingam, 1980). The fungus is widely distributed in tropical and sub-tropical regions and occurs on a wide range of plants hosts (Punithalingam, 1976), mainly woody plants including fruit and tree crops, such as coconut, mango, peach, avocado, and eucalyptus spp (Mohali *et al*., 2005). It can occur in nature either as a saprophyte, endophyte, or parasite (Machado *et al*., 2014; Slippers and Wingfield, 2007; Alves *et al*., 2008). *B. theobromae* is a threat to crops because it avoids detection by quarantine. Among other pathogenic characteristics, *B. theobromae* produces low molecular weight secondary metabolites (botryodiplodin inclusive), which play a role in the initial stages of plant infection, whereby it kills plant tissue creating necrotic areas through which the fungi pathogen can easily penetrate the host (Shier *et al*., 2012). To be precise, botryodiplodin is a simple ribose analog mycotoxin produced by several species of fungi (Alam *et al*., 2022; Salvatore *et al*., 2020). Botryodiplodin has received attention due to its potent antibiotic (Bladt *et al*., 2013), anticancer (McCurry *and* Abe, 1973), mutagenic and cytotoxic (Moule *et al*., 1981), and phytotoxic activities (Shier *et al*., 2007), as well as the ability to induce protein-DNA crosslinking in mammalian cells (Salvatore *et al*., 2020; Moule and Darracq, 1984; Moule *et al*., 1981).

Botryodiplodin chemical nature is simply a 12-membered benzenediol lactones, octaketides possessing a resorcinol aromatic ring and a macrocyclic lactone (Shen *et al*., 2015). The base compound of this class of natural products is (3R)-botryodiplodin (Ibrahim *et al*., 2018), which was isolated from a *B. theobromae* strain together with its (3R)-de-O-methyl-analogue (Aldridge *et al*.,1971). Techniques have been developed to detect botryodiplodin produced by pathogenic fungi isolated from plant materials, contaminated foods, and feeds (Alam *et al*., 2022; Khambhati *et al*., 2020), but these techniques are not easily practicable for screening numerous samples, especially in developing countries, due to the cost and lack of required equipment in carrying out these techniques. This initiated interest in developing in-culture pigment formation methods to identify and differentiate toxigenic and non-toxigenic pathogenic fungal isolates in plant materials, contaminated foods, and feeds (Alam *et al*., 2022; Khambhati *et al*., 2020). The principle behind in-culture pigment formation technique to detect toxigenic fungi isolates is based on the fact that botryodiplodin produced by fungi reacts with amino group-containing substances in some selected amino acids (such as glycine, alanine, leucine, or asparagine) in culture medium containing sucrose, to form a reddish or pinkish pigment (Alam *et al*., 2022; Dunlap

and Bruton, 1986). Therefore, this study was designed to evaluate the use of in-culture pigment formation technique to identify and differentiate toxigenic and non-toxigenic pathogenic *B. theobromae* isolated from diseased coconut fruits.

Materials and methods

Culture media

Potato dextrose agar (PDA), Czapek Dox agar (CDA), sucrose, and glycine were purchase from Sigma-Aldrich, St. Louis, MO, USA, and used for the study.

Fungal strains and culture conditions

B. theobromae strains used in the study were isolated from diseased coconut fruits (in coconut plantation in NIFOR Main Station and Coconut Garden, Isihor, Ovia Northeast L.G.A, Edo State, Nigeria). The coconut palms in both plantations were exhibiting symptoms of fruit rot and premature nut fall diseases, which include a light or dark brown lesion turning into whitish grey colour with shriveled appearance. The infected fruit latter turn blackish with cracks at the basal part of the fruit, and sometimes it causes the fruit to fall off from the bunch before maturity or harvesting called premature-nut-fall (Dheepa *et al*., 2018; Venugopal and ChandraMohanan, 2006; Phipps and Porter, 1998). *B. theobromae* cultures were maintained on PDA medium at 28 ± 2^0C and stored on PDA slants at -80^0C for future use. Eight isolates of *B. theobromae* were initially used for this study. All eight isolates of *B. theobromae* were coded as Bt.NGD, Bt.NOD, Bt.NYD, Bt.NRD, Bt.IGD, Bt.IOD, Bt.IYD, and Bt.IRD in the study.

Detection of botryodiplodin in culture medium

To detect botryodiplodin produced by eight isolates of *B. theobromae*, PDA, CDA and modified CDA medium {2g of sodium nitrate ($NaNO_3$), 1g of dipotassium phosphate (K_2HPO_4), 0.5g of potassium chloride (KCl), 0.5g of magnesium sulphate ($MgSO_4$), 0.01g of ferrous sulphate ($FeSO_4$), 15g of sucrose, 10g of glycine, and 15g of agar} was formulated (Alam *et al*., 2022). Agar plug of each isolates of *B. theobromae* were inoculated on the center of 9 cm Petri dishes containing sterilized PDA, CDA and modified CDA medium. The Petri dishes inoculated in duplicates were incubated for 7 days at room temperature (Alam *et al*., 2022). The effect of each composition or formulation of modified CDA medium (that is, one component of modified CDA medium was selectively removed in the formulation, except glycine and agar) in botryodiplodin detection by two selected isolates of *B. theobromae* were also determined. The effect of sucrose and glycine concentrations in modified CDA medium by two selected isolates of *B. theobromae* were also determined.

Effect of modified CDA medium composition in the detection of botryodiplodin produced by two selected isolates of Botryodiplodia theobromae.

Agar plugs of two isolates of *B. theobromae* (Bt.NYD and Bt.IRD) were inoculated differently on the center of 9 cm Petri dishes containing sterilized individual selected composition of modified CDA medium (that is, CDA medium with water,

glycine, and agar but lacking one of the following other compositions: $NaNO_3$, K_2HPO_4, KCl, $MgSO_4$, $FeSO_4$ or sucrose). The medium inoculated in duplicates were incubated for 7 days at room temperature (Alam *et al*., 2022).

Effect of sucrose concentration in the detection of botryodiplodin produced by two selected isolates of Botryodiplodia theobromae.

Botryodiplodin detection in the presence of different range of sucrose concentrations (0, 1, 5, 10, 15, 20, and 25 g/L) in modified CDA medium was determined. Agar plug of two selected isolates were used to inoculate plates with the modified CDA medium containing the different sucrose concentrations. The Petri dishes inoculated in duplicates were incubated at room temperature for 7 days.

Effect of glycine concentration on botryodiplodin detection by two selected isolates of B. theobromae

Botryodiplodin detection in the presence of different range of glycine concentrations (0, 1, 5, 10, 15, 20, and 25 g/L) in modified CDA medium was determined. Agar plug of two selected isolates were used to inoculate the Petri dishes with the modified CDA medium containing different glycine concentrations. The Petri dishes inoculated in duplicates were incubated at room temperature for 7 days.

Results

The presence of reddish or pinkish pigmentation in modified CDA medium after incubation reveals the detection or presence of botryodiplodin in the culture medium. All isolates of *B. theobromae* were grouped into 4 different categories based on the size (or no detection) of botryodiplodin in the modified CDA medium after 7 days' incubation, as follows; 1) High: Bt.IRD and Bt.NYD; 2) Moderate: Bt.IYD and Bt.NOD; 3) Minimal: Bt.NRD; 4) None: Bt.NGD, Bt.IGD and Bt.IOD (Plate 2).

Table 1: Detection of botryodiplodin in stored culture (PDA slant)

Location	Isolates	Botryodiplodin detection in PDA slant
NIFOR Main Station	Bt.NGD	-
	Bt.NOD	+
	Bt.NYD	+
	Bt.NRD	-
Coconut Garden, Isihor	Bt.IGD	-
	Bt.IOD	-
	Bt.IYD	-
	Bt.IRD	+

Key: Botryodiplodin (red pigmentation): -: absent; +: present; PDA: potato dextrose agar.

Table 2: Detection of botryodiplodin in culture media

Location	Isolates	Botryodiplodin detection in PDA	Botryodiplodin detection in CDA	Botryodiplodin detection in CDA + Glycine
NIFOR Main Station	Bt.NGD	-	-	-
	Bt.NOD	-	-	+
	Bt.NYD	-	-	+
	Bt.NRD	-	-	+
Coconut Garden, Isihor	Bt.IGD	-	-	-
	Bt.IOD	-	-	-
	Bt.IYD	-	-	+
	Bt.IRD	-	-	+

Key: Botryodiplodin (red pigmentation): -: absent; +: present; PDA: potato dextrose agar; CDA: Czepek dox agar.

Table 3: Incubation period in the detection of botryodiplodin in modified CDA

Location	Isolates	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
NIFOR Main Station	Bt.NGD	-	-	-	-	-	-	-
	Bt.NOD	-	-	-	+	+	++	++
	Bt.NYD	-	-	+	+	++	++	+++
	Bt.NRD	-	-	-	-	-	+	+
Coconut Garden, Isihor	Bt.IGD	-	-	-	-	-	-	-
	Bt.IOD	-	-	-	-	-	-	-
	Bt.IYD	-	-	-	+	+	++	++
	Bt.IRD	-	+	+	++	++	+++	+++

Key: -: absent; +: minimal; ++: moderate; +++: high.

A **B**

Plate 1. A: no botryodiplodin detected in PDA slant; B: botryodiplodin (brownish pigmentation) detected in PDA slant.

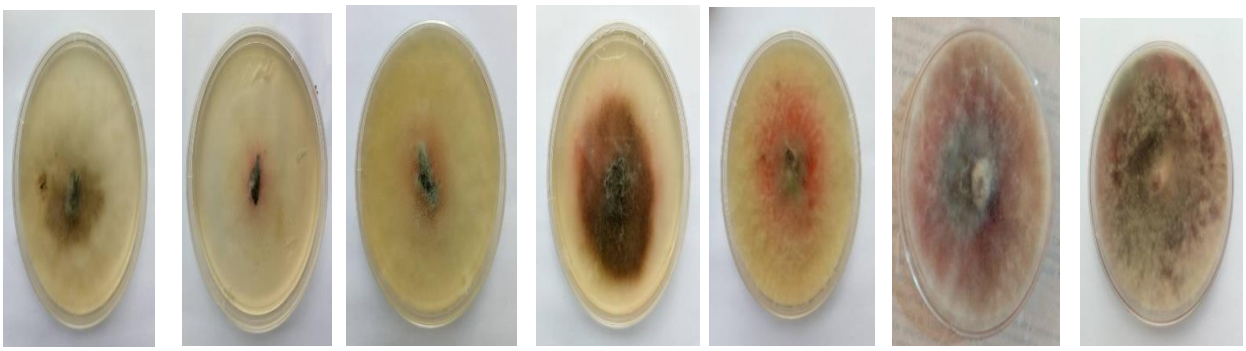

Plate 2. Botryodiplodin detection in modified Czapek Dox agar medium; A: no botryodiplodin detected in 3-day old *B. theobromae*; B: minimal botryodiplodin (reddish pigmentation) detected in 2-day old *B. theobromae*; C: minimal botryodiplodin (reddish pigmentation) detected in 6-day old *B. theobromae*; D: moderate botryodiplodin (reddish pigmentation) detected in 3-day old *B. theobromae* E: moderate botryodiplodin detected in 7-day old *B. theobromae*; F and G: high botryodiplodin detected in 7-day old *B. theobromae*.

Effect of modified CDA composition on botryodiplodin detection by two selected isolates of B. theobromae

The results of the modified CDA composition or formulation on the detection of botryodiplodin reveals that only sucrose stimulated botryodiplodin detection by the selected botryodiplodin-competent *B. theobromae* isolates growing on Petri dish containing the modified CDA medium (Table 4).

Table 4: Effect of composition of modified CDA medium on botryodiplodin detection.

Isolates	Composition of modified CDA medium	Botryodiplodin detection
Bt.NYD	Sodium nitrate + glycine + agar	-
	Dipotassium phosphate + glycine + agar	-
	Potassium chloride + glycine + agar	-
	Magnesium sulfate + glycine + agar	-
	Ferrous sulfate + glycine + agar	-
	Sucrose + glycine + agar	+
Bt.IRD	Sodium nitrate + glycine + agar	-
	Dipotassium phosphate + glycine + agar	-
	Potassium chloride + glycine + agar	-
	Magnesium sulfate + glycine + agar	-
	Ferrous sulfate + glycine + agar	-
	Sucrose + glycine + agar	+

Key: -: absent; +: present.

Effect of sucrose concentration on botryodiplodin detection by two selected isolates of B. theobromae

The results of the effect of sucrose concentration on botryodiplodin detection reveals that at 0 and 1 g/l (botryodiplodin was not detected in the modified CDA), at 5 g/l (minimal botryodiplodin was detected in the modified CDA), at 10 g/l (moderate botryodiplodin was detected in the modified CDA), while at 15, 20 and 25 g/l (high botryodiplodin was detected in the modified CDA) (Table 5).

Table 5: Effect of sucrose concentration on botryodiplodin detection.

Isolates	Sucrose concentration (g/l)	Botryodiplodin detection
Bt.NYD	0	-
	1	-
	5	+
	10	++
	15	+++
	20	+++
	25	+++
Bt.IRD	0	-
	1	-
	5	+
	10	++
	15	+++
	20	+++
	25	+++

Key: -: absent; +: minimal; ++: moderate; +++: high

Effect of glycine concentration on botryodiplodin detection by two selected isolates of *B. theobromae*

The results of the effect of glycine concentration on botryodiplodin detection reveals that at 0 and 1 g/l (botryodiplodin was not detected in the modified CDA), at 5 g/l (minimal botryodiplodin was detected in the modified CDA), at 10, 15, 20 and 25 g/l (high botryodiplodin was detected in the modified CDA) (Table 6).

Table 6: Effect of glycine concentration on botryodiplodin detection.

Isolates	Glycine concentration (g/l)	Botryodiplodin detection
Bt.NYD	0	-
	1	-
	5	+
	10	+++
	15	+++
	20	+++
	25	+++
Bt.IRD	0	-
	1	-
	5	+
	10	+++
	15	+++
	20	+++
	25	+++

Key: -: absent; +: minimal; +++: high.

Discussion

In the study, the presence of brownish or reddish pigmentation (botryodiplodin) after 30 days in PDA slant led to the screening of the eight isolates of *B. theobromae* on their ability to produce botryodiplodin (a mycotoxin) using the in-culture method. More so, three isolates (Bt.NOD, Bt.NYD, and Bt.IRD) out of the eight isolates of *B. theobromae* showed brownish or reddish pigmentation (botryodiplodin) after 30 days in PDA slant, while five isolates (Bt.NGD, Bt.NRD, Bt.IYD, Bt.IGD and Bt.IOD) did not show pigmentation in PDA slant (Plate 1 and Table 1). Furthermore, to detect botryodiplodin produced by the eight isolates of *B. theobromae*, PDA, CDA and modified CDA media were used in the study. Only the modified CDA medium enhances the detection of botryodiplodin produced by isolates of *B. theobromae* due to the addition of glycine into the medium (Table 2). As mentioned earlier, all isolates of *B. theobromae* were grouped into 4 different categories based on the size (or no detection) of botryodiplodin in the modified CDA medium after 7 days' incubation, as follows; 1) High: Bt.IRD and Bt.NYD; 2) Moderate: Bt.IYD and Bt.NOD; 3) Minimal: Bt.NRD; 4) None: Bt.NGD, Bt.IGD and Bt.IOD (Plate 2). Botryodiplodin detected in isolates Bt.IRD and Bt.NYD were categorized as "high" because the botryodiplodin produced by both isolates covers the entire 9 cm Petri dish. Isolates Bt.IYD and Bt.NOD were categorized as "moderate" because the botryodiplodin produced by both isolates covers about 3 – 4 cm of the 9 cm Petri dish, while isolate Bt.NRD was categorized as "minimal" because the botryodiplodin produced covers about 0.5 – 1 cm of the 9 cm Petri dish (Plate 2). Botryodiplodin detected in Bt.IRD isolate was higher as compared to other isolates tested based on the size of reddish or pinkish pigmentation in the modified CDA medium. The size or ranking of botryodiplodin detected (in the modified CDA) by the eight isolates of *B. theobromae* tested was higher in this order; Bt.IRD > Bt.NYD > Bt.IYD = Bt.NOD > Bt.NRD.

Botryodiplodin was detected on day-2 for Bt.IRD, day-3 for Bt.NYD, day-4 for Bt.IYD and Bt.NOD, and day-6 for Bt.NRD after incubation (Table 3). This explains why the size of botryodiplodin in the modified CDA medium increases at two-day interval. The botryodiplodin produced by isolates of *B. theobromae* did not follow the mycelia growth pattern except for Bt.IRD isolates. Study on the effect of composition or formulation of the modified CDA medium in the detection of botryodiplodin produced by the two selected isolates of *B. theobromae*, revealed that only sucrose stimulated the detection of botryodiplodin produced by the two selected botryodiplodin-competent *B. theobromae* isolates in comparison with other ingredients in the modified culture medium (Table 4). This is simply because botryodiplodin produced by *B. theobromae* reacts with the amino group-containing substances in glycine in the modified culture medium containing sucrose, to form the reddish or pinkish pigment (Alam *et al*., 2022; Dunlap and Bruton, 1986). Furthermore, this prompted the quest to know the effect of sucrose and glycine concentration in the detection of botryodiplodin produced by two selected botryodiplodin-competent *B. theobromae* isolates.

On the effect of sucrose concentration in the detection of botryodiplodin produced by the two selected botryodiplodin-competent *B. theobromae* isolates, increased botryodiplodin detection with increase in sucrose concentration, with the maximum size detected at 15 g/l and above. Moderate pigment detection was observed at 10 g/l, while minimal pigment detection was also observed at 5 g/l sucrose (Table 5). On the effect of glycine concentration in the detection of botryodiplodin produced by the two selected botryodiplodin-competent *B. theobromae* isolates, increased botryodiplodin detection with increase in glycine concentration, with the maximum size detected at 10 g/L and above. While minimal pigment detection was also observed at 5 g/l sucrose (Table 6). In the study, the effect of sucrose and glycine in botryodiplodin detection revealed that increasing sucrose and glycine concentrations directly enhanced the detection of botryodiplodin produced by the two selected botryodiplodin-competent *B. theobromae* isolates. For sucrose, 15 g/l was sufficient to detect botryodiplodin produced by the two botryodiplodin-competent *B. theobromae* isolates, while for glycine 10 g/l was sufficient to detect botryodiplodin produced by the two botryodiplodin-competent *B. theobromae* isolates. Hence, there is no need to increase the concentration of both sucrose and glycine above this established concentrations when preparing an in-culture medium for screening *B. theobromae* isolates capable of producing botryodiplodin. The findings agree with the work of Alam *et al*. (2022), Khambhati *et al*. (2020), and Dunlap and Bruton, (1986).

Conclusion

The results obtained from this study affirmed the use of in-culture technique for rapid detection of botryodiplodin using Czapek Dox agar medium supplemented with glycine. This technique is a reliable research tool for screening both toxigenic and non-toxigenic *B. theobromae* strains in plant materials.

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