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Original Research Articles

In-vitro Evaluation of some Fungicides against *Botrydiplodia theobromae*: Causal Pathogen of Pineapple Dieback

Abstract

Dieback is an economically important disease known to cause major losses in food and tree crops. The causal pathogen of pineapple dieback was investigated and the efficacies of some fungicides were evaluated *in-vitro*. Infected leaf and soil samples were collected from the rhizosphere of the infected pineapple at National Horticultural Research Institute field for pathogen isolation. Pathogens isolated from diseased leaves were *Botrydiplodia theobromae* and *Aspergillus niger* while *Aspergillus niger*, *Aspergillus tamari*, *Botrydiplodia theobromae* and *Fusarium verticilliodes* were isolated from the rhizosphere. On infected leaf samples, *B. theobromae* had the highest occurrence (93.3%) while *Aspergillus niger* had the least occurrence (6.7%). Similar trend was observed in the soil samples where *B. theobromae* had the highest occurrence of 80% while *A. tamari* and *F. verticilliodes* had the least frequency of occurrence of 4%. Result from pathogenicity test revealed that *B. theobromae* was the causal pathogen of pineapple dieback. The efficacies of three fungicides namely: Z- force (a. i 80% Mancozeb), Forcelet (a.i 50% carbendazim), and Funguforce (63% mancozeb + 12.5% carbendazim) were tested *in-vitro* on mycelial growth inhibition of *B. theobromae*. The three fungicides evaluated were able to inhibition the mycelial growth of *B. theobromae*.

Keywords: Chemical control; Die-back; disease; Fungicide; Growth inhibition; In-vitro; Pathogenicity; Pineapple; Rhizosphere

1. Introduction

Pineapple (*Ananas cosmosus*) is one of the most important commercial fruit crops in the world. It is a short herbaceous perennial plant which is available throughout the year (David, 1986).

Pineapple fruit is a good source of Vitamin B1 and brometin. It was originally consumed only as a fresh fruit but with the development of the processing industry, the fruit is now prepared and consumed in various forms such as pineapple chunks, slices, juice, syrups, jams, crushed pineapple, sliced pineapple (Shu, 1999). Pineapple is the third most important tropical fruit in world after Banana and Citrus (Bartholomew *et al*., 2003). Thailand is the largest producer of pineapple, accounting for 13% of global output, followed by Brazil and Costa Rica. Nigeria ranked 7th on the list of world producers, as well as the leading pineapple producer ~~in~~ Africa with a production of 1,400,000 MT of fresh pineapple having the largest land area of about 180, 000 ha for pineapple production in the world and yield of 77778 tons/ha (FAOSTAT, 2011). Despite this ranking, many constraints are still facing pineapple production in Nigeria. These include low soil fertility, poor cultivar, pest and diseases attack among others.

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Botryodiplodia theobromae is a cosmopolitan soil-borne fungus causing both field and storage diseases on more than 280 plant species including crops, fruits and plantation trees (Domsch *et al.,* 1980). ~~*Botryodiplodia theobromae* is a fungal pathogen of great economic importance.~~ It is an opportunistic plant pathogen that causes different types of plant diseases with worldwide distribution within tropical and subtropical regions (Faber *et al*., 2007). It has a wide host range with varied pathological effects on its host (Domsch *et al*., 1980). It is an economically important fungus known to cause major losses to mango, cocoa, banana and yam farmers (Rieger, 2006). It is widely distributed in tropical and subtropical regions and has been associated with approximately 500 hosts. ~~It has been responsible for causing pre-harvest dieback and gummosis and/or post-harvest fruit rot diseases.~~ It has been associated with diseases such as dieback, root rot, fruit rot, blights, gummosis, stem necrosis, leaf spot and witches' broom disease (Punithalingam, 1980). This fungus has also been associated with dieback and necrosis at the grafting site of cashew (*Anacardium occidentale* L.) (Freire *et al*., 2002), guava (Cardoso *et al*., 2002), citrus (Davis *et al*., 1987) and grapevine (Aroca *et al*., 2008). The objectives of this work ~~were~~ to isolate and identify the causal pathogen of pineapple dieback and to evaluate the *in-vitro* efficacies of some fungicides in the control of the causative pathogen.

2. Materials and methods

2.1 Experimental site

The experiment was conducted at National Horticultural Research Institute (NIHORT), Ibadan, Oyo State, Nigeria (Latitude 7.54 N; Longitude 3.54 E and 213m above sea level) in 2014. Sample collection was done in two seasons in the same year on the pineapple field of NIHORT, Ibadan. The spacing was 0.5m x 0.5m and separated by 1.0m.

2.2 Isolation of pathogens from diseased pineapple leaves

Pineapple leaves showing symptoms of dieback were collected randomly from 30 plants. Diseased portions were cut into smaller pieces and surface sterilized by immersing in 2% sodium hypochlorite solution for one minute, washed in several changes of sterile distilled water and wipe dried with sterile cotton wool. The surface sterilized tissues were placed on solidified chloraphenicol-modified (50 mg/L) Potato Dextrose Agar medium (PDA) in 90 mm petri dishes. The inoculated plates were incubated at room temperature (28±2 ^{0}C) for 5 days. Hyphae that grew from diseased tissue on the culture media were sub-cultured on PDA amended with chloramphenicol (50 mg/L) to suppress bacterial contamination and to obtained pure cultures of isolates.

2.3 Isolation of pathogens from rhizosphere of infected pineapple plants

Soil samples were collected from the rhizosphere of diseased plants where leaf samples were taken. The samples were bulked to form a composite and air dried on the laboratory bench for 24 hrs. Composite was crushed and allowed to pass through 2mm sieve. One gram soil sample was weighed and added to 9.0 ml sterile distilled water. The mixture was shaken vigorously and serially diluted. One millilitre supernatant was pipetted on solidified

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potato dextrose agar, spread using sterile spreader and incubated at room temperature for 4 days. Each colony forming units were transferred into newly prepared PDA to obtain pure culture of each isolates.

2.4 Identification of Pathogens

Colonies of isolates were identified using both cultural and morphological features according to Barnett and Hunter (1972). The frequencies of occurrence of each fungal isolate associated with diseased pineapple plants were determined by counting and recording the number of times each fungus was encountered. The percentage frequency of occurrence was calculated according to Ebele (2011).

$$\text{Percentage frequency} = \frac{\text{Number of times a fungus was encountered}}{\text{Total fungal isolations}} \times 100$$

2.5 Pathogenicity test

Healthy~~9~~, uninfected pineapple leaves were surface sterilized with 70% ethanol, washed under tap water, rinsed with sterile distilled water and blotted dry with sterile cotton wool. Pathogenicity test was confirmed using dipping method (Marlatt *et al*., 1996). Conidial suspension of pathogen was harvested by flooding conidia of *B. theobromae* and *A. niger* with sterile distilled and gently scraped with spatula. Thereafter the conidia was filtered through three layers of cheese cloth and adjusted to a final concentration of 10^6 microconidia/ml using heamocytometre. Leaves were dipped in spore suspensions for 5 minutes while leaves dipped in sterile distilled water serve as the control. Both inoculated and control treatments were incubated for 7 day at room temperature for disease development. To fulfil koch's postulate, re-isolation of the pathogenic fungi was done and compared with the original isolate.

2.6 Evaluation of fungicides against Botryodiplodia theobromae

The efficacies of three fungicides namely: Z- force (a. i 80% mancozeb), Forcelet (a.i 50% Carbendazim), and Funguforce (63% mancozeb + 12.5% carbendazim) were tested. The fungicides were suspended in sterile water and 0.1 ml was dispensed in 90 mm diameter perti dishes with sterile needle and syringe. Ten millilitres Potato Dextrose Agar (PDA) was dispensed, gently swirled and allowed to solidify. PDA without fungicides served as control. After solidification, each dish was inoculated with a 5-mm diameter disc obtained from an actively growing margin of the fungal colony. There were 5 replicates of each treatment. The Petri dishes were incubated at room temperature with radial mycelial growth measured daily until the control treatment was fully covered with the mycelia growth of the fungus. Radial growth was measured along two axes on pre- drawn perpendicular lines on the reverse side of the plate. Fungitoxicity was also expressed as percentage inhibition of mycelia growth using the formular adopted from Awuah (1989);

$$Mp = \frac{M_1 - M_2}{M_1} \times 100$$

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Where Mp = percentage inhibition of mycelia growth

M_1 = mycelia growth in control plate

M_2 = mycelia growth in fungicide treated plate

2.7 Statistical analysis

Data obtained from the radial growth were subjected to statistical analysis and means were compared using Duncan Multiple Range Test at 5% level of probability.

3. Results

3.1 Isolation of pathogens from diseased leaves

Pathogens that were isolated from diseased leaf samples were *B. theobromae* and *Aspergillus niger.* The frequency of occurrence of *B. theobromae* was 28 while those of *Aspergillus niger* was 2. The percentage occurrence of *B. theobromae* and *A. niger* was 93.3% and 6.7% respectively (Table 1).

TABLE 1 Frequency of occurrence of *Botryodiplodia theobromae* and *Aspergillus niger* isolated from infected pineapple leaves

Pathogen	No of samples	Frequency of occurrence	% occurrence
B. theobromae	30	28	93.3
A. niger	30	2	6.7

3.2 Isolation of pathogens from diseased pineapple rhizosphere

The pathogens that were isolated from the diseased pineapple rhizosphere include; *A. niger, A. tamari, Fusarium verticilliodes* and *B. theobromae.* The frequency of occurrence of *A. niger* was 4. *Aspergillus tamari* and *F. verticilliodes* had frequency of occurrence of 1 while the frequency of occurrence of *B. theobromae* was 20. *Botryodiplodia theobromae* had the highest percentage occurrence of 80% followed by *A. niger* with percentage occurrence of 16. However, *A. tamari* and *F. verticilliodes* had percentage occurrence of 4% (Table 2).

TABLE 2 Frequency of occurrence of pathogens isolated from diseased pineapple rhizosphere

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Pathogen	Sample size	Frequency of occurrence	% occurrence
A.niger	25	4	16
A.tamari	25	1	4
F. verticilliodes	25	1	4
B. theobromae	25	20	80

3.3 Pathogenicity test

When the pineapple leaves were dipped into the fungal suspensions, results revealed that *B. theobromae* was responsible for the dieback infection on the leaves. There was symptomatic drying of leaves which resulted into the characteristic dieback from the top of the leaves towards the base (Fig. 1a). However, this symptom was not noticed on the leaves dipped into the fungal suspensions of the other ioslated pathogens. Both the young (a) and old (b) cultures of *B. theobromae* were shown in Fig. 1b.

Fig. 1. (a) Diseased pineapple plant (b) Pure cultures of *Botryodiplodia theobromae*

3.4 Evaluation of fungicides against Botryodiplodia theobromae

The fungicides used for the control of *B. theobromae in-vitro* were Z-force, forcelet and funguforce. Z-force and funguforce had 80% and 63% active mancozeb respectively. In addition, funguforce had 12.5% carbenda~~zine~~ while forcelet had 50% carbenda~~zin~~ (Table 3).

Z-force and funguforce totally inhibited the growth of *B. theobromae*. Radial growth of 5 mm was obtained when forcelet was used and 94.25% inhibition was obtained with this fungicide. The radial growth of *B. theobromae* in the control plate was 87 mm ~~and there was no inhibition on this plate~~ (Table 4, Fig. 2).

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Table 3 List of fungicides with their description

Product/ trade name	Active ingredient (a.i)	Formulation	Rate (kg/ ha)
Z-force	80% active mancozeb	80wp	2.0kg
Forcelet	50% carbendazin	50wp	0.5 – 1.5kg
Funguforce	63% mancozeb and 12.5% Carbendazin		2.5kg

TABLE 4 Inhibitory effect of fungicides on radial mycelial growth of *B. theobromae*

Product/ trade name	Radial growth (mm)	Percentage inhibition
Z-force	0.00a	100.00
Forcelet	5.00b	94.25
Funguforce	0.00a	100.00
Control	87.00c	0.00

Values are means of 4 replicates per treatment. Means with similar alphabet along the column are not significantly different ($P \leq 0.05$) according to Duncan Multiple Range Test.

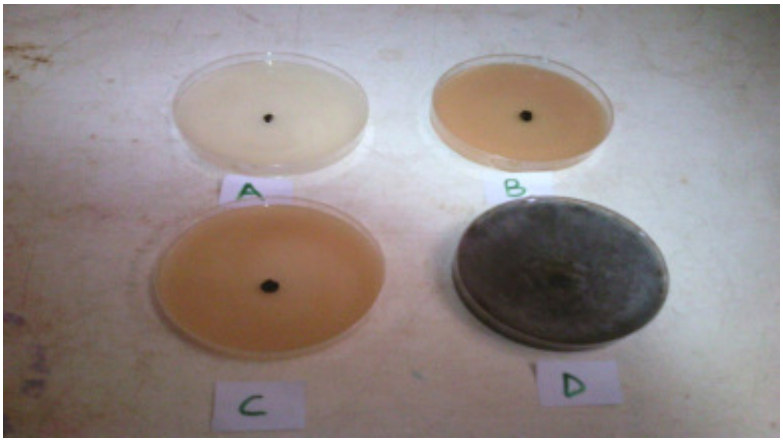

Fig. 2. Inhibitory effect of different fungicides on *Botryodiplodia theobromae* ; A = Forcelet, B = Z-force, C = Funguforce, D = Control without fungicide

4. Discussion

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The study showed that two pathogens (*A. niger* and *B. theobromae*) were isolated from diseased pineapple leaf samples. Multiple infections of agricultural crops by two or more pathogens are not uncommon in nature. The infections of horticultural crops with two or more pathogens have been reported by several authors (Diedhiou *et al*., 2007; Syed *et al*., 2014).

Aspergillus niger, A. tamari, F. verticilloides and *B. theobromae* were isolated from the pineapple rhizosphere. ~~Microorganisms are cosmopolitan in nature~~, soil having the highest number of these microbes because of the photosynthates that are released from the plant roots. This statement corroborates the report of Cook (2002) which stated that the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral and detrimental effects on plant growth. Barth *et al*. (2009) also reported that most microorganisms that are initially observed on whole fruit or vegetable surfaces are soil inhabitants and vectors for disseminating these microbes include soil particles, airborne spores, and irrigation water.

Botryodiplodia theobromae had the highest frequency of occurrence as well as the highest percentage occurrence both in the leaves and the rhizosphere. This could be attributable to the cosmopolitan nature of *B. theobromae*. Domsch *et al*. (1980) and Sutton (1980) reported that *B. theobromae* is a cosmopolitan soil-borne fungus causing both field and storage diseases on more than 280 plant species including crops, fruits and plantation trees. This result is also in agreement with the report of Ambreen *et al*. (2014) which stated that the infection frequency of *B. theobromae* in three cultivars of 300 mango samples was 67.7%.

Pathogenicity test revealed that *B. theobromae* was the only pathogen that produced similar symptoms that were expressed by diseased pineapple samples. This is because pineapple leaves dipped in the suspension of *B. theobromae* could produce the typical dieback symptoms in pineapple on the field. Ko *et al*. (2004) have reported *B. theobromae* as the causal pathogen of dieback of Kumquat. The result of this study is also in agreement with the work of Anthony *et al*. (2004) in which it was stated that *B. theobromae* was associated with dieback in lemon fruits. The disease has also been reported to cause blight and dieback of small branches in sugar apple (Farr *et al*., 1989); root rot of *Brachychiton poplneus* seedlings (Sandlin and Ferrin, 1992); leaf necrosis and stem cankers on Proteas (*Protea magnifica*) (Denman *et al*., 2003) and Cashew gummosis in Brazil (Cardoso *et al*., 2004). However, this result did not agree with the report of Vasquez and Mata (2014) who observed that the causal agent of pineapple dieback in Costa Rica was *Fusarium oxysporum*.

Infected pineapple plants showed dieback symptoms on pineapple leaves. The dryness of the leaves may be explained by the pathogenic effect of the causal organism on the pineapple leaves. This affects photosynthesis and also affects the normal functioning of the vascular bundles, thereby, preventing the flow of water and nutrients. This result corroborates the work of Obregon and Mata (2008) who stated that the causal pathogen is in the vascular system of the plants, leading to blockage and /or translocation deficient water and nutrients to the upper portions of the plant.

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The effectiveness of the fungicides against *B. theobromae* could be due to the fact that the active ingredients in the fungicides are capable of inhibiting the growth of the organism. This conforms to the report of Sharma *et al*. (1994) which stated that carbendazin was the most effective fungicide for the control of *B. theobromae*. This result also corroborates the work of Wang *et al*. (2007) who found that the mixture of cyprodinil and fludioxinil inhibited mycelial growth of *B. theobromae* isolates from papaya. Bester *et al*. (2007) reported that prochloraz and tebuconazole at low EC50 (< 0.6 μgmL^{-1}) inhibited mycelial growth of *L. theobromae* isolates from grapevines.

5. Conclusion

This study revealed that *B. theobromae* was the causal pathogen of pineapple die-back. All the fungicides evaluated were effective in the control of ~~the~~ pathogen. Further studies are, however needed to confirm the efficacies of these fungicides on the field.

References

Ambreen, M., Rehman, A., Ahmad, ~~I,~~ Nafees, M., Ashraf, I., Qureshi, R., Jamil, M., Rafay, M. & Hussain, T. (2014). Physiological attributes of fungi associated with stem end rot of mango (*Mangifera indica* L.) cultivars in postharvest fruit losses. Pakistan Journal of Botany, 46(5):1915-1920.

Anthony, S., Abeywickrama, K., Dayananda, R., Wijeratnam, S. & Arambewela, L. (2004). Fungal pathogens associated with Banana fruit in Sri Lanka, and their treatment with essential oils. Mycopathologia, 157: 91-97.

Aroca, A., Raposo, R., Gramaje, D., Armengol, J., Martos, S . & Luque, J. (2008). First report of *Lasiodiplodia theobromae* on rootstocks mother grapevines in Spain. Plant Disease, 92:832.

Awuah, R.T. (1989). Fungitoxic effects of some West African plants. Annals of Applied Biology, 115:451-453.

Barth, M., Hankinson, T.R., Zhuang, H. & Breidt, F. (2009). Microbiological Spoilage of fruits and vegetables. W.H. Sperber, M.P. Doyle (eds.), *Compendium of the Microbiological Spoilage of Foods and Beverages*, Food Microbiology and Food Safety. C Springer Science+Business Media, LLC, pp135-183.

Barnett, H.~~L,~~ and B.B. Hunter (1972). Illustrated General of Imperfect fungi, 3rd edition. Burgess Publishing Co. Minnesota. 241 Pp.

Bartholomew, D.P., Paul, R. E. & Rorbach, K.G. (2003). The pineapple "Botany, Production and Uses", University of Hawaii Manoa Honolulu, USA. CABI Publishing, CABI International.

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Bester, W., Crous, P.~~W~~ & Fourie, P.H. (2007). Evaluation of fungicides as potential grapevine pruning wound protectants against Botryosphaeriaspecies. ~~Australia~~ Plant Pathology, 36:73-77.

Cardoso, J.~~E.~~, Maia, C.M. & Pesso, M.N.G. (2002). Occurrence of *Pestalotiopsispsidii* and *Lasiodiplodia theobromae* causing stem rot of guava plants in the State of Ceará, Brazil. Fitopatol. Brasil. 27:320.

Cardoso, J.E., Santos, A.A., Rossetti, A.G. & Vidal, J.C. (2004). Relationship between incidence and severity of cashew gummosis in semiarid north-eastern Brazil. Plant Pathology 53: 363-367.

Cook, R.J. (2002). Advances in plant health management in the twentieth century. Annual Reviews of Phytopathology, 38: 95-116.

David, W. (1986). Pineapple production, Mon Repos, East Coast Demerara. National Agriculture Research Institute.

Davis, R.M., Farrald, C.J. & Davila, D. (1987). *Botryodiplodia* trunk lesions in Texas citrus. Plant Disease, 71:848-849.

Denman, S., Crous, P.~~W.~~ Groenwald, J.~~Z.~~ Slippers, B., Wingfield, D. & Wingfield, M.J. (2003). Circumscription of *Botryospheria* species associated with Proteaceae based on morphologyand DNA sequence data. Mycologia, 95: 294-307.

Diedhiou, P.M., Mbaye, N., Drame, A. & Samb, P.I. (2007). Alteration of post harvest diseases of mango *Mangifera indica* through production practices and climatic factors. African Journal of Biotechnology, 6(9): 1087-1094.

Domsch, K.H., Gams, W. & Anderson, T.H. (1980). Compendium of Soil Fungi. Academic Press, NY.

Ebele, M.I. (2011). Evaluation of aqueous plant extracts to control rot fungi in paw paw. Journal of Applied Biosciences, 37:2419-2424.

Faber, G.A., Bender, G.S. & Ohr, H.D. (2007). Diseases.UC IPM Pest management Guidelines. UC ANR publication. P. 3436.

FAOSTAT, (2011). [Online] Available: http://www.fao.org/ (August 30, 2013).

Farr, D.F., Bills, G.F., Chamuris, G.P. & Rossman, A.Y. (1989). Fungi on Plants and Plant Productin the United States. APS Press, Minnesota, USA.

Freire, F., Cardoso, C .O., dosSantos, J.E.A. & Viana, F.M.P. (2002) Diseases of cashew nut plants (*Anacardium occidentale* L.) in Brazil. Crop Protection, 21:489-494.

Ko, W.H., Wang, I.T. & Ann, P.J. (2004). *Lasiodiplodia theobromae* as a causal agent of Kumquat Dieback in Taiwan. Plant Disease, 88: 1383.

Marlatt, M.L., Correll, J.C. & Kaufmann, P. (1996). Two genetically distinct populations of *Fusarium oxysporum* f.sp.lycopersici Race 3 in the United States. Plant Disease, 80: 1336-1342.

Obregon, G.M. & Mata, G.X. (2008). Enfermedades emergentes en el cultivo de la pina. In: X congreso Mundial de *Trichoderma* y *Gliocladium*, pp 10. San Jose, Costa Rica. Asociacion de Profesionales en Enfermedades de Plantas.

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Punithalingam, E. (1980). Plant disease attributed to *Botryodiplodia theobromae.* Cramer, Vaduz, Litchtnstein Berlin. Pp 113.

Rieger M. (2006). Introduction to fruit crops. The Haworth Press. New York.P. 87.

Sandlin, C.M. & D.M. Ferrin. (1992). Root rot of *Brachychiton populneus* seedlings caused by *Lasiodiplodia theobromae*. Plant Disease, 76: 883-885.

Sharma, I.M., Raj, H. & Kaul, J.L. (1994). Studies on postharvest diseases of mango and chemical control of stem end rot and anthracnose. India Phytopathology, 47(2): 197-200.

Shu, S. (1999). Pineapple Processing http://pineapple.org.com.

Sutton, B.C. (1980). The Coelomycetes.Common Wealth Mycological Inst. Kew, Surrey.

Syed, R.N., Mansha, N., Khaskheli, M.A., Khanzada, M.A. & Lodhi, A.M. (2014). Chemical control of stem end rot of mango caused by *Lasiodiplodia theobromae.* Pakistan Journal of Phytopathology, 26(2): 201-206.

Vasquez, J.J. & Mata, G.X. (2014). Diagnosis of *Fusarium oxysporum* in the cultivation of pineapple *Ananas comosus* (L)Merr. Net Journalof Agricultural Science, 2(3): 107-112.

Wang, H.L., Chen, P.H., Ni, H.F. & Chen, R.S. (2007). Physiological characterization and screen of control chemicals for *Lasiodiplodia theobromae* of papaya. Plant Pathology Bulletin, 16:71-77.