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In-vitro Evaluation of Some Selected Fungicides against *Pestalotio psis clavispora* and *Pseudocochliobolus eragrostidis* Isolated from *Vitellaria paradoxa*Seedlings.

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Abstract: *Preliminary investigation into the nursery diseases of Vitellaria paradoxa in the Nigerian Institute fo r Oil Palm Research substation, Bida, Niger state, revealed mainly the presence of leaf pustules and leaf blight disease. Symptoms of the leaf pustules disease results in a total defoliation of the leaves of the plant while that of the leaf blight is characterized by fire burnt edges around most of the leaves in a single seedlings or shrub. Mic roorganisms isolated from the infected leaf samples using the direct plating techniques on potato dextrose agar include Pestalotiopsis clavispora and Botryodiplodia (Lasiodiplodia spp) from the leaf pustules while P. clavisp ora and Pseudocochliobolus eragrostidis were isolated from the leaf blight. The result of the effect of the fungici des captan, benlate dithane M-45and difolatan at 500 and 250ppm on the in-vitro mycelia growth of P. clavispo ra and P. eragrostidis for a test period of nine days shows benlate at 500ppm to completely inhibit the mycelia g rowth of P. clavispora and P. eragrostidis. The fungicide dithane did not completely inhibit the pathogens at sa me concentration of 500 and 250ppm. The mode of action of the fungicide benlate on P. clavisopra and P. eragr ostidis was fungicidal. It's therefore recommended that the fungicide benlate be used for field trials.*
Key words: *Blight, Pustules Pestalotiopsis clavispora, Pseudocochliobolus eragrostidis, Vitellaria paradoxa*

I. Introduction

The Shea butter tree (*Vitellaria paradoxa* C. F. Gaertn.) is a tree of economic importance as it contributes to red uction of rural poverty, hunger and disease and enhancing environmental sustainability. The fruit pulp, which ha s excellent nutritional content (Ugese *et al.,* 2008) is widely consumed among indigenous peoples of Africa (Ma ranz*et al.*,2004) while among some ethnic groups, the flowers are made into fritters (ICRAF, 2000).Locally, the oil is used as a cooking fat while in Europe and Japan; it is used in chocolate manufacture (Umali and Nikiema, 2002).Caterpillars of *Cirinabutyrospermi*, associated with the species are eaten by some ethnic groups in Nigeri a such as the Yoruba, Nupe and Tiv (Ande, 2004; Ugese *et al*., 2005).Shea butter is also used in production of c osmetics (Boffa *et al*., 1996). The increasing popularity of the butter in cosmetics the world over is attributed to its skin protecting and rejuvenating properties (FAO, 2006). The tree confers stability on the land resource there by curtailing degradation (Boffa *et al*., 1996). This is achieved partly through improvement of soil structure by t he slow decomposing leaf litter. Trade in Shea tree products has been reported to improve the incomes and livin g standards of rural farm families and the economies of exporting countries (Popoola and Tee 2001).

The tree provides many different uses. Shea bark is used for medicine to cure ailments in skin treatment in child ren- especially newborns and treat minor scratches and cuts, the leaves are eaten as a vegetable in Yewa, North Central Nigeria, and Benue (George, 2011, Abidemi, 2009). They also reported that the extract from the leaves has been used to relieve headaches and as an eye bath; the nutshell has a built- in mosquito repellant; and the but ter which has been a central element of this report. Butter made from the Shea nut is used for skin moisturizing c reams and lotions, cooking, soap, and in manufacturing chocolate.

The objective of this study was to assess the incidence of the leaf spot diseases of *V. paradoxa*, identify the caus al organisms and proffer possible control measures using fungicides application.

II. Material And Methods

The survey report was conducted in the Nigerian Institute for Oil Palm Research Substation, Bida and Dzujagi c ommunity of Niger State, Nigeria.

Isolation of pathogens

Seedlings of *V. paradoxa* were inspected for various forms of disease symptoms of seedling diseases. Portion of tissue segments of the advancing margin of lesions were excised, surface sterilized by washing samples with sod ium hypochlorite solution and then rinsed about three times in sterile distilled water, blotted dry with sterile tiss ue paper, and plated on potato dextrose agar. The cultures were incubated under room temperature.

Identification of fungal isolates
Molecular identification of the isolated pathogens was carried out by the Commonwealth Mycological Institute (UK).

Fungicidal trials
In-vitro experiments were carried out to find effective fungicides against the isolated pathogens. Four fungicides namely, Benlate Dithane M-45, Captan, and Difolatan were evaluated at 500 and 250ppm concentrations using the food poison technique. The desired concentrations of the fungicides were obtained by adding appropriate am ount of stock solution of individual fungicide in potato dextrose agar (PDA) medium in conical flask. Amended PDA medium was poured separately into Petri dishes and replicated five times for each treatment. PDA medium without fungicide served as control.
Each plates were inoculated with the mycelial disc of each pathogens (5 mm) taken from the periphery of 7 day old culture grown on PDA. The inoculated plates were incubated at 28±1°C till the fungus growth covered the pl ate in case of control. The average growth was determined for each isolates at day 3, day 6 and day 9.

Determination of mycelial radial growth
The centre of each of five replicates PDA plates was inoculated with a 0.5 cm diameter mycelial disc c ut from the periphery of a 7 day-old culture of each isolates and was incubated at room temperature for 9 days. At 3 days interval, the radial growth of mycelium was measured along the intersecting lines and the mean of the two measurements was recorded for each of three replicates. By subtracting 0.5cm initial diameter of inoculum f rom the mean, the total growth of mycelium was thus determined.

Determination of the fungistatic and funigicidal activity of the fungicides
Growth medium
Modified Czapek dox broth (MCDB) was used as the basal medium for culturing the fungi. The composition of the medium consisted of glucose, 30.0; Yeast extract, 1.0; $NaNo_3$, 2.0; KH_2PO_4, 1.0; $MgSO_3$ 0.5; KCL, 0.5 and $FeSO_4$ 0.01 (g/l distilled water).The medium was dispensed into a 250mls conical flask and then sterilized in an autoclaved at 121^0c for 15 minutes and then evaluated at 250ppm and 500ppm with the respective fungicides be nlate, captan difolatan and dithane M45. MCBD medium without fungicides serve as the control.

Inoculation and incubation procedure
The fungi species was cultured on PDA at 28 ± 2^0c for 7 days. A cork borer of 0.5 cm diameter was used to cut a mycelial disc from the periphery of the 7 day old isolates of *P. clavispora* and *P. eragrostidis* then inoc ulated into conical flask containing the MCDB medium. The conical flasks were then incubated for 3, 6 and 9 da ys.The cultures were placed in a rotary shaker to break up the mycelia mat. All experiments were conducted wit h five replicates

Fungistatic and funigicidal assay procedure
At the end of each incubation period, the MCDB medium were observed for mycelia growth by harvest ing the inoculated mycelia disc, inoculated on PDA without any fungicides before incubating under room tempe rature.

III. Results

Leaf pustules disease
The study found that all stages of infection of the leaf pustules spot to occur both at the nursery and young field of the plant. The sequence of infection and lesion development were different in all categories of the disease. Inf ections of the leaf pustules in the nursery was observed to have started as small pinhead points from which the le sions enlarged and thus forming a black pustules as shown on plate ii above. The acute stage of the diseases resu lts in a total defoliation of the leaves of the plant (plate iii above). The distribution of the lesions on the leaves di d not follow any regular pattern. However, lesions traversed all ribs on the lamina, except the midrib. These sy mptoms gradually progress and leads to the death of the plant.
The resulting colonies were white with a regular margin and rough edges. Identifications were undertaken by co mparing the sequence obtained from the sample with those available from the European Molecular Biology Lab oratory (EMBL) database via the European Bioinformatics Institute (EBI). Where matches of 99-100% identity were obtained, identification was provided to species level or where appropriate to species aggregate, provided t hat matches included a sequence derived from type or other validated culture and when there was a clear sequen ce distinction between taxa. The sequence from this sample showed 100% identity to ITS sequences reported fro m various species of *Pestalotiopsis* including *P. clavispora* and *P. mangiferae* with IMI No 502905.

Leaf blight

The leaf blight disease is characterized by fire burnt edges around most of the leaves in a single seedlings or shr ub. Acute stage can result in a complete death of the branch showing the symptoms or the seedlings (shrubs stag e)

The ITS sequence obtained from this sample showed top matches at >99% identity to ITS sequences from strain s of *Curvularia clavata*, *Curvularia eragrostidis* with IMI No 502907and its teleomorphic state *Pseudochliobol us eragrostidis* (syn. *Cochliobolus eragrostidis*) and to members of the genus *Curvularia* which have not been f ully named to species level. Top matches include 99.8% identity to a sequence from *P. eragrostidis* (under its sy nonym *Cochliobolus eragrostidis*). From the morphological examination, conidia were observed to be in the ran ge 20-24 x 11-14µm, broadest in the centre, three-septate with a markedly thickened second septum.

Efficacy of in-vitro fungicidal trial

The results of the effect of the fungicides benlate, captan, difolatan and dithane M45 at 500 and 250ppm on the *i n-vitro* mycelia growth of *P. clavispora*, for a test period of nine days shows captan at 500 and 250ppm, benlate at 500ppm and difolatan at 500ppm to completely inhibit the *in-vitro* mycelia growth of *P. clavispora* as shown in figure i below. The fungicides dithane did not completely inhibit the same pathogen at the same concentration of 500 and 250ppm. However, signs of growth inhibition were observed by the fungicide dithane M45 for the p athogens *P. clavispora* when compared to the control during the test period as shown in figure i below.

The results of the effect of the fungicides benlate, captan, difolatan and dithane M45 at 500 and 250ppm on the *i nvitro* mycelia growth of *P. eragrostidis*, for a test period of nine days shows benlate at 500ppm to completely i nhibit the *in-vitro* mycelia growth of *P. eragrostidis* as shown in figure ii below. The fungicides captan, difolata n and dithaneat 500 and 250ppm did not completely inhibit the pathogen. However, signs of growth inhibition w ere observed by the fungicides on the pathogen *P. eragrostidis* when compared to the control during the test peri od as shown in figure ii below.

IV. Discussion

Pestalotia clavisopora and *Botryodiplodia* (*Lasiodiplodia* spp) were isolated as microorganisms associ ated with the leaf pustules. This report was also in agreement with the work of Akrofi and Amoah, (2009), who also isolated *Pestalotia* and *Botryodiplodia* (*Lasiodiplodia* spp) from the leaf spot disease of *V. paradoxa* in Gha na.

The *P. clavispora* isolated from the *V. paradoxa* seedlings in this study were however morphologically similar to that isolated from blueberry in China which was described by Luan *et al*., (2008) as having a black an d globular acervuli with a diameter of 100 to 200µm long. The base of each was also described to have a swolle n conidiophores and globose with phialides growing from the apical base. This characteristic morphological feat ures were however observed in the *V. paradoxa* isolate when the pH of the growth medium (PDA) was slightly akaline.

Pestalotoiopsis Steyaert is an appendage-bearing conidial anamorphic form (coelomycetes) in the famil y Amphisphaeriaceae (Kang *et al.* 1999), and molecular studies have shown that *Pestalotiopsis* is monophyletic (Jeewon *et al.* 2004). Species of *Pestalotiopsis* are common in tropical and temperate ecosystems (Bate-Smith a nd Metcalfe 1957) and may cause plant disease (Das *et al*. 2010), are often isolated as endophytes (Watanabe *et al*., 2010), or occur as saprobes (Liu *et al*. 2008). The genus has received much attention from the scientific com munity. However, this is not because of its pathogenic nature (Yasuda *et al*. 2003), but rather because its species have been shown to produce many important secondary metabolites (Xu *et al*. 2010).

Pseudocochliobolus eragrostidis belongs to the phylum Ascomycota and family Pleosporaceae, and is described as the teleomorph of *Cochliobolus eragrostidis* (from rice and *Eragrostis tef*) Tsuda and Ueyama, (1985). *C. e ragrostidis* has also been reported to cause brown spot disease of asparagus (Salleh *et al*., 1996), leaf tip blight o f spider lilly causing straw yellow coloured leaf spot which later form necrotic blight, with dark yellow halo inte rfacing diseased. The description of brown leaf spot disease caused by *P. eragrostidis* in *V. paradoxa* seedlings r ecorded in this study was quite similar to those of the tip leaf blight of spider lilly.

This is the first report of *P. eragrostidis* causing leaf blight disease in *V. paradoxa* seedlings.

The *in-vitro* control of *P. clavisopra* and *P. eragrostidis* using chemical based fungicides showed captan at 500a nd 250ppm, benlate at 500ppm and difolatan at 500ppmto significantly reduce the mycelia growth of the fungi *P .clavisopra* from the very first day of the experiment; showing 100% level of mycelia inhibition by these chemi cals and 0% level of tolerance by the organism. While the fungicides dithane at 500 and 250ppm did not record any significant mycelia inhibition. Captan, difolatan and dithane M-45, were not significantly effective in the re duction of the pathogenic activities of *P. eragrostidis* compared to the control experimental set up; as 100% leve l of tolerance to the chemical based fungicides was recorded on potato dextrose agar treated with these fungicide s.

There was an increased inhibition of the mycelia growth of the pathogens *P .clavisopra* and *P. eragrostidis* with increase concentration of the active ingredients. Benlate (Benomyl) was the most effective and potent while cap tan and dithame M45 performed poorly. Pandey (1988) also showed that captan performed poorly, but it had an adverse effect on the phylloplane of guava. The effectiveness of captan, dithane M45, and difolatan may be impr oved with further increase in their concentration.

Oruade-Dimaro, (2010), reported that the ability of the fungal discs to resume growth when taken from inhibitor y level to PDA without fungicides showed that the fungicides were fungistatic. In this study, *P. eragrostidis* and *P. clavispora* did not resume growth when taken away from PDA containing benlate, showed that benlate had a fungicidal effect on *P. eragrostidis* and *P. clavispora*.

The fungicides benlate (Benomyl) is a systemic benzimidazole fungicide that is selectively toxic to microorgani sms and invertebrates. It functions by interfering with meiosis and intracellular transportation.It is used against a wide range of fungal diseases of fields' crops, fruits, nuts ornamentals, mushroom and tuff. Formulations includ e wettable powder, dry flowable powder and dispersible granules (Wierzbicka, 1987).

The ability of the fungicides benlate to completely inhibit the growth of *P. clavispora* and *P. eragrostidis* maybe attributed to it functions of interfering with meiosis and intracellular transportation.

In the control of the leaf pustules and leaf blight diseases of *V. paradoxa,* it is recommended that the fungicides benlate be used for field trials.

However due to the high risk of fungicides to humans, animals, environmental and the increasing rate at which p athogens develop resistance against fungicides, it therefore necessary to developed a more integrated approach which needs to be used simultaneously so as to reduce economic and environmental problems.

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Plate i: Healthy Shea seedlings


Plate ii: Acute stage of the leafspot disease with pustules


Plate iii: Chronic stage of the leafspot disease with pustules

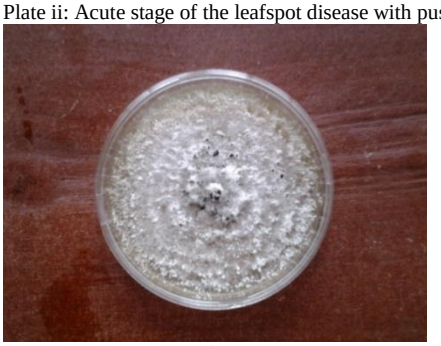


Plate iv: Culture plate of *P. clavisopra*grown on PDA under room temperature for 7 days


Plate v: Leaf blight disease of Shea seedlings

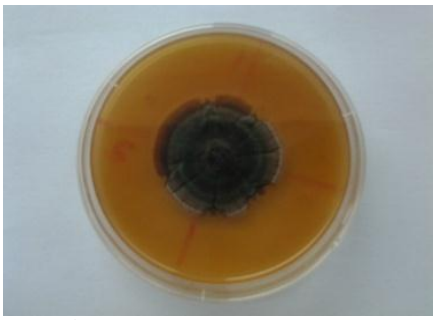


Plate vi: Culture of*P. eragrostidis* grown on PDA under room temperature for 7 days

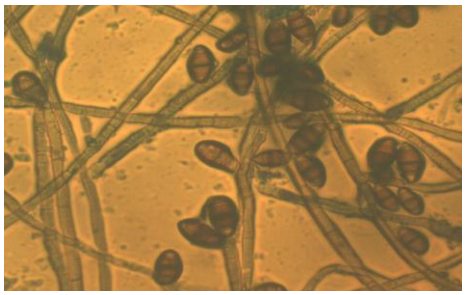
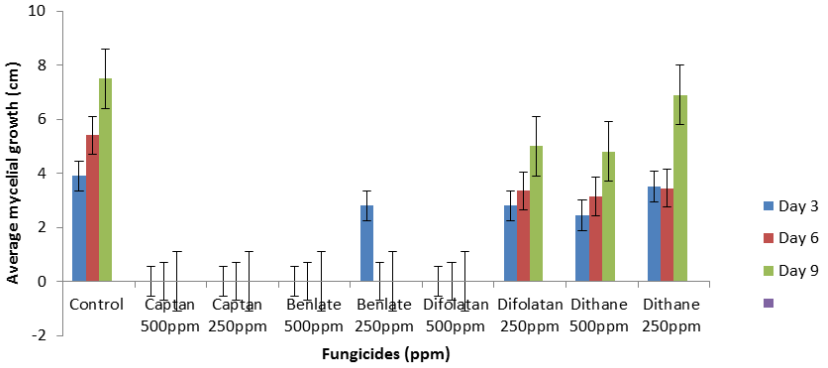


Plate vii: Photomicrograph of *P. eragrostidis* grown on PDA under room temperature for 7 days

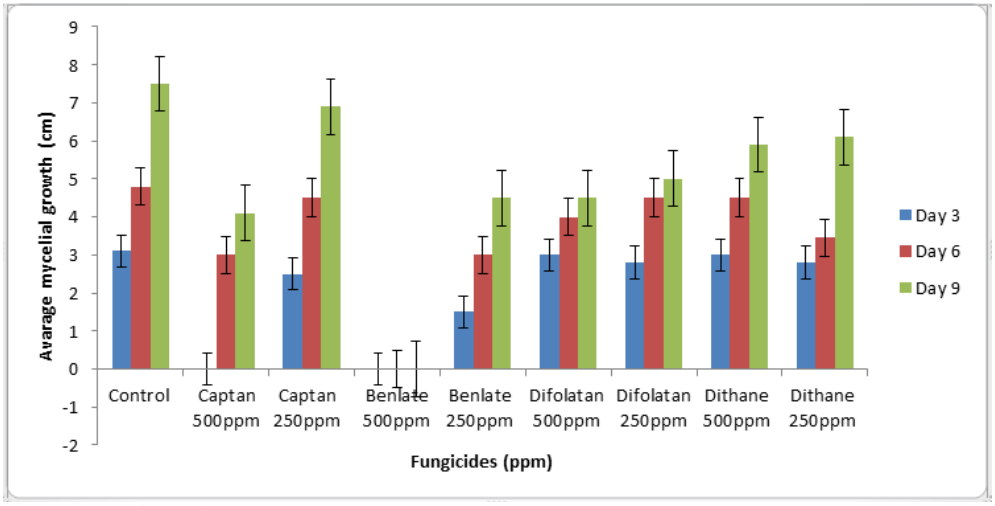
Table i: **Morphological description of the microorganisms associated with *V. paradoxa*seedlings**

Disease type	**Organisms suspected**	**Morphological description**
Leaf pustules	*Pestalotiopsisclavispora*	White mycelia growth
	Botryodiplodia(*Lasiodiplodia*sp)	Black fluffy mycelia growthLe
af blight	*Pestalotiopsis clavispora*	White mycelia growth
	Pseudocochlioboluseragrostidis	Black mycelia growth



Error bars represent the S.E for the five replicates

Figure i: Effect of Captan, Dithane, Difolatan, and Benlate fungicides at 500 and 250ppm on the *in-vitro* mycelia growth of *P. clavisp ora* grown on PDA under room temperature for 9 days.



Error bars represent the S.E for the five replicates

Figure ii: Effect of Captan, Dithane, Difolatan, and Benlate fungicides at 250ppm on the *in-vitro* myelial growth of *P. eragrostidis* g rown on PDA under room temperature for 9 days.