**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 for 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 single seedlings or shrub. Microorganisms 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. clavispora and Pseudocochliobolus eragrostidis were isolated from the leaf blight. The result of the effect of the fungicides captan, benlate dithane M-45 and difolatan at 500 and 250ppm on the in-vitro mycelia growth of P. clavispora and P. eragrostidis for a test period of nine days shows benlate at 500ppm to completely inhibit the mycelia growth of P. clavispora and P. eragrostidis. The fungicide dithane did not completely inhibit the pathogens at concentration of 500 and 250ppm. The mode of action of the fungicide benlate on P. clavispora and P. eragrostidis was fungicidal. It’s therefore recommended that the fungicide benlate be used for field trials.

**Key words:** Blight, Pustules Pestalotio psis clavispora, Pseudocochliobolus eragrostidis, Vitellaria paradoxa

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**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 has excellent nutritional content (Ugese et al., 2008) is widely consumed among indigenous peoples of Africa (Maranz 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 Cirinabutyrospermia, associated with the species are eaten by some ethnic groups in Nigeria such as the Yoruba, Nupe and Tiv (Ande, 2004; Ugese et al., 2005). Shea butter is also used in production of cosmetics (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 reducing soil compaction (George et al, 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 repellent; and the butter which has been a central element of this report. Butter made from the Shea nut is used for skin moisturizing, 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 causal 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 communities 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 sodium hypochlorite solution and then rinsed about three times in sterile distilled water, blotted dry with sterile tissue paper, and plated on potato dextrose agar. The cultures were incubated under room temperature.
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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 amount 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 plate 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 cut 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 from the mean, the total growth of mycelium was thus determined.

Determination of the fungistatic and fungicidal 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; NaNo2, 2.0; KH2PO4, 1.0; MgSO4 0.5; KCL, 0.5 and FeSO4 0.01 (g/l distilled water).The medium was dispensed into a 250mls conical flask and then sterilized in an autoclaved at 121°C for 15 minutes and then evaluated at 250ppm and 500ppm with the respective fungicides benlate, 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°C 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 inoculated into conical flask containing the MCDB medium. The conical flasks were then incubated for 3, 6 and 9 days. The cultures were placed in a rotary shaker to break up the mycelia mat. All experiments were conducted with five replicates.

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

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. Infections of the leaf pustules in the nursery was observed to have started as small pinhead points from which the lesions enlarged and thus forming a black pustules as shown on plate ii above. The acute stage of the diseases resulted in a total defoliation of the leaves of the plant (plate iii above). The distribution of the lesions on the leaves did not follow any regular pattern. However, lesions traversed all ribs on the lamina, except the midrib. These symptoms 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 comparing the sequence obtained from the sample with those available from the European Molecular Biology Laboratory (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 that matches included a sequence derived from type or other validated culture and when there was a clear sequence distinction between taxa. The sequence from this sample showed 100% identity to ITS sequences reported from 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 shrubs. Acute stage can result in a complete death of the branch showing the symptoms or the seedlings (shrubs stage).

The ITS sequence obtained from this sample showed top matches at >99% identity to ITS sequences from strains of Curvularia clavata, Curvularia eragrostidis with IMI No 502907 and its teleomorphic state Pseudochliobolus eragrostidis (syn. Cochliobolus eragrostidis) and to members of the genus Curvularia which have not been fully named to species level. Top matches include 99.8% identity to a sequence from P. eragrostidis (under its synonym Cochliobolus eragrostidis). From the morphological examination, conidia were observed to be in the range 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 in 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 difolatan 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 pathogens 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 in vitro mycelia growth of P. eragrostidis, for a test period of nine days shows benlate at 500ppm to completely inhibit the in-vitro mycelia growth of P. eragrostidis as shown in figure ii below. The fungicides captan, difolatan and dithane at 500 and 250ppm did not completely inhibit the pathogen. However, signs of growth inhibition were observed by the fungicides on the pathogen P. eragrostidis when compared to the control during the test period as shown in figure ii below.

IV. Discussion
Pestalotia clavispora and Botryodiplodia (Lasiodiplodia spp) were isolated as microorganisms associated 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 Ghana.

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 and globular acervuli with a diameter of 100 to 200µm long. The base of each was also described to have a swollen conidiophores and globose with phialides growing from the apical base. This characteristic morphological features were however observed in the V. paradoxa isolate when the pH of the growth medium (PDA) was slightly alkaline.

Pestalotiopsis Steyaert is an appendage-bearing conidial anamorphic form (coelomycetes) in the family Amphiphaericaeae (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 and 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 community. 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). Pseudochliobolus 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 of the lily causing straw yellow coloured leaf spot which later form necrotic blight, with dark yellow halos interfacing diseases. The description of brown leaf spot disease caused by P. eragrostidis in V. paradoxa seedlings recorded in this study was quite similar to those of the tip leaf blight of spider lily. This is the first report of P. eragrostidis causing leaf blight disease in V. paradoxa seedlings.

The in-vitro control of P. clavispora and P. eragrostidis using chemical based fungicides showed captan at 500a nd 250ppm, benlate at 500ppm and difolatan at 500ppm to significantly reduce the mycelia growth of the fungi P. clavispora from the very first day of the experiment; showing 100% level of mycelia inhibition by these chemicals 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 reduction of the pathogenic activities of P. eragrostidis compared to the control experimental set up; as 100% level 1 of tolerance to the chemical based fungicides was recorded on potato dextrose agar treated with these fungicides.
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There was an increased inhibition of the mycelia growth of the pathogens *P. clavispora* and *P. eragrostidis* with increase concentration of the active ingredients. Benlate (Benomyl) was the most effective and potent white cap tan and dithane 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 improved with further increase in their concentration.

Oruade-Dimaro, (2010), reported that the ability of the fungal discs to resume growth when taken from the 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 microorganisms 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 include wettable powder, dry flowable powder and dispersible granules (Wierzbiacka, 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 pathogens 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.

Acknowledgement

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References

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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. clavispora 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

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<thead>
<tr>
<th>Disease type</th>
<th>Organisms suspected</th>
<th>Morphological description</th>
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<tbody>
<tr>
<td>Leaf pustules</td>
<td>Pestalotiopsis clavispora</td>
<td>White mycelia growth</td>
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<tr>
<td></td>
<td>Botryodiplodia (Lasiodiplodia)</td>
<td>Black fluffy mycelia growth</td>
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<tr>
<td>Leaf blight</td>
<td>Pestalotiopsis clavispora</td>
<td>White mycelia growth</td>
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<td></td>
<td>Pseudocochliobolus eragrostidis</td>
<td>Black mycelia growth</td>
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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.

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