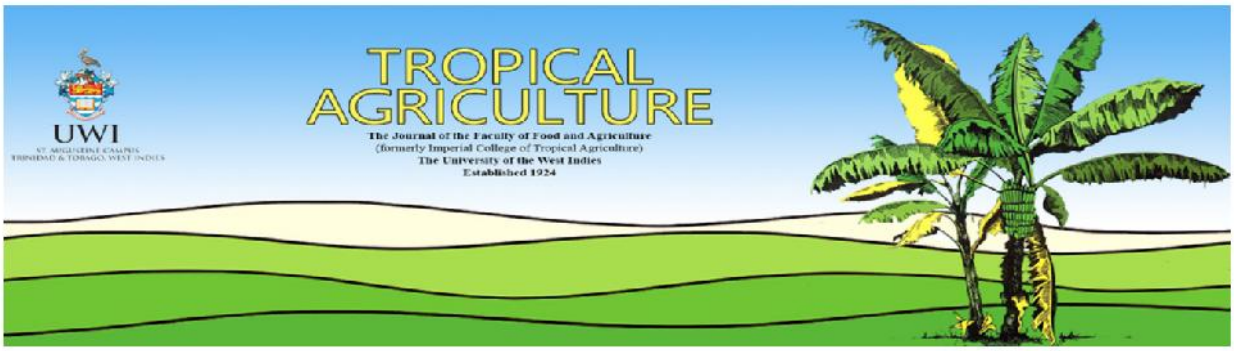

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Cotonou Benin

Dear Dr. Bachabi,

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Laboratory assessment of the antibacterial effect of *Moringa oleifera* Lam., *Azadirachta indica* L. and *Jatropha curcas* L. *on Pantoea spp* and S*phingomonas spp* in rice seed in Benin

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The antibacterial effect of *Moringa oleifera* Lam., *Azadirachta indica* L. and *Jatropha curcas* L. on *Pantoea* spp and *Sphingomonas* spp was assessed in rice seeds in Benin Republic. Isolates of three bacteria - *Pantoea stewartii* (IIIV11BE2_Sterw), *Pantoea ananatis* (IIIV6LIB1_Anan) and *Sphingomonas* spp (IIIV27BE_Sphingo) - were used. The treatments were neem oil (*Azadirachta indica*) and neem seed powder, *Moringa oleifera.* seed and leaf powder, and *Jatropha curcas*. oil. The diameter of the inhibitory zone for each treatment was measured at 72, 96, 120 and 144 hours after incubation (HAI). The effects of the treatments on the size of the inhibition zone of IIIV11BE2_Sterw and IIIV27BE_Sphingo were not significant 72 HAI but were significant on both isolates 96, 120 and 144 HAI. The largest diameters of the inhibition zone of IIIV11BE2_Sterw (0.27 cm at 96 HAI; 0.23 cm at 120 HAI and 0.23 cm at 144 HAI) were recorded with moringa seed extract. For *Sphingomonas* spp (IIIV27BE_Sphingo), the highest values of the inhibition zone were 0.20 cm with moringa seed extract at 96 and 144 HAI and 0.20 cm with moringa leaf extract at 120 HAI. *M. oleifera* Lam. has a great potential as antibacterial and its active ingredients should be identified for exploitation.

Keywords: Antibacterial plant extracts, moringa, neem, jatropha

Rice is one of the most important staples cultivated around the world. In 2015, Africa produced 28,798,202 tons of rice on 11,206,813 ha (FAOSTAT 2015). Seck et al. (2012) estimated that sub-Saharan Africa will require 30 million tons of milled rice by 2035, representing a 130% increase in rice consumption. Effective control of pests is necessary to reach this production level. Diseases constitute one of the most important constraints to rice production (Lopez et al. 2011). *Pantoea* spp and *Sphingomonas spp* have recently been isolated from rice seeds collected from several fields in Benin, Togo and Mali (Okunishi et al. 2005; Kini et al. 2016; AfricaRice 2010) - *Pantoae* spp occur only inside the seeds while *Sphingomonas* spp occur both inside and on the surface of seeds (Mano et al. 2006). Bacterial strains closely related to both genera were isolated from rice leaves (Mano et al. 2007; Okunishi et al. 2005).

Although some levels of losses have been noticed in fields where seed samples were collected, especially in Benin Republic, the extent of losses associated with these bacteria has not yet been estimated, neither have the symptoms of their attack been described. Little is known about the impact of *Sphingomonas* spp. on rice production, especially in Africa. However, *Pantoae* spp have been associated with many symptoms and losses on a variety of cereals including rice. *Pantoea ananatis* Serrano and *P. stewartii* Smith were isolated from sudangrass [*Sorghum drummondii* (Nees ex Sttroud) Millsp. and Chase) synonym *Sorghum sudanense* Stapf.]. Similar levels of disease severity were recorded for both bacteria on sorghum and sudangrass. *P.*

ananatis Serrano was found to be pathogenic on corn and oats, while *P. stewartii* Smith was pathogenic on corn, oats and tricicale (Azad et al. 2000). Stewart's bacterial wilt and leaf blight are the most important bacterial diseases of sweet corn in the north-central and eastern United States (Hyun Ham et al. 2006). On maize and wheat, *Pantoea spp* induce a decrease in productivity and grain quality in addition to lesions on leaves, stems, seeds, and roots. A decline in the resistance of crops to the diseases has been reported (Brake et al. 2000; Denli and Perez 2010). Hyun Ham et al. (2006) indicated that symptoms of both bacteria after inoculation were similar initially but differed subsequently – while *P. ananatis* induced white streaks or irregular necrotic blotches often surrounded by a reddish or purplish hue, *P. stewartii* was associated with light-colored necrotic streaks. Prevailing conditions in the tropics, such as high temperature (around and beyond 37°C) and high humidity, are favourable to *Pantoea* spp. The bacteria incubate for 2 to 20 days after inoculation depending on environmental conditions of temperature and relative humidity, as well as the inoculum level and method of inoculation. *P. ananatis* is seed-borne and seed-transmitted in rice (Tabei et al. 1988; Cottyn et al. 2001), sudangrass (Azad et al. 2000), buckwheat (Iimura and Hosono 1996) and onion (Goszczynska et al. 2006); *P stewartii* is transmitted by the corn flea beetle, *Chaetocnema pulicaria* Melsheimer, through feeding wounds. The pathogen thereafter grows in the xylem vessels of the plants and in the intercellular spaces of the leaves, causing, respectively, wilt and "water-soaked" lesions (Hyun Ham et al. 2006; Mark et al. 2002; Walcott et al. 2002). *P. ananatis* can cause up to 100% loss in onions while infestation of corn by *P stewartii* at the 9, 7 and 5 leaf stages reduced losses by 15, 35 and 100%, respectively (Pataky et al. 1988; Suparyono and Pataky 1989). Due to the large volume of field corn seed exchange across the world, *P. stewartii* poses a serious economic problem (Block et al. 1998).

Based on its transmission via seed, diverse host range, mechanism of development, short period of incubation under certain environmental conditions, and high potential yield losses associated with its attack at the leaf stages, *Pantoae spp* is a serious threat to cereals and innovative management measures are required, especially under tropical conditions. Some insecticides (clothianidin, imidacloprid and thiamethoxam) were successfully applied at 1.25 mg ai/kernel) to control Stewart's wilt (Pataky et al. 2005). However, seed treatment prior to germination has given better control of *P. stewartii*. Wopereis et al. (2008) reported that seed treatment before sowing with preferably organic products is the first component of the integrated management of rice diseases. Many recent studies have focused on the assessment of plant products for the control of harmful pathogens (Cavaleiro et al. 2006; Shivalingaiah et al. 2012). The insecticidal properties of many plant extracts are already known, exploited and widely recommended, but their bactericidal effects are still not well-known. However, neem (*Azadirachta indica*) seed oil is known for its bactericidal effects while moringa (*Moringa oleifera* Lam.) and *Jatropha curcas L.* reportedly have positive effects on human health by controlling many diseases and may be able to successfully control pathogens. Their use as a component of the integrated management of bacteria could, therefore, provide an alternative to synthetic pesticides to which many pests have become resistant (Razak et al. 2009; Zhao et al. 2007). This study assessed the bactericidal effects of *Moringa oleifera, Azadirachta indica* and *Jatropha curcas* in the control of *Pantoea spp* and *Sphingomonas spp*.

Materials and methods

Isolation of the bacteria

Bacteria were obtained from infected seeds collected from a field experiment conducted in the Sowé area, Glazoue district, in the Collines Department, Benin Republic (Lat. 8°1.21'N, Long. 2°15.37'E and Alt. 173 m). From a batch of harvested rice seeds, 100 untreated seeds were counted, cleaned with 5% hypochlorite, dried on Whatman filter paper, and plated on Nutrient Broth Yeast (NBY) comprising 8 g/l nutrient broth, 2 g/l yeast extract, 0.5 g/l KH_2PO_4, 2 g/l $KHPO_4$, 2 g/l glucose, 15 g/l agar powder, and 1000 ml of sterile distilled water. The seeds were divided into 4 replicates of 25 seeds per Petri dish and incubated in the LAB-LINE L.C OVEN at 28°C. After three days of incubation, the colony of bacteria growing around each seed was isolated with sterile Needle and then purified on a semi-selective peptone-sucrose-agar (PSA) medium (Poulin et al. 2014) containing 10 g/l sucrose, 1 g/l Na-glutamate, 15 g/l Agar, and 1000 ml of sterile distilled water. Physicochemical catalase and gram tests (Schaad et al. 2001) were performed to separate negative gram bacteria from positive ones. The negative gram bacteria were submitted to a Polymorphism Chain Reaction (PCR). The primers *KK_PANST_RPOB-rev GTC*-CTG-AGG-CAT-CAA-TGT-GT, *KK_PANST_RPOB-fwd* CAC-CGG-TGA-ACT-GAT-TAT-CG at 559bp (Kini et al. 2016) were used to identify *P. stewartii* species, *KK_PANAN_GYRB-rev* GAT-CTT-GCG-GTA-TTC-GCC-AC, *K_PANAN_GYRB-fwd* GAT-GAC-GAR-GCC-ATG-CTG-C at 423 bp *(*Kini et al. 2016) were used to identify *P. ananatis* species, while *Sphingomonas* species were identified using sphingo 108F GCGTAACGCGTGGGAATCTG and sphingo 420 R TTACAACCC TAAGGCCTTC primers. Single bacteria colonies were pale-yellow, yellow, flat transparent, and developed at medium growth pace. *P. stewartii* III V11 BE-2, *P. ananatis* IIIV6 Lib1 and *Sphingomonas* IIIV27BE isolates were used in the laboratory trials.

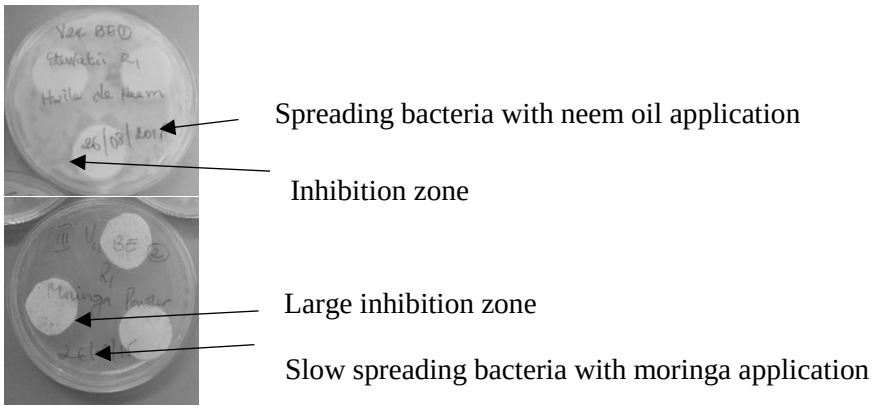
Biopesticide products

Neem (*A. indica*.) oil and seed powder, *M. oleifera* seed and leaf powder, and *J. curcas* oil were tested as biopesticides for the control of the growth of the bacteria. Neem seed, *M. oleifera* seed and leaves, and *J. curcas* seed were obtained from the International Institute of Tropical Agriculture (IITA) - Cotonou station. Neem and jatropha oil were obtained from seed kernels by cold-press-extraction (Togbe et al. 2015). The leaves and seeds were cleaned with 5% hypochlorite and dried in a warehouse before being crushed with pestle and mortar and conserved in sterilized bottles. Seeds were peeled before being crushed.

Treatment applications

Laboratory tests were conducted on a Peptone-Sucrose-Agar PSA medium in Petri dishes. For each of the five test materials, three Petri dishes, representing three replications, were prepared and kept in a completely sterilized hood at 4 °C for 72 hours before use, to check for contamination. Thereafter, bacterial isolates were spread over the PSA medium, one per three of the 15 Petri dishes. Discs of sterilized filter paper were soaked with each biopesticide product and applied before the spreading of bacteria on the medium the same day and incubated at 28°C. Each product was applied to three Petri dishes of the same species of bacteria. The Petri dishes were arranged in a randomized complete block design with three blocks.

Illustrations



Picture 1: Culture medium showing inhibition zone

Data collection and analysis

The diameter of the inhibition zone in each Petri dish was measured at 72, 96, 120 and 144 hours after incubation (HAI). Data were recorded for each biopesticide and tabulated in an Excel sheet. The most effective biopesticides had the widest inhibition zone.

Analysis of variance (ANOVA) was performed using repeated measures (Crowder and Hand 1990) with the GLM procedure (SAS 9.2, SAS Institute, Cary, NC, USA). Factors such as observation time and treatments were considered as fixed factors and replicates as a random factor. Interactions such as treatment*time were used as fixed whereas repetitions*times were random. Whenever the F-tests for fixed effects were found to be significant, a Tukey's test (α = 0.05) was performed for multiple comparisons among treatments.

Results

The effect of treatments on the inhibition zone of isolates of *P. stewarti* (IIIV11BE2_Sterw) and *Sphingomonas* spp. (IIIV27BE_Sphingo) at the various observation times was very significant as shown by the interaction treatment*time ($p<0.0.5$) but the effect of the treatments on the isolate of *P. ananatis* (IIIV6LIB1_Anan) appeared not to be significant ($p>0.05$) (Table 1). Thus, we concluded that IIIV6LIB1_Anan was not sensitive to any of the biopesticides tested.

The effect of the biopesticides on the inhibition zone of IIIV11BE2_Sterw and IIIV27BE_Sphingo was significant only 96, 120 and 144 hours after incubation but not earlier. Moringa seed extract had the widest inhibition zone on IIIV11BE2_Sterw (0.27 cm after 96 hours, 0.23 cm after 120 hours, and 0.23 cm after 144 hours) (Figure1). The widest inhibition zones for *Sphingomonas* spp (IIIV27BE_Sphingo) were obtained with moringa seed extract and moringa leaf extract (0.20 cm after 96 hours and 0.20 cm after 120 hours). The spreading bacteria with moringa is very low compared to the others biopesticides (Picture1). Moringa seed extract also had the widest inhibition zone on IIIV27BE_Sphingo (0.20 cm after 144 hours) (Figure1).

P. stewartii (IIIV11BE2_Sterw) was more sensitive than *Sphingomonas* spp. (IIIV27BE_Sphingo) and *Pantoea ananatis* IIIV6LIB1_Anan) to moringa seed extract (Figure1). Table 1 shows that the differences observed in the responses of *Pantoea stewartii* Smith were due to treatment effects, observation time, interaction between treatment and interaction between replicate. It also indicates that differences for *Sphingomonas* spp could be ascribed to

treatment effects, time of observation, interaction between treatment and observation time.

Surprisingly, the inhibition zone of IIIV27BE_Sphingo decreased from 0.20 cm after 96 hours to 0.14 cm after 120 hours with moringa seed extract and from 0.14 cm after 120 hours to 0.13 cm after 144 hours with jatropha oil. Similarly, the inhibition zone of IIIV11BE2_Sterw decreased from 0.27 cm after 96 hours to 0.24 cm after 120 hours with moringa seed extract and from 0.11cm after 120 hours to 0.10 cm after 144 hours with neem oil.

Table 1: Analysis of variance of the effect of treatments and observation time on the inhibition zone of isolates of the bacteria species *P. ananatis* (IIIV6LIB1_Anan), *P. stewarti* (IIIV11BE2_Sterw) and *Sphyngomonas* spp. (IIIV27BE_Sphingo)

Sources	Df	F value		
		IIIV6LIB1_Anan	IIIV11BE2_Sterw	IIIV27BE_Sphingo
Blocks	2	0.20 ns	2.34 ns	1.04 ns
Treatment	5	2.76 ns	27.37 ***	302.83***
Time	3	4.00 *	15.21 ***	34.40 ***
Treatment*time	15	1.03 ns	5.80 ***	14.25 ***
Repeat*time	6	0.40 ns	2.91 ***	1.00 ns
Residual	30			

ns : non-significant or significant at p <0.05 : *, p<0.01 : ** or p<0.001 : ***, respectively

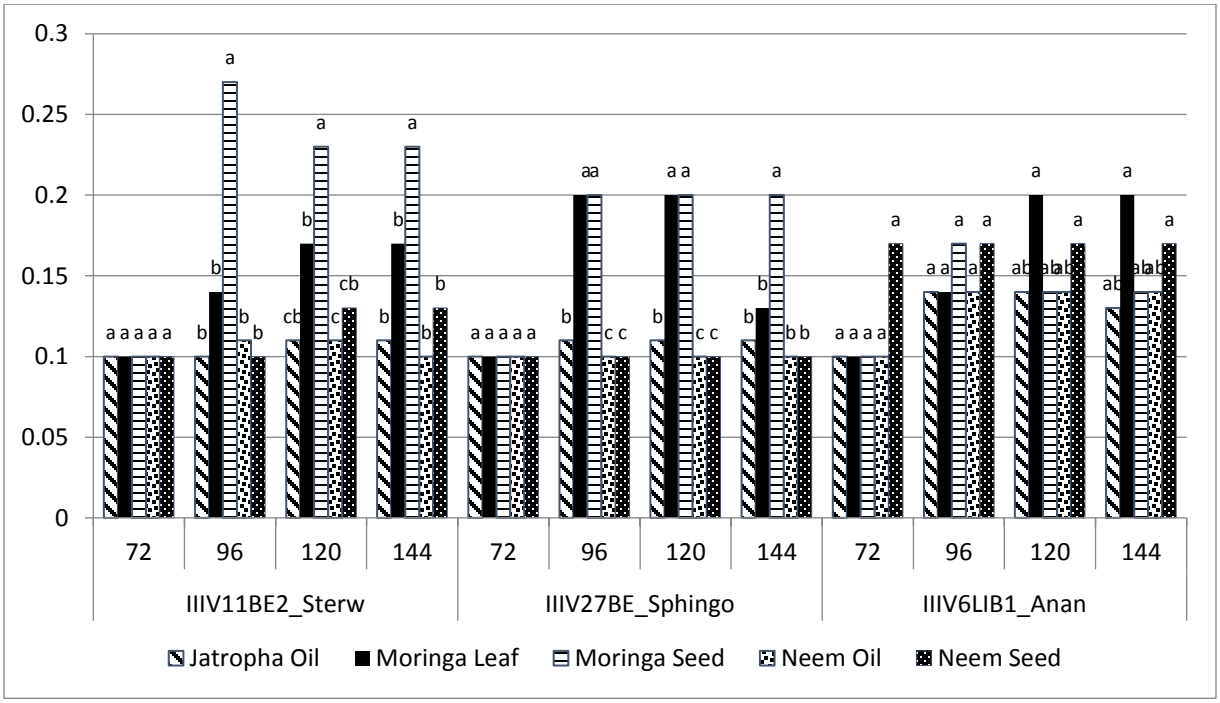


Figure 1: Mean diameter (mm) of the inhibition zone with various biopesticides applied on isolates of bacteria species *P. ananatis* (IIIV6LIB1_Anan), *P. stewarti* (IIIV11BE2_Sterw) and *Sphyngomonas* spp. (IIIV27BE_Sphingo)

Means followed by the same letter within a column are not significantly different at P<0.05 according to Tukey's test.

Discussion

The presence of inhibitory zones indicated that all the five materials tested (neem seed, moringa seed, moringa leaves; jathropha oil and neem oil) have antibacterial effects on the three bacteria (*P ananatis*, *P stewartii* and *Sphyngomonas* spp Yabuuchi). Several earlier studies have demonstrated the antibacterial effect of neem extract (Biswas et al. 2002, Banerjee et al. 2013; Mamman et al, 2013). Banerjee et al. (2013) identified the Minimum Inhibitory Concentration (MIC) of 3.13 % for *Vibrio parahaemolyticus* and 6.25% for *V. alginolyticus* and the Minimum Bactericidal Concentration (MBC) of 12.50% for *Vibrio parahaemolyticus* and 25% for *V. alginolyticus*. This effect appeared to be induced by the properties of some specific molecules present in the neem, moringa and jatropha extracts. Biswas et al. (2002) identified Nibidin as the main active ingredient in *A. indica* L. responsible for the antibacterial properties.

Also, Suhaili et al. (2011), Dada et al. (2014), and Priadarshini et al. (2013) have demonstrated the antibacterial effect of *J. curcas* and *M. oleifera*. The significant differences in the inhibitory zone of the various strains of bacteria indicate that the content of the active antibacterial ingredient in *M. oleifera* and neem oil was higher in the seed than in the leaves. The differential distribution of concentration of the antibacterial ingredients in the various parts of *A. indica*, moringa and jatropha has been alluded to by Rampadarath et al. (2016) and was strongly associated with the solvent used for extraction (Orhue et al. 2014; Priadarshini et al. 2013). Biswas et al. (2002) showed that Nibidin, the antibacterial active ingredient in *A. indica*, was more concentrated in the bark than in the leaves and concluded that this may explain why the bark is more bitter than the leaves.

Various solvents are used for extraction, including water, ethanol, 1% HCl, chloroform, acetone and petroleum ether. In the present work, only water was used for extraction, suggesting that this solvent could have limited the antibacterial effect of the active ingredients present in each part of the plants tested. Priadarshini et al. (2013) showed the higher effect of chloroform extracts of neem and moringa than petroleum ether extracts. Rampadarath et al. (2016) reported that crude ethyl acetate extract of *J. curcas* L. bark and mature seed oil had the highest efficacy. Likewise, Abalaka et al. (2012) indicated higher antimicrobial effect of crude chloroform extract against the growth of *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* compared to *M. oleifera* Lam. aqueous leaf extract. These results indicate that the efficacy of active antimicrobial ingredients strongly depends on the solvent used for the extraction. Future tests could vary the extraction solvents to determine the most appropriate method for each species and plant part.

The observed differences in the sensitivity of *P. stewarti* and *Sphingomonas* spp isolates to various biopesticides and to different doses of the active ingredients point to the need to identify the active ingredients and determine their MIC and MBC (Biswas et al. 2002; Suhaili et al. 2011; Rampadarath et al. 2016). Earlier phytochemical screening of *M. oleifera* revealed the presence of secondary metabolites, such as alkaloids, flavonoids, saponins and tannins (Abalaka et al. 2012; Ajiako 2014), and represent a great step toward the determination and characterization of the active principle responsible for antibacterial activity in *M. oleifera*. The non-sensitivity of the *P. ananatis* Serrano isolate to the various treatments applied could be explained by the development of resistance in this isolate. In fact, the seed from which these bacteria were isolated were collected from the fields where synthetic pesticides are used routinely.

The decrease with time in the mean diameter of the inhibition zone suggests a degradation of the active ingredients in these two plant species after a period of time. There is, therefore, a need to improve the

stabilization of antibacterial active ingredients in *M. oleifera*. and *J. curcas* after their identification, extraction and characterization.

Conclusions

The aqueous extracts of *Azadirachta indica*, *Moringa oleifera* and *Jartropha curcas* possess antibacterial effects which varied according to the isolates of bacteria on which they were tested. Among the three species, *M. oleifera* appeared to be the most effective and the active ingredients should be identified, screened and characterized. *P. ananatis* did not show any sensitivity to the various biopesticides. This may be a response to the development of resistance in those bacteria isolated from the seeds collected from fields where farmers are accustomed to an indiscriminate use of synthetic pesticides. This work also highlights the instability of biopesticides over time which could limit their use. This study provides the basis for exploiting *Azadirachta indica*, *Moringa oleifera* and *Jartropha curcas* as antibacterial agents in rice seed treatment.

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