

## Fungal Leaf Spot Diseases of Mango (*Mangifera indica* L.) in Southeastern Nigeria and Biological Control with *Bacillus subtilis*

RALPH N. OKIGBO<sup>1</sup> and MARIA I. OSUINDE<sup>2</sup>

<sup>1</sup>Department of Biological Sciences, Michael Okpara University of Agriculture, Umudike, Nigeria;

<sup>2</sup>Department of Microbiology, Ambrose Alli University, Ekpoma, Nigeria

### Abstract

OKIGBO R.N., OSUINDE M.I. (2003): **Fungal leaf spot diseases of mango (*Mangifera indica* L.) in Southeastern Nigeria and biological control with *Bacillus subtilis*.** Plant Protect. Sci., **39**: 70–77.

The incidence of fungal leaf spot diseases on mango (*Mangifera indica*) in Southeastern Nigeria and application of a biological control measure was investigated. The survey proved that the incidence of leaf spot diseases was greatest in Umuahia (72%) followed by Okigwe and Ojoto, with a peak at the beginning of the rainy season (February–March). Three pathogenic fungi, *Pestalotiopsis mangiferae*, *Botryodiplodia theobromae* and *Macrophoma mangiferae*, were isolated from leaf spots. Other fungi, *Fusariella* spp., which are well known saprophytes of dead plant materials, and the fungus *Meliola* sp., were also isolated from diseased leaves. Pathogenicity tests showed that *P. mangiferae*, *B. theobromae* and *M. mangiferae* were the causal agents of the fungal leaf spot diseases. Symptoms developed 5 weeks after inoculation of healthy leaves. *Bacillus subtilis* NCIB 3610, isolated from soil under a mango tree, inhibited *P. mangiferae*, *B. theobromae* and *M. mangiferae* by 57%, 61% and 58% respectively on agar plates. Also, in *in vivo* experiments the symptoms were considerably reduced in the field by the application of the antagonist. The importance of the biological control method for rural mango farmers is emphasised.

**Keywords:** *Pestalotiopsis mangiferae*; *Botryodiplodia theobromae*; *Macrophoma mangiferae*; biological control; *Bacillus subtilis*

Mango (*Mangifera indica* L.) is a tropical tree crop belonging to the family of *Anarcadiaceae*. Mango occupies relatively the same position in the tropics as enjoyed by the apple in temperate America and Europe (SINGH 1960). The cultivars of mango commonly found in Nigeria are Edward, Early Gold, Local Alphonso, July Haden and Ogbomosho (BRUNO & GOLDBERG 1963; OKIGBO 2001).

Microorganism associated with post harvest spoilage of fruits have engaged the attention of many mycologists for years (OKIGBO 2001). It has been reported that *Macrophoma mangiferae* Higorami and Sharma caused foliage blight of young seedlings and young grafted plants, while *Botryodiplodia theobromae* Pat. was the agent of die-back and bark

canker, and the gray blight of leaves is caused by *Pestalotiopsis mangiferae* (VERMA *et al.* 1991).

The attention of mycologists is presently focused on control measures of mango diseases (VERMA *et al.* 1991; OKIGBO 2001). The use of chemicals to control plant pathogens, especially foliage pathogens, has had only limited success in the past in Africa due to lack of suitable methods of application, or lack of an effective chemical and prohibitive cost. There is also the added concern about chemical residue in the environment and the development of resistance by the pathogen (SPOTTS & CERVANTES 1986; KORSTEN 1995; OSUINDE *et al.* 2001).

Biological control of plant pathogens could reduce or eliminate some of those concerns. It is

also potentially more durable and much cheaper (OKIGBO & IKEDIUGWU 2000, 2001; OSUINDE *et al.* 2001). More recently, an increasing number of reports have focussed on the potential of *Bacillus subtilis* as a bio-control agent (FERREIRA *et al.* 1991; IKEDIUGWU *et al.* 1994; KORSTEN 1995; OKIGBO 2002). The presence of endospores in *Bacillus* sp. would allow it to persist on the leaf surface of mango, especially in the hot tropics of Africa. In this study, the fungi responsible for leaf spot diseases of mango were isolated, identified and pathogenicity tests carried out. The potential to use *Bacillus subtilis* (Ehrenberg) Cohn as a control agent was also investigated.

## MATERIALS AND METHODS

### Survey, isolation and identification

Surveys were carried out in 1999–2001 in Umuahia, Enugu, Nsukka, Ojoto and Okigwe (all in Southeastern Nigeria) to determine the frequency of occurrence of fungal leaf spot disease of mango. At each site, 10 randomly selected and 4 year old mango were inspected. The frequency of occurrence was taken as the number of mango trees affected by the disease expressed as percentage of the total number of mango trees at a location. A visual assessment technique was used with which many plantations can be evaluated in a relatively short time (DERSO 1999). Analysis of variance was used to compare the mean occurrence of the disease at different locations. Diseased leaves, stems and branches were collected and brought to the laboratory for identification of the pathogen.

Two centimetres of the infected mango leaf tissue was excised with a sterilised cork borer and surface sterilised by dipping into 0.1% mercuric chloride solution for 1.5 to 2.0 min. The tissues were rinsed in three changes of sterilised distilled water and comminuted onto PDA (potato dextrose agar) in Petri dishes. These were incubated at room temperature (25–28°C) under light for 6 d to enhance fungal growth and sporulation. Subcultures were made until pure cultures were obtained. All isolates were identified using the methods of SNEATH (1978), and BARNETT and HUNTER (1972). Stock cultures of all the isolates were maintained on PDA slopes in McCartney bottles at 4°C in the dark. These were subcultured at monthly intervals. A fresh subculture was used for each experiment.

### Pathogenicity tests

Colonies of *Botryodiplodia theobromae*, *Macrophoma mangiferae* and *Pestalotiopsis mangiferae* from mango leaves with spot disease were incubated for 10–15 d on a laboratory bench at room temperature (26–28°C). Spore suspensions were prepared by centrifuging and re-suspending the spores in three changes of sterile distilled water. Healthy leaves on a young mango tree were surface sterilised with 5% mercuric chloride and then rinsed with sterile distilled water. The lower epidermis of the leaves were sprayed separately with a spore suspension from each of the three fungi, using a rocking sprayer. A mixture of the spore suspensions of the three fungi was also used as inoculum by mixing 50 ml of each suspension. There were three replicates of the experiment on different branches of a tree. One healthy branch was sprayed with sterile distilled water and served as control. All branches were covered with sterile cellophane bags for 5 weeks until symptoms of the diseases were apparent, at which the bags were removed to expose the leaves to natural conditions. The leaves were inspected daily to check for symptoms or any other effect of the pathogens on the leaves.

### Source of prospective antagonist

Samples of surface soil collected from under a mango tree were plated out for isolation of species of *Bacillus*. In each case 2 g of the soil was shaken in 10 ml of water and maintained at 90°C for 15 min in a water bath to select for endospore formers. Samples (0.1 ml) of a  $10^{-2}$  dilution of the soil suspension was spread on PDA and incubated at room temperature (28–30°C) for 48 h before colonies with the characteristic features of *Bacillus* were isolated and stored in slant cultures at 4°C. Isolates were subjected to microbial analysis and preliminary identification was done by standard procedures (GORDON *et al.* 1973; BUCHANAN & GIBBONS 1974). One of the isolates was later confirmed as *Bacillus subtilis* NCIB 3610. Six replicate plates were prepared for each soil sample and the experiment was repeated thrice. Three loops of two – day old culture on PDA was mixed with 5 ml of Potato dextrose broth and 0.1 ml of the suspension was used as inocula for the *Bacillus* isolates. The concentration of the bacterial suspension used was  $10^6$  cfu/ml.

### Antagonism of *Bacillus subtilis* against fungal leaf spot pathogens

*Bacillus subtilis* NCIB 3610 from the soil under a mango tree was used for this investigation. The more important fungal leaf spot pathogens, *Pestalotiopsis mangiferae*, *Botryodiplodia theobromae*, and *Macrophoma mangiferae* were isolated from diseased mangoes (cv. Ogbomosho) for the sensitivity test against *B. subtilis*.

**In vitro antagonism test.** For this the plate pairing method of FERREIRA *et al.* (1991) was adopted. One of the pathogens and *Bacillus subtilis* were inoculated 25 mm apart on PDA Petri plates which were then incubated at room temperature ( $26 \pm 2^\circ\text{C}$ ) for up to 5 d. The interface between the two colonies was examined and if there was inhibition it was measured. Its magnitude, i.e. the difference between mycelial growth away and towards the antagonist, was expressed as a percentage of the growth away from the antagonist as described by FERREIRA *et al.* (1991). Four replicate plates were prepared for each pair of organisms and the experiment was repeated three times.

**In vivo antagonistic trials.** Young leaves on a 3-year old mango tree cv. Ogbomosho from Umuahia were surface sterilised with 70% ethanol for 30 s and rinsed with sterile water. The lower surfaces of leaves from three mango plants for each experiment were treated according to the following regimen:

- i. inoculated with one of the pathogens;
- ii. inoculated with *B. subtilis* alone;
- iii. inoculated with a pathogen and *B. subtilis* simultaneously;
- iv. inoculated with *B. subtilis* and a day later with a pathogen.

All branches were covered with sterile cellophane bags for 1 week to maintain high relative humidity for spore germination. The bags were then removed to expose the branches to ambient environmental conditions for 12 weeks. Five replicate treatments were set up in a randomised block design for each of the three mango trees.

## RESULTS

### Survey and identification

The survey showed that the incidence of leaf spot diseases was highest at Umuahia (72%), and less at Okigwe and Ojoto in descending order (Table 1).

Table 1. Frequency of occurrence (%) of fungal leaf spot diseases of mango in Southeastern Nigeria

Location	Frequency of occurrence (%)
Umuahia	72.3 a
Okigwe	62.4 b
Ojoto	44.6 c
Enugu	43.1 c
Nsukka	31.4 d

Values within the same column followed by the same letter are not significantly different at  $P > 0.05$  by Duncan's Multiple Range Test

Although Umuahia and Okigwe are at the same altitude, they had a significant difference ( $P < 0.05$ ) in occurrence of the diseases. Also, their incidence was more frequent at the beginning of the rainy season which corresponds to February–March, with a relative humidity of 80%.

At Umuahia and other places, differences in susceptibility between individual trees of local varieties were seen. Leaf spot diseases were noticed in all developmental stages of the mango tree, but they were most pronounced on older leaves.

The fungi isolated from diseased leaves were *Pestalotiopsis mangiferae*, *Botryodiplodia theobromae* and *Macrophoma mangiferae* (Table 2). Direct scrapings from the leaf surface revealed the presence of two other fungi, *Fusariella* sp. and *Meliola* sp.. The spores of these two fungi were either washed away from the leaf surface during surface sterilisation or did not germinate *in vitro*.

### Pathogenicity test

Leaves of the mango tree that were sprayed with spores of the different fungal isolates started showing lesions after 5 weeks. The lesions were very conspicuous for the three fungi and the mixture of their spores (Table 2). Infection was established fast, maybe as a result of the hot weather. Young leaves generally escape the disease during the rainy season, but in the dry season exposed mango leaves on very dry land show infected leaves.

Branches whose leaves were sprayed with the spore suspension of *Botryodiplodia theobromae*, gave rise to the characteristic blue stain of *B. theobromae* after 56 d when some were cut and examined. Leaves infected with either *B. theobromae* or *Pestalotiopsis mangiferae* showed the same symptoms

Table 2. Symptom induction and re-isolation of fungus after incubation of inoculated mango leaves for 5 weeks

Inoculum	Re-isolation from leaf tissues after 5 weeks	Leaves exhibiting symptoms (%)
<i>Pestalotiopsis mangiferae</i>	+ve	54 a
<i>Botryodiplodia theobromae</i>	+ve	45 b
<i>Macrophoma mangiferae</i>	+ve	67 c
Mixture of the three pathogens	+ve	58 d
Control	–ve	0 e

Values within columns followed by the same letter are not significantly different at  $P > 0.05$  by Duncan's Multiple Range Test

+ve = fungus re-isolated

–ve = fungus not re-isolated

5 weeks after inoculation. There were black spots of about 0.5 mm on both the upper and lower epidermis, but the uninfected areas on the same leaf remained dark green, thus maintaining their normal colour. The lesions engulfed a considerable area of the leaf surface (Table 3). After 12 weeks the black spots on leaves infected with *P. mangiferae* started developing chlorotic margins, which enlarged with time (Table 3).

Leaves sprayed with spores of *Macrophoma mangiferae* showed lesions characteristic of the fungus; they first appeared as small yellow spots, which gradually enlarged. Although circular at first, the lesions later became irregular in shape and involved the entire surface of affected leaves. Fruiting bodies, light brown in colour, were produced mostly on the undersurface of the leaves.

Leaves sprayed with a mixture of the spores of the three fungi showed a combination of the symptoms exhibited by each of the three fungi isolates separately in the fifth week. However, the chlorotic

spots associated with infection by *M. mangiferae* did not appear. Only its fruiting bodies were found scattered among the numerous black spots, with the uninfected areas retaining their original dark green colour.

The leaves sprayed with sterile distilled water as control did not show any sign of infection throughout the period of observation.

**Identity of the bacterial isolate.** The bacterial isolate was identified and confirmed as *Bacillus subtilis* NCIB 3610.

#### Antagonism by *Bacillus subtilis* against pathogens of mango leaf

The *in vitro* assessment of antagonism showed that *Bacillus subtilis* NCIB 3610 inhibited strongly on agar plates the three leaf spot pathogens of mango *Pestalotiopsis mangiferae*, *Botryodiplodia theobromae* and *Macrophoma mangiferae* by 57%, 60% and 58% respectively (Table 4). The *in vivo* experiment

Table 3. Percentage area of mango leaf occupied by lesions 5 and 12 weeks after inoculation

Inoculum	Leaf area (%) covered by lesions	
	5 weeks	12 weeks
<i>Pestalotiopsis mangiferae</i>	19 d	23 h
<i>Botryodiplodia theobromae</i>	17 d	25 g
<i>Macrophoma mangiferae</i>	39 c	60 f
Mixture of the three pathogens	28 b	40 e
Control	0 a	0 a

Values within columns followed by the same letter are not significantly different at  $P > 0.05$  by Duncan's Multiple Range Test

Table 4. *In vitro* antagonism between *Bacillus subtilis* NCIB 3610 and leaf spot pathogens of

	<i>B. subtilis</i> NCIB 3610
<i>Pestalotiopsis mangiferae</i>	57.4 a
<i>Botryodiplodia theobromae</i>	60.6 b
<i>Macrophoma mangiferae</i>	58.4 a
Mixture of spores	68.6 c

Values within columns followed by the same letter are not significantly different at  $P > 0.05$  by Duncan's Multiple Range Test

showed that symptoms were considerably reduced by application of the antagonist (Table 4). Plants on which *B. subtilis* and a pathogen were sprayed simultaneously showed considerable reduction in the symptoms at a significant level ( $P > 0.05$ ). Trees sprayed with *B. subtilis* first and the pathogen a day later also showed a significantly lower ( $P > 0.05$ ) level of symptoms than the control (Table 5). Plants sprayed with *B. subtilis* alone did not show any leaf spot. The results indicated that *B. subtilis* has some fungicidal properties.

## DISCUSSION

Leaf spot diseases of fungal origin on mango were serious in some areas, and sometimes led to a poor fruit yield as seen in the survey which covered only two seasons. Among the surveyed areas, Umuahia and Okigwe at the same altitude

(OKIGBO 2001) and with a common boundary were seriously affected areas, but in Okigwe the mango stems were less affected by the disease. The incidence of the disease at Nsukka was quite low. Similar observations were made by OKIGBO (2001) with respect to infection by *M. mangiferae*, the causal agent of mango blight. Many leaf spot diseases manifest more at the onset of the rainy season since the high relative humidity helps in faster development (DERSO 1999; OKIGBO 2001) and rainfall aids in the dispersal of fungal spores (CHANG *et al.* 1996). This confirms the report of OKIGBO (2001) that high humidity furthers the dispersal of spores of *M. mangiferae*. The susceptibility of individual trees depended on that of local cultivars.

Among the cultivated mango in Nigeria (BRUNO & GOLDBERG 1963; OKIGBO 2001) the most susceptible was cv. Ogbomosho. The leaf spot disease that affected all developmental stages was found more on older mango leaves. DERSO (1999) reported that phaeoramularia leaf spot disease occurred more on younger leaves in Ethiopia. In *Macrophoma mangiferae* there were abundant pycnidia that developed on the leaf veins and they are the best means of survival (VERMA *et al.* 1996; OKIGBO 2001).

A combination of isolates of the three fungi sprayed on healthy mango leaves produced symptoms of the disease after 8 weeks. The slight differences in the lesions produced might have arisen as a result of the failure of *Meliola* and *Fusariella* spp. to be reintroduced on the surfaces of healthy mango leaves along with *P. mangiferae*, *B. theobromae* and *M. mangiferae* in the pathogenicity tests.

Table 5. Effect of *Bacillus subtilis* on the diameter (mm) of spot lesions in mango leaf caused by pathogens of mango

	<i>B. subtilis</i> or pathogen alone	<i>B. subtilis</i> and pathogen simultaneously	<i>B. subtilis</i> first, pathogen a day later
<i>P. mangiferae</i>	5.1 ± 1.0		
<i>B. theobromae</i>	4.1 ± 0.8		
<i>M. mangiferae</i>	10.8 ± 1.3		
Mixture of pathogen spores	3.6 ± 0.9		
<i>B. subtilis</i> alone	0		
<i>P. mangiferae</i> + <i>B. subtilis</i>		2.1 ± 0.4	1.5 ± 0.4
<i>B. theobromae</i> + <i>B. subtilis</i>		1.8 ± 0.3	1.1 ± 0.5
<i>M. mangiferae</i> + <i>B. subtilis</i>		3.6 ± 0.9	1.9 ± 0.8
Mixture of pathogen spores		1.4 ± 0.6	1.1 ± 0.3



*Fusariella* is a saprophyte on most crop plants, so that its invasion of a plant tissue depends on the level of damage to that tissue or organ by a parasitic fungus that will prepare the grounds for it (BARNETT & HUNTER 1972). *Pestalotiopsis mangiferae* is a parasitic fungus causing a leaf spot disease on mango which had been reported to cause a serious grey blight of mango in India (VERMA *et al.* 1996). *B. theobromae* is a wound parasite of many crops which was implicated in the leaf spot diseases of mango. This fungus is widespread in Nigeria and affects both food and tuber crops (ALASOADURA 1971; APPAH 1983; OKIGBO 2003).

The symptoms observed after inoculation of the lower surface of healthy mango leaves with conidia of *M. mangifera* is consistent with earlier reports (COOK 1975; OKIGBO 2001). Depletion of some of the essential elements used for the synthesis of chlorophyll in the infected leaves by the pathogens may account for the observed general chlorosis of the leaves. *M. mangiferae* was the strongest pathogen attacking the leaf of mango when compared to *B. theobromae* and *P. mangiferae*. A considerable leaf area was occupied within 12 weeks of inoculation (Table 3).

The use of specific microbial agents has brought remarkable success in the control of plant pathogens (OKIGBO & IKEDIUGWU 2000, 2001). Many *Bacillus* spp. including *B. subtilis* exhibit antifungal activity against many plant pathogenic fungi (IKEDIUGWU *et al.* 1994; CHUANG & ANN 1997; OKIGBO 2002). In the present study, *B. subtilis* proved to be a strong antagonist against *Pestalotiopsis mangiferae*, *Botryodiplodia theobromae* and *Macrophoma mangiferae* on agar plates. IKEDIUGWU *et al.* (1994) reported that *B. subtilis* isolated from soil controlled choanephora shoot diseases of the vegetable crop *Amaranthus hybridus* in the greenhouse. The rapid colonisation of the shoot tips when inoculated onto plants was the basis for extended protection over time. Also, the post harvest treatment of mango fruit with *B. subtilis* decreases anthracnose development (CHUANG & ANN 1997). Our *in vivo* experiment proved that the leaf spot lesions on mango were reduced by application of the antagonist. This confirms a similar study used to control *Eutypa lata* on grapevine (FERREIRA *et al.* 1991; SCHMIDT *et al.* 1997) and on yam tubers (OKIGBO 2002). The fact that mango leaf sprayed only with *B. subtilis* did not show symptoms of the disease is an indication that *B. subtilis* is not a pathogen of mango. Hence, it can be another candidate for microbial pesticides

(TSUGE *et al.* 1995). Several workers have reported that *B. subtilis* produces antibiotics (FERREIRA *et al.* 1991) which include iturin A and surfactin (TSUGE *et al.* 1995; ASAKA & SHODA 1996) and Bacillopeptins and Bacillomycin (KAJIMURA *et al.* 1995; ESHITA *et al.* 1995) and this could have contributed to the biological control activity observed in the present study. It is also important to note that the naturally occurring microflora on plant surfaces can impart resistance to pathogen infection.

Fungal leaf spot diseases of mango, especially that caused by *M. mangiferae*, have become a menace to many orchards in Nigeria, sometimes resulting in complete loss of the crop in some areas. This study has now shown that antagonists can be artificially introduced on to plant surfaces to impart resistance or reduce the incidence of the disease. The most difficult problem that has to be overcome lies in the commercialisation of an antagonist due to a low profit margin and possible difficulties in finding a natural antagonist which may produce antibiotics in the leaves. *B. subtilis* is clearly shown in this work to have the ability for persistence in the leaf surface, even under ambient environmental conditions. The repeated spraying that is often required for chemical pesticides would thus be unnecessary.

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## Souhrn

OKIGBO R.N., OSUINDE M.I. (2003): **Výskyt skvrnitostí na mangu (*Mangifera indica* L.) v jihovýchodní Nigérii a ověření biologické ochrany na bázi *Bacillus subtilis*.** *Plant Protect. Sci.*, **39**: 70–77.

Byla sledována četnost výskytu listových skvrnitostí na mangu (*Mangifera indica* L.) v jihovýchodní Nigérii a ověřována možnost biologické ochrany. Průzkum prokázal, že četnost listových skvrnitostí byla nejvyšší v oblasti Umuahia (72 %) a dále v klesající řadě v oblastech Okigwe a Qjoto. Nejvyšší četnost listových skvrnitostí se objevuje na počátku deštivého období (únor, březen). Listové skvrnitosti, jak bylo izolací patogenů a zpětným testem patogenity prokázáno, způsobují houby *Pestalotiopsis mangifera*, *Botryodiplodia theobromae* a *Macrophoma mangiferae*. Vedle původců listových skvrnitostí se vyskytuje houba *Fusariella* spp., která kolonizuje mrtvá pletiva listových skvrnitostí, a houba *Meliola* sp., která kolonizuje exsudáty svého hmyzu (kříšů). Testy patogenity prokázaly,

že houby *P. mangiferae*, *B. theobromae* a *M. mangiferae* jsou původci listových skvrnitostí. Příznaky po inokulaci zdravých listů se objevily po pěti týdnech. Z půdy pod stromy manga s příznaky choroby byla izolována antagonistická bakterie *Bacillus subtilis* NCIB 3610, která potlačuje růst patogenních izolovaných hub *P. mangiferae*, *B. theobromae* a *M. mangiferae* na úrovni 57 %, 61 % a 58 %. Také podle testů *in vivo* v polních podmínkách se jeví tato biologická ochrana jako významná pro pěstitele manga v oblastech průzkumu.

**Klíčová slova:** *Pestalotiopsis mangiferae*; *Botryodiplodia theobromae*; *Macrophoma mangiferae*; biologická ochrana; *Bacillus subtilis*

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Corresponding author:

DR. RALPH N. OKIGBO, Michael Okpara University of Agriculture, Department of Biological Sciences, Umudike, PMB 7267, Umuahia, Abia State, Nigeria  
e-mail: okigborn17@yahoo.com

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