African Crop Science Journal, Vol. 23, No. 3, pp. 203 - 210
ISSN 1021-9730/2015 $4.00

OCCURRENCE AND CHARACTERISATION OF *Xanthomonas axonopodis* pv. *glycines*, CAUSING BACTERIAL PUSTULES ON SOYBEAN IN GUINEA SAVANNA OF BENIN

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(Received 28 July, 2014; accepted 3 August, 2015)

ABSTRACT

To improve soybean (*Glycine max* L.) production in Benin, knowledge of bacterial diseases is needed. The objective of this study was to establish the disease occurrence and to identify *Xanthomonas axonopodis* pv. *glycines*, the causal agent of soybean bacterial pustule. Soybean bacterial pustule disease was studied during the flowering and at pod maturation, in thirty four sites of the main soybean cultivation areas in eight districts of the Guinea Savanna in Benin. Twenty and 10 soybean plants were randomly inspected for incidence and severity, respectively, on two diagonals through the field. Seven isolates from 6 different locations with highest severity, were submitted for virulence tests. The most virulent was characterised by Polymerase Chain Reaction. Bacterial pustules were present in 33 of 34 sites. Incidence and severity per district ranged from 15.8 to 70%, and 6.0 to 26.14%, respectively. Severity was highest at Binassi (34.33%) in Perere and Baka (33.2%) in Parakou. Among the seven strains isolated, UP-PK-S3 from Baka (Parakou) was the most virulent, and was characterised as *Xanthomonas axonopodis* pv. *glycines*.

Key Words: *Glycine max*, incidence, severity, *Xanthomonas axonopodis*

RESUME

Une prospection a été conduite sur la pustule bactérienne du soja durant la phase de floraison et de formation des gousses sur trente-quatre sites de production du soja localisés dans 8 Communes de la savane guinéenne du Bénin en 2011. L'objectif était d'établir la présence de la maladie et d'identifier *Xanthomonas axonopodis* pv. *glycines*, l'agent responsable de la pustule bactérienne du soja. Trois à six sites ont été prospectés par commune et sur chaque site 20 et 10 plants sont respectivement inspectés au hasard sur deux diagonales du champ pour évaluer l'incidence et la sévérité. Sept isolats provenant de 6 différentes localités à fortes valeurs de sévérités ont été soumis à un test de virulence et l'isolat le plus virulent a été caractérisé par PCR et séquencé. Les résultats ont montré que la pustule bactérienne était présente sur 33 des 34 champs prospectés. L'analyse statistique de la moyenne d'incidence et de sévérité par commune a révélé une variabilité de 15,8 à 70% pour l'incidence et de 6,0 à 26,14% pour la sévérité. Parmi les sept souches isolées, la souche UP-PK-S3 provenant de Baka (Parakou) était la plus virulente et a été caractérisée comme *Xanthomonas axonopodis* pv. *glycines* et pourrait être utilisée pour le criblage pour la résistance en milieu contrôlé.

Mots Clés: *Glycine max*, incidence, sévérité, *Xanthomonas axonopodis*

INTRODUCTION

Bacterial pustule, caused by *Xanthomonas axonopodis* pv. *Glycines,* is among the main diseases in several major soybean (*Glycine max* L.) producing countries (Wrather *et al*., 2001). The symptoms are characterised visually by small pustules, surrounded by haloes. Spots of bacterial pustule vary from minute specks to large, irregular and mottled brown areas that arise when smaller lesions coalesce. Later, dried and broken remnants of pustules may be seen on small brown necrotic areas, surrounded by yellowing haloes. The symptoms may develop on the stems and pods of susceptible varieties. Symptoms on resistant cultivars are small chlorotic spots, without well-defined pustules. On susceptible cultivars, the disease causes 15-50% yield losses (Prathuangwong and Amnuaykit, 1987).

It is difficult to distinguish symptoms of this disease from bacterial leaf blight disease and leaf rust fungus, especially in the early phase of disease progression (Frederick *et al*., 2002). The morphological and physiological characteristics are the first step of diagnosis, but for purposes of detecting pathogens in the field, are not effective because the test takes a long time (Suwanto, 1994). Along with technological developments in the field of microbiology, identification and detection techniques have been developed, among others, by Polymerase Chain Reaction (PCR). A wide variety of PCR methods have been developed for the identification and detection of plant pathogenic bacteria, such as gene amplification HRP (Leite *et al.,* 1995) and the use of PCR with specific primers of pathogenicity genes in the plasmid (Verdier *et al*., 1998).

The distribution pattern and diversity of *X. axonopodis* pv. *glycines* in Benin remain largely unknown, thus hindering progress in designing interventions. The objective of this study was to establish the occurrence of the disease and identify *Xanthomonas axonopodis* pv. *glycines.*

MATERIALS AND METHODS

A disease survey was conducted in thirty-four sites of the eight main soybean cultivation districts (Dassa, Glazoué, Savè, Ouessè, Tchaourou, Parakou, N'Dali and Pèrerè) of the Guinea Savanna in Benin, during the flowering and pod maturation period (August to September) in 2011. The districts were selected on the basis of production potential (at least 1000 metric tonnes per district). Three to six sites were surveyed per district. The sites with at least 0.25 ha, and 5 to 10 Km distance from each other, were selected.

The Guinea savanna comprises the Northern Guinea Savanna and the Southern Guinea Savanna. In the Northern Guinea Savanna (dry savanna), with a unimodal rainfall pattern, rains begin in June and remain until November, with 800-1300 mm per year. In the Southern Guinea Savanna (wet savanna) rainfall averages 1000-1200 mm per year and is distributed in a bimodal pattern from late April until November. The relative humidity fluctuates seasonally between a mean minimum of 60% during the dry season (January), to a mean maximum of 80% during the rainy season (July) (Wydra and Verdier, 2002).

Disease incidence and severity. At each site, 20 soybean plants were randomly selected on two diagonals through the field, to evaluate the disease incidence. Ten of these plants were used to score disease severity as described by Prathuangwong *et al*. (1993). A stencil card of 4 cm x 7 cm, with 9 puncked-out circles of 1 cm diameter sections per leaflet (27 sections per leaf), was placed over infected leaves. The number of holes with lesions was recorded and divided by the total number of holes. Three leaves from the apical, middle or basal portions were evaluated for each plant.

These quantitative survey data were coupled with global position systems (GPS) and geographic information systems (GIS) technologies to map incidence and severity data (ESRI, 2008). Leaves with symptoms were collected and strains isolated for further characterisation.

Bacterial isolates and pathogenicity test. The bacterial inocula were prepared by culturing 8 strains from Benin and one from Thailand (reference strain) at 30 °C for 48 hr, on Yeast Peptone Glucose Agar (YGPA) medium, and then suspending cells in sterile distilled water to obtain

aqueous cell suspensions (OD_{600} = 0.2,≈10^8 CFU ml^{-1}). The suspensions were infiltrated into leaves of 3 weeks old plants of soybean, variety Merlin, maintained in the greenhouse at 22 °C and 70% relative humidity. Sterile distilled water served as negative control in each trial. At 3 to 7 days after inoculation, disease severity was assessed according to Prathuangwong *et al*. (1993). Severity was calculated based on the number of pustules observed in nine sections of 1 cm diameter per leaf. For each isolate, at least three leaves were used and sections collected from the middle and basal portion were considered.

DNA isolation and sequencing. The isolates from 8 different locations of the Guinea Savanna of Benin were grown for 48 hr on YPGA medium at 30 °C. Pure bacterial colonies of each isolate were suspended in 400 µl buffer AP1 and 4 µl RNase A (Qiagen). Bacterial DNA was extracted and purified using the DNeasy Plant Mini kit, according to the protocol proposed by the manufacturer (Qiagen Company).

The 16S rRNA genes were PCR-amplified using primers described by Maes (1993) for fast classification of plant associated bacteria in the *Xanthomonas* genus: 17-mer homologous to position 461/477 (5'-AAGGATCGGGTATTAAC-3') and 16S rDNA primer position 8/27 (5'-AGAGTTTGATCTTGGCTCAG- 3'). Bacterial DNA (1 µl) of each of the isolates, coming from the DNA extraction, was added to a final volume of 25 µl containing buffer Invitrogen (1,5 µl 50 mM $MgCl_2$ Invitrogen), 0,5 µl 40 mM dNTP'S, 0,5 µl of each primer, 0,25 µl Taq Polymerase and 18,5 µl H_2O. *Xanthomonas campestris* pv. *musacearum* strain originated from Kenya, previously characterised at DSMZ (DSMZ, AS-1039), was used as positive control, and banana plant extract used as negative control.

The following PCR programme was run: initial denaturation for 2 min at 95 °C and 29 cycles of 45 s at 95 °C, 1 min at 37 °C and 2 min at 62 °C. The final extension step took 10 min at 62 °C.

The PCR products were visualised under UV after running the mix of 5 µl of each sample and 4 µl of the marker of the reaction in an agarose gel (1%) electrophoresis stained with ethidium bromide. The PCR products were sequenced at Sequiserve GmbH in Vaterstetten (Germany). The generated sequence and annotation of the genome were submitted to Basic Local Alignment Search Tool (BLAST) for the similarities identification.

Statistical analysis. Analysis of variance (ANOVA) was performed using the GLM procedure of SAS (SAS Institute Inc, Release 6 12, Cary, NC, USA) on disease incidence and severity data, which were log-transformed to stabilise the variance. The student Newmann-Keuls test was used to compare mean values of disease incidence and severity. Values given in tables are original means with corresponding standard errors.

RESULTS

Disease incidence and severity. Bacterial pustule was present in 33 of the 34 sites surveyed in the Guinea Savanna in Benin. Disease incidence, as presented in the map, was recorded in four classes (0-15, 15-35, 35-55, >55%) (Fig. 1). Infection was most severe at Degue-Degue (100%) in Save, followed by Badekparou (70%) in Tchaourou and Ouesse centre (68%) (data not shown). Disease severity was distributed in four classes (Fig. 2). Severity was highest at the sites in Binassi (34.33%) in Perere, followed by Baka (33.2%) in Parakou, Bougnankou (32.35%) in Perere, Gbegourou (32.43%), Alafiarou-Dourbe (31.68%) in N'dali, and Bethel (31.8%) in Save.

Disease incidence and severity of sites per district were variable ranging from 15.82 to 70% for incidence and 5.95 to 26.14% for severity, with the highest in Save (Table 1).

Virulence of isolates. Seven strains of *X. axonopodis* pv. *glycines* were isolated from leaves with bacterial pustule symptoms coming from sites with the highest severity, in 6 districts of Guinea Savanna (Dassa, Ouesse, Parakou, N'dali, Save and Tchaourou). The virulence test showed a significant variability among strains, with the most virulent strains being UP-PK-S3, originating from Baka (Parakou) in Benin and the reference strain Thai 12.2 from Thailand (Table 2).

Molecular characterisation. Among the strains, UP-PK-S3, UP-TC-S6 and saprophytic bacteria

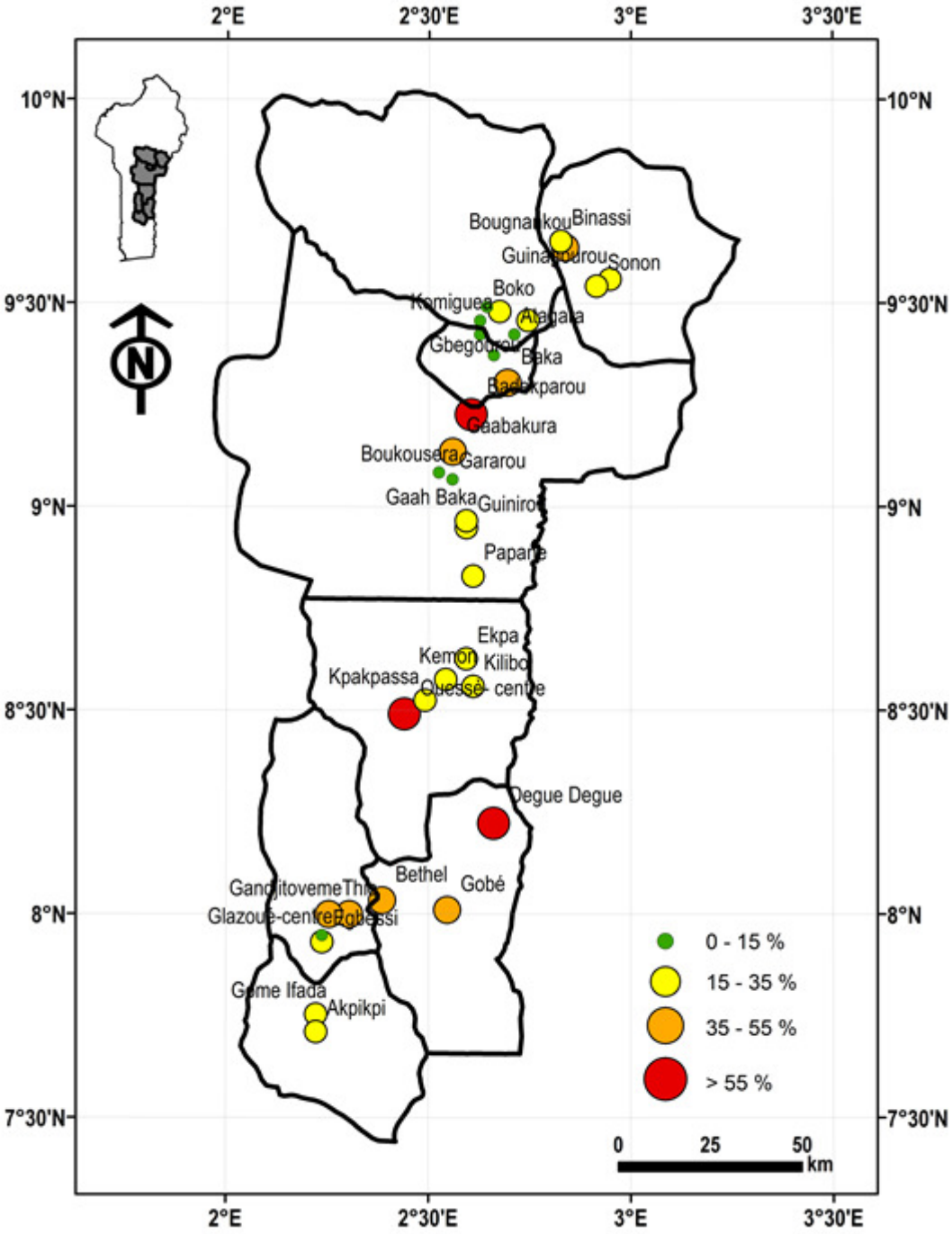

Figure 1. Disease incidence of soybean bacterial pustule in 34 sites of 8 districts of the Guinea savanna of Benin.

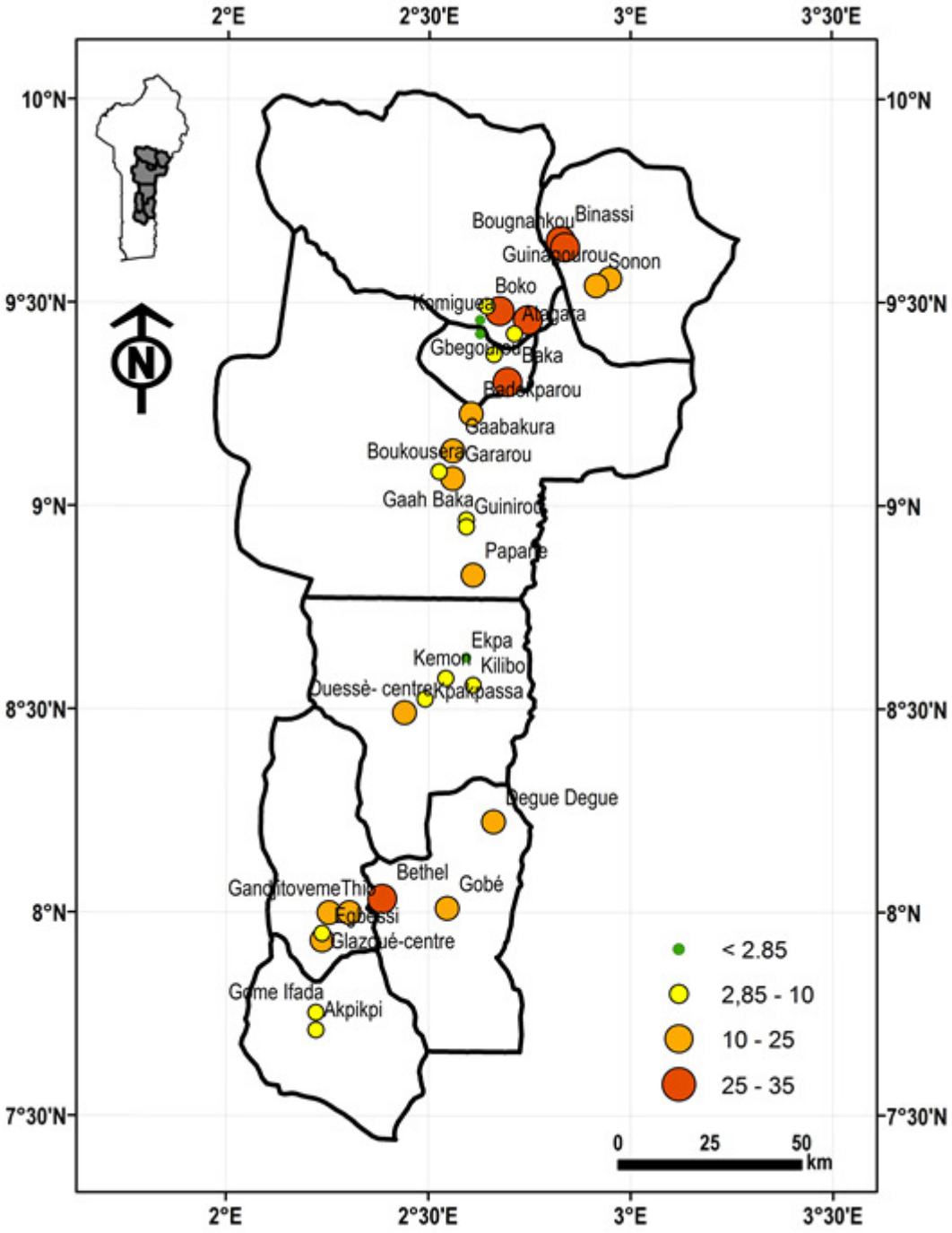

Figure 2. Disease severity of soybean bacterial pustule in 34 sites of 8 districts of the Guinea savanna of Benin.

TABLE 1. Incidence and severity of soybean bacterial pustule of *X. axonopodis* pv. *glycines* per district in the Guinea Savanna of Benin

District	Incidence (%)	Severity (%)
Glazoue	32,00±4,66b	14,27±2,71b
N'Dali	16,42±3,13a	16,14±3,41b
Save	70,00±5,91d	26,14±5,31c
Perere	30,00±3,87b	24,13±3,08c
Parakou	15,82±3,07a	12,78±3,52b
Dassa	26,66±5,70b	5,95±2,10a
Tchaourou	33,33±3,51b	11,51±1,85b
Ouesse	42,37±4,50c	9,83±2,19ab

Means marked with the same letter are not significantly different (P<0.05

TABLE 2. Virulence of eight soybean bacterial isolates of *X. axonopodis* pv. *glycines* from Benin and Thailand on variety Merlin

Isolate	Origin	Disease severity (%)
UP-PK-S3	Parakou	75,9±4,55 a
Thai 12.2	Thailand	65,42±8,53 a
UP-OU-S2-1	Ouesse	52,15±3,27b
UP-OU-S2-2	Ouesse	51,35±4,02 b
UP-ND-S5	N'Dali	50,18±2,05 b
UP-TC-S6	Tchaourou	49,99±5,67 bc
UP-SV-S7	Save	44,02±3,54 cd
UP-DA-S1	Dassa	42,03±2,01 cd

UP : University of Parakou DA : Dassa OU : Ouesse PK : Parakou, ND : N'Dali, TC : Tchaourou, SV : Save, S : soja. Means marked with the same letter are not significantly different (P<0.05, by ANOVA)

species and *X. campestris* pv. *musacearum* tested, only UP-PK-S3, UP-TC-S6 and *X. campestris* pv. *musacearum* produced a single intense 480-bp fragment (Fig. 3).

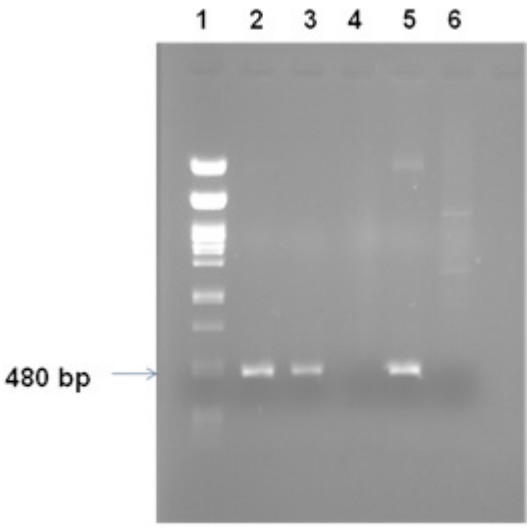

Figure 3. Comparative PCR amplification of *Xanthomonas* related bacteria and a saprophytic isolate from plants. 1: Marker; 2: Isolate UP-PK-S3; 3: Isolate UP-TC-S6; 4: saprophytic bacteria; 5: *X. campestris musacearum*; 6: negative control (banana plant)

DISCUSSION

Severity was highest in five locations of the Guinea Savanna of Benin, with Parakou district having the most virulent strain (Table 1). The observed diversity in disease reaction could be due to soybean varieties susceptibity to bacterial pustule, or to strain virulence in the specific environment being favourable to the disease development. The main soybean varieties recommended in Benin are Jupiter, TGx1985-11F, TGx1984-17F, TGx1910-2F, IRSA 2972, IRSA 44A/73, TCx536-02D (CaBEV, 2011), but there is no specific soybean variety for this agroecological zone. Cockerman (1963) and Falconer (1990) demonstrated that diversity of reaction of soybean obtained under different environments was due to differential responses from the same group of genes or expression of different groups. Several factors which may contribute to the virulence of *X. axonopodis* pv. *glycines* have been described, such as production of indoleacetic acid and cytokinin by Fett *et al.* (1987), extracellular polysaccharide (Jones and Fett, 1985), toxin (Hokawat and Rudolph, 1993), bacteriocins (Fett *et al.*, 1987) or cellulase and protopectinase (Hokawat and Rudolph, 1993). The bacterium also secretes amylase, gelatinase and proteinase. The synthesis of extracellular enzymes and EPS is controlled by a cluster of genes called the *rpf* cluster, for regulation of pathogenicity factors (Dow and Daniels, 1994; Slater *et al.*, 2000). Pirhonen *et al.* (1996) showed that virulence of *X. axonopodis* pv. *glycines* is

associated to different genetic determinants which are regulated by *HrpG* and *HrpX* important for pathogenesis and induction of hypersensitivity reaction within certain plants. Bacterial pustule was present in 33 of 34 sites of the Guinea Savanna in Benin, with mean incidence varying from 15.82 to 70%. This is the first report on the occurrence of bacterial pustule on soybean in Benin.

Bacterial pustule occurs also in many soybean producing areas of the world with warm temperature and sufficient moisture (Moffett and Croft, 1983; Sinclair, 1984; Bradbury, 1986). The disease is more important in tropical areas and is an important yield reducing factor in soybean (Hokawat, 1993; Prathuangwong, 1985).

Molecular characterisation by PCR and sequencing combined with pathogenicity test allowed to identify our strains. Indeed, the result of PCR product sequencing allows concluding that these species belong to the genus *Xanthomonas*. Combining the pathogenicity test and characterization results, we can confirm that the strains belong to *X. axonopodis* pv. *glycines*. *X. axonopodis* pv. *glycines*. was also characterised by Prathuangwong and Ketsuwan (2003) and Kaewnum *et al*. (2005) using rep-PCR.

ACKNOWLEDGMENT

This work was supported by the TWAS-DFG Cooperation Visits Programme for scientists from Sub-Saharan Africa, DSMZ and the University of Parakou. Thanks to Prof. Prathuangwong for providing the bacterial strain from Thailand. The technical staff of Plant Viruses Department of DSMZ in Germany and Dr Y.C. Hountondji of Faculty of Agronomy from University of Parakou in Benin.

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