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Characterization and insecticidal potential of bacteria symbiotic with native *Heterorhabditis* sp. (Nematoda: Heterorhabditidae) against the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)

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ABSTRACT

The current study aimed to identify symbiotic bacteria associated with previously identified native entomopathogenic nematodes from southern Benin and evaluate their biocontrol potential on fifth instar larvae of *Spodoptera frugiperda*. Colony characteristics, bioluminescence capacity, and a molecular approach using the 16S rRNA gene were used to identify the symbiotic bacteria. Pathogenicity tests against fifth instar larvae of *S. frugiperda* were also conducted. The results indicated that all the eighteen bacterial isolates isolated from strains of *Heterorhabditis sonorensis* are identical (99% sequence similarity) to *Photorhabdus* isolate R-66822 (accession number MF353507). The symbiotic bacteria associated with the single strain of *H. indica* are identical (98% sequence similarity) to the *Photorhabdus* bacterial isolate R-52361 (accession number MF353497). With regard to pathogenicity tests, nine bacterial isolates were pathogenic to *S. frugiperda* larvae at 35°C, with insect mortalities varying between 0.24 and 78.47%. Better yet, seven of them could still kill the insects at an exposure temperature of 40°C, inducing insect mortalities ranging from 4.86 to 63.65%. Moreover, bacterial densities of 0.450 x 10^6 CFU/mL and 0.483 x 10^6 CFU/mL were sufficient for the bacterial isolates BSKassehlo and BSZoundomey, respectively, to kill 50% of the fifth instar larvae of *S. frugiperda*, whereas BSKissamey, BSAzohoue2, and BSZe1 needed 3.173 x 10^6 CFU/Ml and 2.900 x 10^6 CFU/mL, respectively. These findings are sufficiently encouraging to initiate further investigations into the potential of these native bacterial isolates for sustainable management strategies against *S. frugiperda* in agricultural areas.

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INTRODUCTION

Bacteria of the genera *Photorhabdus* and *Xenorhabdus* are symbiotically associated with the entomopathogenic nematodes (EPNs) belonging to the genera *Heterorhabditis* and *Steinernema*, respectively (Thomas and Poinar, 1979). These bacteria live in the gut of the infective juveniles (IJs), which is the only free-living stage of EPN (Torres-Barragan *et al.,* 2011). The IJs infect the larval stage of a diverse range of insects by entering into their body through natural openings (mouth, anus, and spiracles) or, in some cases, the cuticle using a buccal tooth-like structure (Thomas and Poinar, 1979). Once inside the insect host, they recover and release the bacterial symbionts into the insect's hemocoel. The bacteria then multiply and release toxins and antimicrobial compounds within the host. The latter dies within approximately 72 h after infection. Upon insect death, the IJs multiply rapidly by feeding on the insect hemolymph and the bacteria, and they complete several generations inside the insect host depending on the availability of food. Once food resources start running low, they carry the symbiotic bacteria in their gut and exit the host cadaver in search of new hosts (Sajnaga and Kazimierczak, 2020). The bacteria of the genus *Photorhabdus*, associated with *Heterorhabditis* species, are the only known terrestrial bacteria with natural luminescence (Hyršl *et al.*, 2004). Currently, the *Photorhabdus* genus consists of 23 species with validly published names (Machado *et al.*, 2024). These bacteria have been used with success as biopesticides in agriculture and are able to kill a wide range of insects (Luca *et al.*, 2013). Mohan and Sabir (2005) have reported the use of *Photorhabdus* species alone as a biopesticide, independent of its nematode symbiont, against the cabbage white butterfly, *Pieris brassicae*; the mango mealybug, *Drosicha mangiferae*; and the pupae of the diamondback moth, *Plutella xylostella*, has been demonstrated successfully. Such bacteria can help reduce the huge chemical inputs used for plant protection (Migunova and Sasanelli, 2021).

The fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a destructive agricultural pest all over the world (Qiu *et al.*, 2023). It has been reported to feed on more than 350 host plants (Chisonga *et al.*, 2023). It was discovered for the first time in Benin in 2016 (Goergen *et al.*, 2016) and has been the major pest in maize (*Zea mays*) crops since then. Damages may be observed on all maize plant parts depending on development stage, causing yield losses estimated at 797 kg/ha, representing 49% of the average farmer's production (Houngbo *et al.,* 2020). Beninese farmers rely on numerous synthetic pesticides in an unsuccessful attempt to control this pest (Houngbo *et al.,* 2020). As the application of harmful chemical pesticides affects the entire environment, there is an urgent need to develop eco-friendly approaches to control the pest (Sun *et al.,* 2021). The use of biopesticides is an important eco-friendly method for pest management, including the use of microorganisms and their metabolites (Migunova and Sasanelli, 2021).

Native EPNs are more suitable for use as biological weapons against local pests because of their adaptations to the local climate and host population (Bedding, 1990). The first surveys for EPNs carried out in southern Benin had allowed the harvest of 32 strains belonging to two species (*H. sonorensis* and *H. indica*), including 29 strains of *H. sonorensis* and three strains of *H. indica* (Zadji *et al.*, 2013). Some exploratory studies have been carried out on these EPN strains, and their biocontrol potential was assessed (Baimey *et al.,* 2015; Zadji *et al.,* 2014a; Zadji *et al.*, 2014b).

However, knowledge of their symbiotic bacteria is lacking, since different EPNs harbor specific bacterial symbionts, which are believed to dictate the virulence of the vector nematode (Ngugi *et al.*, 2021). Hence, the present study was carried out to isolate and characterize the symbiotic bacteria associated with these native EPNs and evaluate their bio-efficacy against *S. frugiperda* under laboratory conditions.

MATERIALS AND METHODS

Source of bacterial isolate hosts

In a previous study, surveys were conducted for EPNs through four agroecological zones (AEZ) located in the south, out of the eight AEZ that

count the country. EPNs were identified as described in Zadji *et al.* (2013). The bacterial isolates associated with nineteen (18 strains of *H. sonorensis* and one of *H. indica*) out of the 32 EPN strains recovered from the surveys were studied in the present study. Fig. 1 illustrates the locations of the retrieved EPNs, which serve as the bacterial isolate hosts. Five bacterial isolate hosts were collected from the Central Benin cotton zone (AEZ V), twelve from the Clayey earth zone (AEZ VI), one from the Depression zone (AEZ VII), and one from the Fishering zone (AEZ VIII) (Fig. 1).

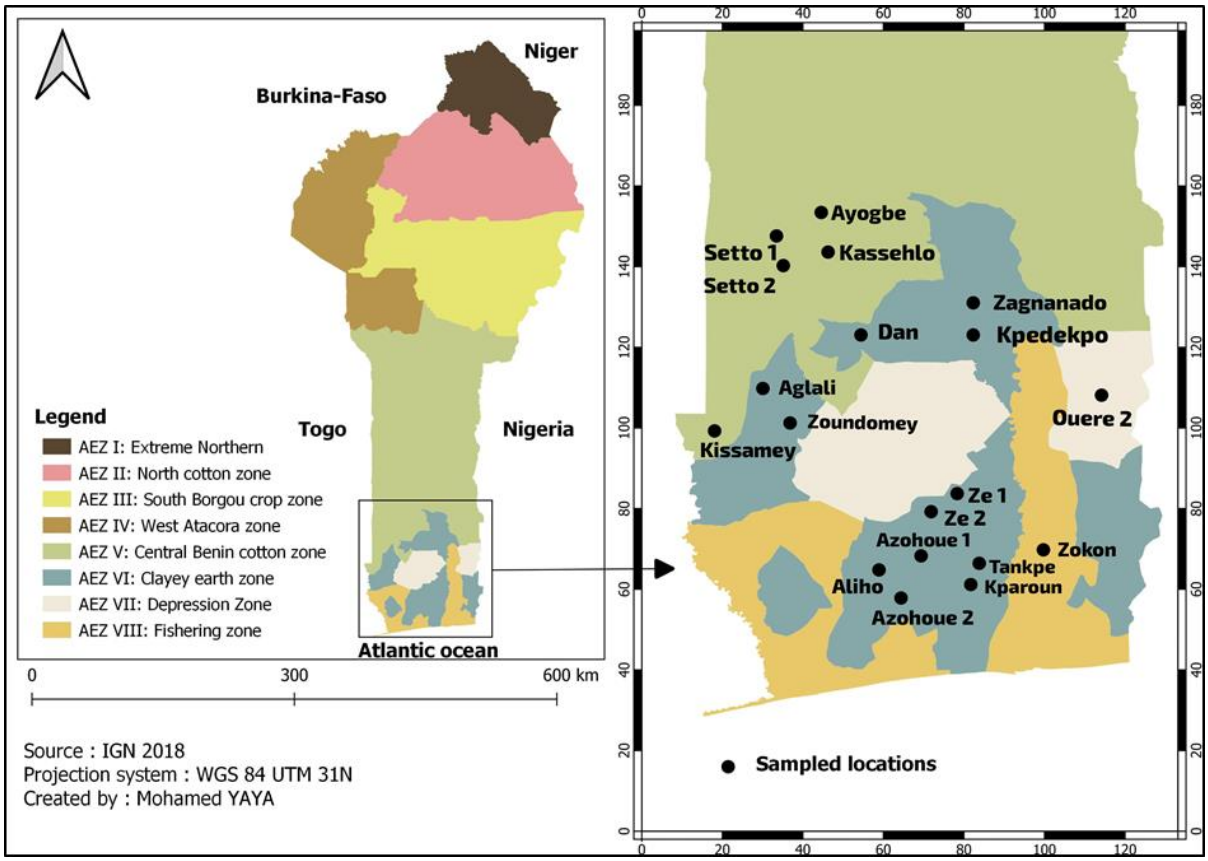

Fig. 1. Map of south Benin showing agro ecological zones and sampled locations for EPNs

Isolation of bacteria associated with EPN isolates

Bacteria were isolated from the hemocoel of the greater wax moth larvae, *Galleria mellonella* (Gm) (Poinar and Thomas, 1966), infected with freshly produced IJs of each EPN strain (at the ratio of 100 IJs/Gm larva) at room temperature (28°C). The resulting insect cadavers were surface sterilized in 75% ethanol prior to dissecting on a sterile petri dish. A drop of hemolymph was collected from the infected larvae, streaked onto sterile plates of Nutrient Bromothymol Agar (NBTA) medium (nutrient agar with 0.004% triphenyl tetrazolium chloride and 0.025% bromothymol blue), and incubated at room temperature (28°C) for 72 hours (Akhurst, 1980). The most prominent form of bacteria turned blue after absorbing bromothymol blue dye. A single colony of the stained blue bacteria was routinely grown on the same medium every 24 hours until pure isolated colonies were observed. The bacterial stock was initiated from a single colony of each of the pure isolated colonies of the nineteen bacterial isolates, inoculated in in 5 mL of liquid media Luria–Bertani Broth, and incubated with shaking at 150 rpm overnight at 27 ± 1 °C.

Colony characteristics of symbiotic bacteria

Distinctive features, including texture, opacity, shape, elevation, color, size, and margin were recorded after 48 hours of incubation at 28°C on NBTA plates. In

addition, the bioluminescence capacity of symbiotic bacteria isolates was visually assessed in darkness (Kazimierczak *et al*., 2017).

Molecular characterization of symbiotic bacteria

Bacterial DNA extraction: Bacterial genomic DNA was extracted starting from a single colony from a 24 h culture of each bacterial isolate, actively growing on nutrient agar (NA), by the method of Baele *et al.* (2000).

Bacterial DNA amplification and sequencing: The 16S rRNA gene was amplified by PCR in a 25 µl reaction mixture that included 1 µl of template DNA, 0.12 µl Top Taq DNA polymerase (Qiagen) in the supplied PCR buffer, and 0.1 µM of each primer. The primers used for amplification of total 16S rRNA were pA (16F271) (5-AGA GTT TGA TCC TGG CTC AG-3') and pH (16R1522) (5-AAG GAG GTG ATC CAG CCG CA-3'). All amplifications were performed using a thermocycler (Eppendorf) with initial denaturation at 96°C for 4 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. PCR products were purified by adding 1 µl Exo-FAP (Fast AP Thermosensitive Alkaline phosphatase 1 unit/µl; Exonuclease I (Exo I) (20 u/µl); Thermoscientific) to 5 µl PCR product and incubating at 37 °C for 15 min, followed by another 15 min at 85° C. Subsequently, partial 16S rRNA was sequenced for all isolates using the primers 16F358 (5'-CTC CTA CGG GAG GCA GCA GT-3') and 16R519 (5'-GTA TTA CCG CGG CTG CTG-3'). Sequencing was performed using a BigDye v3.1 terminator cycle sequencing kit (Applied Biosystems, USA) and run on an ABI 3130XL DNA Sequencer. Identification of the partial fragments obtained was done by blasting them in the National Center for Biotechnology Information (NCBI). Multiple sequence alignments of partial 16S rRNA together with sequences of reference type strains of the *Photorhabdus* group retrieved from GenBank were made using Clustal W included in MEGA 12 of Kumar *et al.* (2024). Alignments were trimmed to the most common size of all sequences and visually inspected to remove non-overlapping sequences. The remaining sequences were analyzed for phylogenetic relations along with 32 accessions of *Photorhabdus* spp. and *Xenorhabdus nematophila* DSM3370 as an outgroup.

Phylogenetic analysis: The evolutionary relationship of the 16S rRNA gene sequences was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with a sum of branch lengths equal to 0.367 is shown. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendant clade is shown next to each internal node in the tree. The analytical procedure encompassed 52 amino acid sequences. The pairwise deletion option was applied to all ambiguous positions for each sequence pair, resulting in a final data set comprising 481 positions. Evolutionary analyses were conducted in MEGA12 (Kumar *et al.*, 2024).

Preparation of bacterial suspensions

The optical densities of bacterial suspensions were determined from the isolated bacterial cultures in LB broth using a UV/VIS spectrophotometer (T80, PG-Instruments), and the populations of cells at the various optical densities were determined by dilution plating (El Fakhouri *et al.*, 2023).

Insect rearing

Field-collected caterpillars of *S. frugiperda* were reared on maize leaves in plastic boxes in the laboratory. Caterpillars were individually deposited into boxes previously filled with soil collected from a maize field. The boxes were incubated in the laboratory conditions until adult emergence. Afterwards, three couples of emerged adults were introduced into the insect egg-laying cage along with a cotton ball soaked in 10% honey solution as a food source for moths. Eggs laid by the female moths were separated and reared in insect-rearing boxes on maize leaves to create a homogenous population for the experiment. The insect culture was therefore maintained during the course of the experiment.

Bioassays

Preliminary screening: An oral toxicity test was performed to determine the pathogenicity of bacterial isolates against the 5th instar larvae of *S. frugiperda* at different temperatures (25, 30, 35, and 40°C). The *S. frugiperda* larvae were obtained from a culture maintained on maize leaves in the laboratory during the course of the experiment. To exclude damaged, infected insects and avoid cannibalism, all larvae were kept in a plastic box (one individual per box) and fed with a leaf of maize for one day prior to the test. Leaf bits of maize (6 cm diam.) individually amended with 1 mL of a bacterial isolate suspension, at a concentration of 1 x 10^8 colony-forming units (CFU)/mL, were individually placed in Petri dishes (60 x 15 mm) and kept at the assigned temperature (25, 30, 35, or 40°C) for one hour. Afterwards, one insect larva was put in each Petri dish. Dishes were sealed with Parafilm before incubation for three days. The leaf that was amended with 0.8 mL of LB medium diluted in saline served as the control. Each treatment (bacterial isolate x exposure temperature) consisted of five Petri dishes and was repeated three times. After exposure, larval mortality was recorded.

Larvae were considered dead if they did not move when lightly touched with a camelhair needle. The experiment was repeated twice.

Concentration-response: Based on the results of the screening of bacterial isolates against *S. frugiperda* at different temperatures, bacterial isolates that were able to kill larvae of *S. frugiperda* at 35°C were considered for virulence tests. Nine bacterial isolateswere identified, including eight (BSZoundomey, BSKissamey, BSAzohoue2, BSZagnanado, BSKpedekpo, BSSetto1, BSKassehlo, and BSZe1) that were hosted by the EPN *H. sonorensis* and one isolate BSAyogbe1 that was hosted by the EPN *H. indica* (Table 1). Fifth instar larvae of *S. frugiperda* were individually exposed to a leaf bit of maize (6 cm diam.) amended with 1 mL of each bacterial isolate suspension at different concentrations (1 x 10^4, 1 x 10^5, 1 x 10^6, 1 x 10^7, and 1 x 10^8 CFU/mL) as described in the previous experiment. Amended leaves were individually placed in Petri dishes (60 x 15 mm) and allowed to dry before a single 5th instar larva of *S. frugiperda* was transferred into each Petri dish. The control was only treated with 1 mL of LB medium diluted in saline. The Petri dishes were sealed and incubated at room temperature (28°C) for three days. Each treatment (bacterial isolate x bacterial concentration) consisted of ten Petri dishes. After exposure, larval mortality was recorded. Larvae were considered dead if they did not move when lightly touched with a camelhair needle. The experiment was repeated twice.

Statistical analysis

Control data were not included in the statistical analysis, since no mortality in control treatments was observed. Data from repeated experiments were similar and were therefore pooled for analysis. To stabilize the variance of means, data obtained as percentages were transformed (arcsine of the square root) prior to analysis. Data were subjected to analysis of variance (ANOVA) using SAS (SAS, 2011), and the differences between means were compared using the Student-Newman-Keuls (SNK) test at $P < 0.05$. Linear regression analyses were performed using the REG procedure of SAS. Lethal Concentration (LC_{50}) values were calculated using probit analysis in MedCalc® Statistical Software version 23.2.1. Differences among bacterial isolates were considered significant when the 95% confidence limits for the LC_{50} values failed to overlap.

RESULTS

Colony characteristics of symbiotic bacteria

All bacterial colonies displayed a smooth texture. The exhibited colors raging from green to dark-green, and their diameters varied among small (<1 mm), medium (= 1 mm), and large (>1 mm). Most of them were circular, raised, and entire edged. Some were irregular, convex, translucent, and undulating edged. After allowing eyes to adjust in a completely dark room for 10 min, the bioluminescence the bacterial isolates was observed (Table 1).

Table 1. Bacterial isolate colony characteristics

Bacteria Isolates*	Closest specie according to identification based on partial 16S rRNA		Bacterial colony characteristics							Biolumi-Nascence feature	Associated EPN strains		
	Species	Strains	Shape	Elevation	Margin	Colour	Texture	Opacity	Size		Species	Strains	Accession numbers
BSOuere2	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Large	w	*H. sonorensis*	Ouere2	KF723800
BSYokon	*Photorhabdus* sp.	R-66822	Irregular	Convex	Undulate	Dark-green	Smooth	Opaque	Medium	+	*H. sonorensis*	Yokon	KF723801
BSAglali	*Photorhabdus* sp.	R-66822	Circular	Raised	Undulate	Green	Smooth	Translucent	Large	+	*H. sonorensis*	Aglali	KF723804
BSZoundomey	*Photorhabdus* sp.	R-66822	Circular	Raised	Undulate	Green	Smooth	Translucent	Large	+	*H. sonorensis*	Zoundomey	KF723805
BSKissamey	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Medium	+	*H. sonorensis*	Kissamey	KF723806
BSAliho	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Large	+	*H. sonorensis*	Aliho	KF723807
BSAzohoue1	*Photorhabdus* sp.	R-66822	Circular	Convex	Entire	Green	Smooth	Translucent	Medium	+	*H. sonorensis*	Azohoue1	KF723808
BSAzohoue2	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Large	+	*H. sonorensis*	Azohoue2	KF723809
BSKpanroun	*Photorhabdus* sp.	R-66822	Circular	Convex	Entire	Green	Smooth	Translucent	Large	w	*H. sonorensis*	Kpanroun	KF723810
BSTankpe	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Medium	w	*H. sonorensis*	Tankpe	KF723812
BSZagnanado	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Large	+	*H. sonorensis*	Zagnanado	KF723814
BSKpedekpo	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Small	+	*H. sonorensis*	Kpedekpo	KF723815
BSSetto1	*Photorhabdus* sp.	R-66822	Irregular	Raised	Undulate	Green	Smooth	Translucent	Large	+	*H. sonorensis*	Setto1	KF723819
BSSetto2	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Dark-green	Smooth	Opaque	Large	+	*H. sonorensis*	Setto2	KF723820
BSKassehlo	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Small	+	*H. sonorensis*	Kassehlo	KF723824
BSDan	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Dark-green	Smooth	Opaque	Large	+	*H. sonorensis*	Dan	KF723825
BSZe1	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Green	Smooth	Translucent	Medium	+	*H. sonorensis*	Ze1	KF723827
BSZe4	*Photorhabdus* sp.	R-66822	Circular	Raised	Entire	Dark-green	Smooth	Opaque	Large	+	*H. sonorensis*	Ze4	KF723829
BSAyogbe1	*Photorhabdus* sp.	R-52434	Circular	Raised	Entire	Green	Smooth	Translucent	Large	+	*H. indica*	Ayogbe1	KF723816

Molecular characterization of symbiotic bacteria

Our colony characteristics as *Photorhabdus* sp. were validated by comparing the partial 16S rRNA gene sequences (481 bp) of all 19 bacteria isolates with the type strains of reference species. The bacteria isolates were distributed within two different clusters of the *Photorhabdus* species groups (Fig. 2). All the bacteria isolates (18) were hosted by EPN *H. sonorensis* strains grouped in cluster A along with *Photorhabdus* isolate R-66822 (deposited in GenBank under the accession number MF353507), with whom they shared 99% sequence similarity. They all shared 98% sequence similarity with *Photorhabdus* bacterial isolate R-52361 (deposited in GenBank under the accession number MF353497) (Fig. 2). The only one bacterial isolate BSAyogbe1, hosted by H. *indica*, grouped in cluster B and shared 98% sequence similarity with the *Photorhabdus* bacterial isolate R-52434 (deposited in GenBank under the accession number MF353501), representing its closest neighbor. They both also shared 98% sequence similarity with *Photorhabdus* bacterial strains R-52391 (deposited in GenBank under the accession MF353499).

Bioassay

Preliminary screening: Temperature had a negative impact on the efficacy of bacterial isolates as indicated by the negative slope of the regression equation Y = 122.8939 - 2.8747 X (data not shown). The relationship between bacterial isolates and larvae mortality varies according to temperature. Bacterial isolates' pathogenicity was therefore assessed separately for each exposed temperature (Table 2). Insect mortality differed significantly among bacterial isolates at each tested temperature.

At 25°C ($F = 43.09$, $p < 0.0001$), insect mortality ranged from 4.13 to 85.51%. The bacterial isolate BSKpedekpo recorded the highest larva mortality, while the bacterial isolate BSSetto1 recorded the lowest. The EPN *H. indica* hosted only one bacterial isolate, BSAyogbe1, which recorded 65.83% insect mortality.

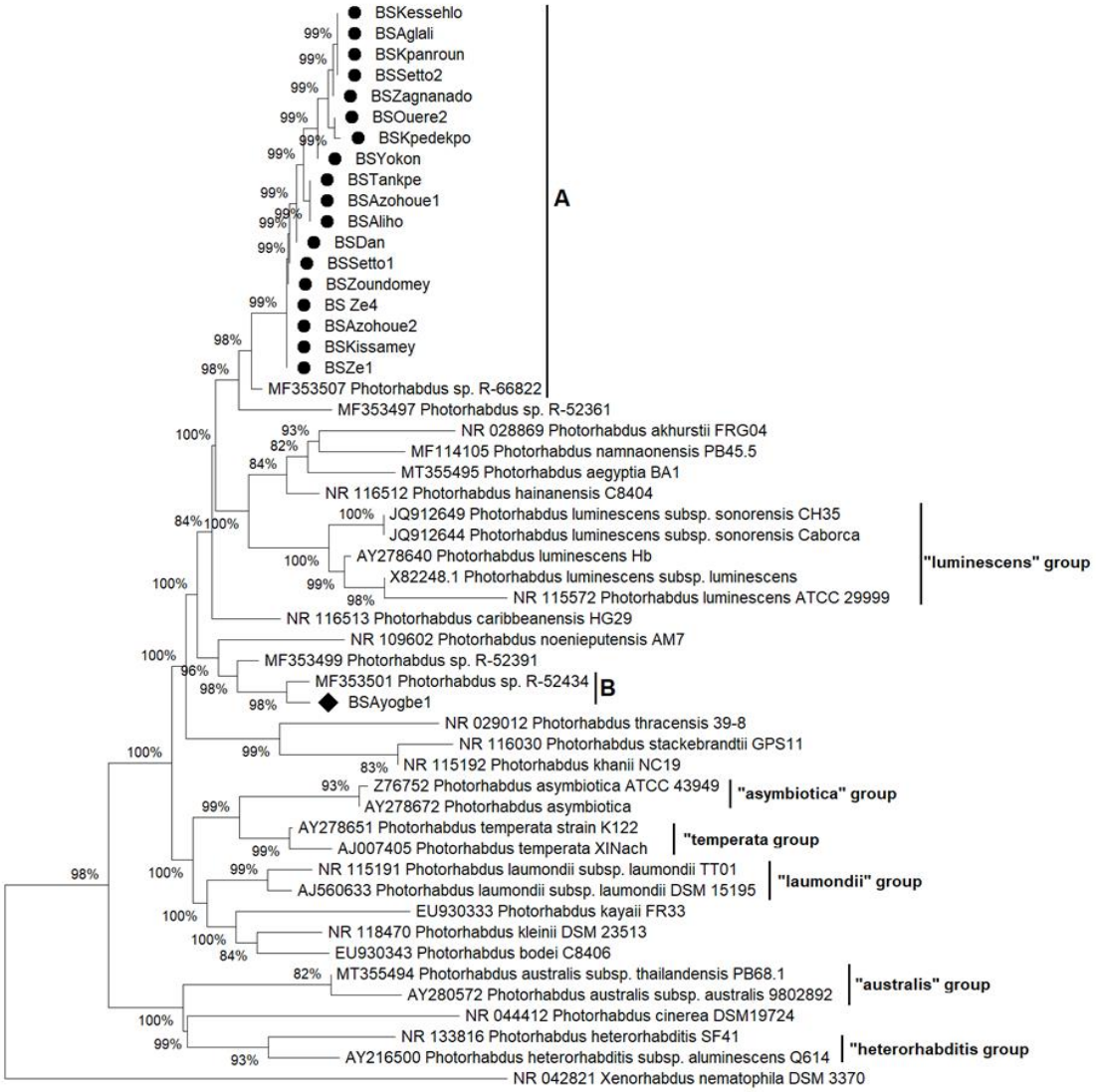

Fig. 2. Neighbor joining tree based on 16S rRNA gene sequences showing the phylogenetic position of Beninese Photorhabdus isolates among type isolates of described Photorhabdus species. Bootstrap values above 50% based on 1000 replicates are indicated at the nodes of each branch. (Beninese bacterial isolate name was composed of its entomopathogenic isolate host name with the prefix "BS)

● = Beninese bacterial isolates hosted by the entomopathogenic nematode *Heterorhabditis sonorensis* strain;

♦ = Beninese bacterial isolates hosted by the entomopathogenic nematode *Heterorhabditis indica* strain.

At 30°C (F = 216.54, p < 0.0001), insect mortality ranged from 0 to 81.63%. The highest larvae mortality was recorded for the bacterial isolates BSZoundomey (81.63%), BSKpedekpo (79.68%), BSZagnanado (78.95%), BSKassehlo (78.22%), BSSetto2 (77.74%), and BSZe1 (71.42%). No insect mortality was induced by the bacterial isolates BSAzohoue1, BSSetto1, BSDan, BSYokon, and BSZe4. The only one bacterial isolate, BSAyogbe1, hosted by the EPN *H. indica*, recorded 1.70% insect mortality.

At 35°C (F = 322.38, p < 0.0001), insect mortality ranged from 0 to 78.47%. The highest larva mortality was recorded by the bacterial isolate BSKpedekpo (78.47%), followed by BSKissamey (75.79%) and BSKassehlo (73.37%). In addition to the aforementioned isolates, the bacterial isolates BSAglali, BSAliho, BSKpanroun, BSTankpe, and BSOuere2 induced no insect mortality. The only one bacterial isolate, BSAyogbe1, hosted by the EPN *H. indica*, recorded 0.24% insect mortality.

At 40°C (F = 94.57, p < 0.0001), insect mortality ranged from 0 to 56.12%. Only seven bacterial isolates, including BSKassehlo (63.65%), BSKpedekpo (56.12%), BSZagnanado (51.99%), BSZoundomey (46.64%), BSSetto1 (7.29%), BSZe1 (5.83%), and BSKissamey (4.86%), were able to induce mortality in insects at this temperature (Table 2).

Table 2. Mortality (% ± SEM) of *Spodoptera frugiperda* exposed to different bacterial isolates associate with Heterorhabditid entomopathogenic nematodes from southern Benin at different temperatures 25, 30, 35 and 40°C

Isolates	Mortality (% ± SEM) at			
	25°C	30°C	35°C	40°C
BSOuere2	78.47 ± 2.3 AB	3.16 ± 1.8 DE	0 ± 0 F	0 ± 0 D
BSYokon	28.18 ± 3.5 E	0 ± 0 E	0 ± 0 F	0 ± 0 D
BSAglali	66.32 ± 4.7 BC	10.69 ± 2.4 C	0 ± 0 F	0 ± 0 D
BSZoundomey*	82.45 ± 1.6 AB	81.63 ± 2.5 A	75.79 ± 3.1 AB	46.64 ± 2.3 B
BSKissamey*	68.26 ± 7.5 ABC	19.19 ± 4.3 B	15.79 ± 2.0 E	4.86 ± 1.8 C
BSAliho	70.45 ± 5.4 AB	7.77 ± 2.5 C	0 ± 0 F	0 ± 0 D
BSAzohoue1	53.69 ± 2.9 CD	0 ± 0 E	0 ± 0 F	0 ± 0 D
BSAzohoue2*	74.09 ± 4.9 AB	5.10 ± 1.5 CD	0.97 ± 0.7 F	0 ± 0 D
BSKpanroun	22.35 ± 2.3 E	0.24 ± 0.2 E	0 ± 0 F	0 ± 0 D
BSTankpe	45.43 ± 7.9 D	0.24 ± 0.2 E	0 ± 0 F	0 ± 0 D
BSZagnanado*	79.44 ± 2.3 AB	78.95 ± 3.7 A	78.47 ± 1.6 A	51.99 ± 4.7 B
BSKpedekpo*	85.51 ± 0.9 A	79.68 ± 2.1 A	68.99 ± 7.2 B	56.12 ± 7.0 AB
BSSetto1	4.13 ± 1.5 G	0 ± 0 E	0 ± 0 F	0 ± 0 D
BSSetto2*	81.63 ± 1.7 AB	77.74 ± 1.3 A	40.81 ± 1.5 C	7.29 ± 2.9 C
BSKassehlo*	82.35 ± 0.8 AB	78.22 ± 1.7 A	73.37 ± 2.4 AB	63.65 ± 3.6 A
BSDan	10.93 ± 2.6 F	0 ± 0 E	0 ± 0 F	0 ± 0 D
BSZe1*	78.71 ± 2.4 AB	71.42 ± 1.2 A	31.09 ± 1.9 D	5.83 ± 2.5 C
BSZe4*	26.24 ± 4.4 E	0 ± 0 E	0 ± 0 F	0 ± 0 D
BSAyogbe1*	65.83 ± 6.2 BC	1.70 ± 1.4 E	0.24 ± 0.2 F	0 ± 0 D
F	43.09	216.54	322.38	94.57
P	<.0001	<.0001	<.0001	<.0001

Means (% ± SEM) within a column followed by the same letter are not significantly different (SNK's test; P < 0.05). An asterisk (∗) indicates isolates selected for virulence studies.

Table 3. Comparison of dose-mortality responses of bacterial isolates associated with Heterorhabditid entomopathogenic nematodes from southern Benin against Spodoptera frugiperda

Isolates	Probit equation[a]	Chi-squared	LC_{50}[b]	95% CL[c]
BSZoundomey	-0.97 + 0.19994 x 10^7 C	445.834	0.483 x 10^6 C	117008.54 – 1969476.61
BSKissamey	-0.68 + 2.13600 x 10^7 C	316.588	3.173 x 10^6 A	2348452.05 – 3996811.38
BSAzohoue2	-0.60 + 2.08510 x 10^7 C	299.737	2.900 x 10^6 A	2066167.78 – 3733941.02
BSZagnanado	-0.46 + 2.22300 x 10^7 C	280.642	2.082 x 10^6 ABC	1305450.00 – 2859641.96
BSKpedekpo	-0.34 + 2.51990 x 10^7 C	267.097	1.355 x 10^6 BC	667176.22 – 2043527.29
BSSetto2	-0.59 + 2.06170 x 10^7 C	296.224	2.883 x 10^6 AB	2042085.68 – 3724480.64
BSKassehlo	-0.96 + 0.21359 x 10^7 C	452.789	0.450 x 10^6 C	85197.13 – 2030947.43
BSZe1	-0.68 + 2.38270 x 10^7 C	333.171	2.876 x 10^6 AB	2125531.62 – 3626634.37
BSAyogbe1	-0.49 + 1.81450 x 10^7 C	261.121	2.705 x 10^6 ABC	1772431.66 – 3636941.34

[a] General responses of percentage killed insect (Y) as a function of bacterial isolate concentration (C), [b] Concentration of bacterial isolate (colony-forming units (CFU)/mL) required to kill 50% of treated insects; LC_{50} values within a column followed by the same letter are not significantly different, based on non-overlapping 95% CL, [c] 95% confident limits (CL) for the LC_{50}.

Concentration-response: Based on the non-overlap of the 95% confidence limits of the LC_{50}, significant differences in LC_{50} were observed among selected bacterial isolates (Table 3). The highest LC_{50} was recorded with the bacterial isolates BSKissamey (3.173 x 10^6 CFU/mL), BSAzohoue2 (2.900 x 10^6

CFU/mL), BSSetto2 (2.883 x 10^6 CFU/mL), and BSZe1 (2.876 x 10^6 CFU/mL). The lowest LC_{50} values were recorded for the bacterial isolate BSZoundomey (0.483 x 10^6 CFU/mL), followed by BSKassehlo (0.450 x 10^6 CUF/mL). The only one bacterial isolate, BSAyogbe1, hosted by *H. indica*, recorded an LC_{50} value of 2.705 x 10^6 CFU/mL (Table 3).

DISCUSSION

Bacterial symbionts associated with EPN play an important role for their ability to control a wide range of soil-dwelling insects and other arthropods (Hiltpold, 2015). In Benin, research works on EPN and their symbiotic bacteria remain embryonic. Our data revealed distinctive colony features displayed by all studied bacterial isolates were typical of the genus of *Photorhabdus*, since they were circular or irregular, green or dark green, raised or convex, and exhibited bioluminescence in the darkness (Thanwisai *et al.*, 2012; Boemare, 2002).

These findings are consistent with those reported in previous work by Godjo *et al.* (2018), where several symbiotic bacterial isolates hosted by EPN *H. taysearae* strains isolated from soils collected in northern Benin have been characterized.

Molecular methods can be used for rapid and accurate identification of bacteria to avoid laborious phenotypic characterization (Adams *et al.*, 2006). Phylogenetic analysis confirmed that all bacterial symbiont isolates, based on their partial 16S rRNA gene sequences (481 bp) definitively belonged to the *Photorhabdus* genus. All the bacterial isolates hosted by *H. sonorensis* strains were close to the *Photorhabdus* isolate R-66822 (99% sequence similarity) previously found to be hosted by *H. taysearae* recovered from soil in northen and central of Benin (Godjo *et al.*, 2018). *H. sonorensis* has been considered as a senior synonym for *H. taysearae* (Hunt and Subbotin, 2016). In addition, a study conducted by Dhakal *et al.* (2020) revealed many cases of *Heterorhabditis* species misidentifications and the presence of reading mistakes in some sequences. These results led into a synonymization of *H. sonorensis* with *H. taysearae*, meaning that *H. sonorensis* and *H. taysearae* consist of the same species (Dhakal *et al.*, 2020). In light of the above, it may be concluded that bacterial isolates hosted by Beninese's *H. sonorensis* in this study are identical to the *Photorhabdus* isolate R-66822. However, this result was inconsistent with previous findings of Orozco *et al.* (2013), who identified *P. luminescens* subsp. *sonorensis* subsp. nov as the bacterial symbiont of the entomopathogenic nematode *H. sonorenis* isolated from the Mexican state of Sonora. The only one bacterial isolate hosted by *H. indica* was close to the *Photorhabdus* bacterial isolate R-52434, with 98% sequence similarity as its closest neighbor. This result agrees with Godjo *et al.* (2018), who isolated the *Photorhabdus* bacterial isolate R-52434 from EPN *H. indica* strains retrieved from soil in northern Benin. Our results are not in line with previous studies, which have reported *P. luminescens* subsp. *akhurstii* to be associated with EPN of *H. indica* (Salazar-Gutiérrez *et al.*, 2017).

H. sonorensis was described as having *P. luminescens* subsp. *sonorensis* as a bacterial symbiont (Orozco *et al.*, 2013), while *H. indica* has been reported to live in association with *P. luminescens* subsp. *akhurstii* (Fischer-Le Saux *et al.*, 1999). Further investigations need to be carried out to fully characterize these *Photorhabdus* bacterial isolates, R-66822 and R-52434, hosted by Beninese EPN *H. sonorensis* and *H. indica*, respectively.

The entomopathogenic potential of the isolated bacteria showed all tested bacterial isolates were pathogenic against *S. frugiperda*, as they were able to infect and kill the larvae. This suggests that the bacterial isolates overcame the immune resistance of *S. frugiperda*, resulting in the insect's death. Even outside the EPN vector, bacterial isolates were pathogenic to *S. frugiperda* larvae. These findings are consistent with those of Salvadori *et al.* (2012), who revealed four *Photorhabdus* species injected into the hemocoel of the 5th instar larvae of *S. frugiperda* (10^4 cells per larva) produced 75-96% mortality three days after bacteria injection.

Moreover, in their studies, Mohan and Sabir (2005) demonstrated the successful use of *Photorhabdus* species alone as a biopesticide, independent of its nematode symbiont, against some insects of the Lepidopteran order, including the cabbage white butterfly, *Pieris brassicae*, and the pupae of the diamondback moth, *Plutella xylostella*. On the other hand, our results displayed different levels of susceptibility of *S. frugiperda* to different bacterial isolates housed by one EPN species, *H. sonorensis*, thus corroborating findings of Salvadori *et al.* (2012). Nevertheless, besides pathogenicity against *S. frugiperda* larvae, the tolerance to environmental stresses is a paramount criterion when selecting isolates for biocontrol purposes (Glazer, 2002). Temperature is one of the important factors affecting both entomopathogenic nematodes and their symbiotic bacteria (Sharmila *et al.*, 2018).

Temperature significantly influenced the infectivity of all isolated bacteria in our study. Insect mortality decreased as exposure temperature increased from 25°C to 40°C. But nine bacterial isolates were pathogenic to *S. frugiperda* larvae at 35°C, and better yet, seven of them could still kill the insects at an exposure temperature of 40°C. This is the first scientific research report on EPNs' symbiotic bacteria pathogenic to *S. frugiperda* larvae in Benin.

Significant differences occurred between bacterial isolates with respect to the LC_{50}. Bacterial densities of 0.450 x 10^6 CFU/mL and 0.483 x 10^6 CFU/mL were sufficient for the bacterial isolates BSKassehlo and BSZoundomey, respectively, to kill 50% of the fifth instar larvae of *S. frugiperda*, whereas BSKissamey, BSAzohoue2, and BSZe1 needed 3.173 x 10^6 CFU/mL and 2.900 x 10^6 CFU/mL, respectively. Our results suggest bacterial isolates retrieved from different strain belonging to one species of EPN exhibited differential efficacy to the 5^{th} instar larvae of *S. frugiperda*. These values are less than that needed by the symbiotic bacteria (9.95 × 10^9 CFU/mL) associated with *Steinernema carpocapsae* to kill 50% of a population of the third-instar larvae and adults of the Red Palm Weevil (Chaojun *et al.*, 2025).

CONCLUSION

The results of this study demonstrated that symbiotic bacteria associated with *Heterorhabditis sonorensis* strains from Benin are identical to the *Photorhabdus* isolate R-66822 deposited in GenBank under the accession number MF353507. Additionally, the symbiotic bacteria associated with the single strain of *Heterorhabditis indica* are identical to the *Photorhabdus* bacterial isolate R-52361, which is deposited in GenBank under the accession number MF353497. All tested bacterial isolates were pathogenic, to various extents, against 5th instar larvae of *S. frugiperda*. These findings are sufficiently encouraging to pursue further investigations of continued investigation into the potential of these native bacterial isolates for sustainable management strategies of *S. frugiperda* in agricultural areas.

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