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ORIGINAL PAPER

Plant growth-promoting rhizobacteria do not pose any deleterious effect on cowpea and detectable amounts of ethylene are produced

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Summary The use of trap crops such as cowpea could reduce the effects of the root parasitic weed, *Striga hermonthica* and its subsequent constraints on the growth of cereals. Certain bacteria could augment the trap crop stimulatory effect. We studied the effect of three bacteria introduced to the rhizosphere of three cowpea varieties at planting. Number of days to cowpea flowering was noted and at harvest, data were collected on pod characteristics and biomass. Means of data subjected to ANOVA were compared using Tukey's Studentized Range Test. We analysed bacterial headspace volatiles for ethylene by gas chromatography and gas chromatography–mass spectrometry. Bacterial type significantly influenced the cowpea varieties with better performance over the non-inoculated control. Average pod weight (g) with bacterial treatment was 37.97 for *Enterobacter sakazakii* 8MR5, 34.38 for *Pseudomonas* 44MS8 and 27.46 for *Pseudomonas* 10M3. Non-inoculated control had an average weight of 20.98 g. Bacteria promoted a significant increase in pod weight (≥30.89%), fresh biomass (≥24.22%), and improved pod number (≥20.54%) and pod wall thickness (≥7.33%) with no deleterious effect on plant health. Ethylene released by the bacteria ranged from trace concentrations in *Pseudomonas* sp. to 210 nmoles/10^8 c. f. u./ml in *Ent. sakazakii* 8MR5.

Keywords Bacteria · Biological control · Cowpea · Ethylene · Gas chromatography · *Striga hermonthica*

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Introduction

Cowpea (*Vigna unguiculata*) is the most popular legume in West Africa, particularly in countries such as Nigeria, Niger, and Burkina Faso where much is consumed. Cowpea is also important in some other regions of Africa and the world (Lambot 2000). Ransom (1997) reported that seed demise of the root-parasitic weed *Striga hermonthica* (witchweed) in suppressive soil might be due to microbiological elements in the soil or biological activity. In Africa, cowpea is one of the commonly used trap crops in areas under *S. hermonthica* infestation. Some other trap crops of *S. hermonthica* documented include bean (*Phaseolus vulgaris*), groundnuts (*Arachis hypogea*), (Kiriro 1998) and soybean (*Glycine max* L.). Most farmers because of the yield obtained prefer trap cropping to other *Striga* control methods. However, it is hoped that if *S. hermonthica* stimulating-bacteria (Babalola et al. 2002, 2003, 2004a, b) are introduced to the rhizosphere at cowpea planting this will augment the suicidal effect

of cowpea on *S. hermonthica* and deplete the *Striga* seed bank in the soil. The use of bacteria for the biological control of *S. hermonthica* is recent (Babalola et al. 2002; Berner et al. 1999). Studies indicate that *Striga asiatica* seed may remain viable in the soil for 14 years or more (Bebawi et al. 1984) indicating that the venture of inducing *Striga* suicidal germination is worth undertaking.

Previous work on the recovery, isolation, and identification of the natural germination stimulants, although with little success, showed that even extremely small amounts of these substances induce maximum germination (Herb et al. 1987; Sugimoto et al. 2003). The induction of suicidal germination stimulants (Babalola et al. 2003; Sugimoto et al. 2003) in very low dosages in the absence of a host will eventually result in a more effective method of controlling *Striga* spp. This approach is environmentally friendly. Knowledge of the phytotoxicity of microorganisms is very useful in developing a sound biological management program for *S. hermonthica*. Numerous studies have been conducted on the use of trap crops in the rotation to deplete *Striga* seed reservoir in the soil (Gbehounou and Adango 2003; Ransom 2000). However, these studies are mostly not on plant health. It is therefore, necessary to conduct more studies on the effect of bacteria on plant health.

Berner et al. (1999) successfully used ethylene-producing bacteria to stimulate *Striga* seed germination in the laboratory. In related screening experiments, several fluorescent pseudomonads suppressive to *S. hermonthica* seeds have been identified (Ahonsi et al. 2002). However, the levels of ethylene produced by these and related rhizobacteria are not known. Therefore, the objectives of this study were to induce *Striga* suicidal germination with bacteria, screen for the effect of bacteria on cowpea health, quantify the levels of ethylene produced in the headspace of three rhizobacteria isolated from soils associated with maize, with the aim of providing additional information to support the use of bacteria in the biocontrol of *S. hermonthica*.

Materials and methods

Inoculum, culture conditions and identification

Bacterial isolate 8MR5 was from the endorhizosphere (Klyuchnikov and Kozhevin 1991) of maize (var. 8338-1) (Babalola et al. 2002). Isolates 44MS8 and 10M3 were from maize exorhizosphere (Klyuchnikov and Kozhevin 1991).

Fluorescent *Pseudomonas* 44MS8 and *Pseudomonas* 10M3 were stored in 25% glycerol at –80°C until 24 h before use and then spotted on King's medium B (KB) agar plates (King et al. 1954) for 24 h. Bacterial isolate 8MR5 was grown on Tryptone Soy Agar (TSA). All cultures were grown for 48 h at 28°C on appropriate agar plates as stated above. Individual plates were harvested by adding 10 ml of 0.01 M phosphate buffer (PBS, pH 7.0) solution and scrapping with a sterile cotton swab. The harvested bacteria were pooled together according to the bacterial type, washed twice, and adjusted to the desired concentration by optical density. The bacteria were serially diluted in buffer and plate counts were made on the inoculum prior to inoculation. The average colony forming units (c. f. u.) from triplicate plates was determined by counting after 48 h incubation, this was used to express the quantity of inoculum added. These isolates were identified by '*Appareils et Procédés d'identification*' (API20E 1988) test.

Seed collection and *S. hermonthica* viability test

Kenya sugar research foundation (KESREF) Kisumu provided the seed of cowpea Rabuor (Kenya) local. The other trap crops IT95K-286-4 and IT94D-437-1 used in the experiments came from the International Institute of Tropical Agriculture (IITA) germplasm collection.

Seed of *S. hermonthica* from parasites on maize in Kibos Kenya were dried, put in paper bags and stored at room temperature. The seed to be preconditioned were sterilized in 1% NaOCl solution for 5 min with continuous stirring. The seed were rinsed, air-dried and kept in glass vials in a dark incubator at 30°C until used.

S. hermonthica seed viability was determined by the method of Eplee and Norris (1987), using 1% aqueous solution of 2, 3, 5-triphenyltetrazolium chloride (TTR) solution (pH 7).

Planting in the screenhouse

The study was conducted at KESREF, in Western Kenya, between latitude 0°02′S and longitude 34°48′E under screenhouse conditions. Soils used were collected from fields under heavy *S. hermonthica* infestation at KESREF. The soils were passed through a 10-mesh sieve and thoroughly mixed for potting. Fourteen days before planting was done, the soil was artificially infested with *S. hermonthica* seeds at the rate of 50 mg/container within the first 0–10 cm depth. All the experimental containers (30-cm diameter,

30-cm depth) were *S. hermonthica*-infested. At planting, isolates 8MR5, 44MS8 and 10M3 were applied to soil by inundation of 50 ml inoculum suspension (see Table 1 for c. f. u./ml), prepared from KB or TSA cultures. Only one isolate was added to a container. Inundation of the inoculum suspension was made into the open planting hole on the top of each container. Three cowpea seeds were evenly spaced in the hole immediately after addition of the inoculum and were covered with about 2 cm of soil. Non-inoculated containers served as control. The cowpea seeds (*Vigna unguiculata* (L.) Walp. were treated in 70% ethanol for 30 s and in 4% NaOCl for 3 min for surface sterilization just before planting. After 2 weeks thinning was done and a stick was put close to the container to support the plants. Watering was done daily using tap water, and weeding was done at appropriate times. Plants were harvested 61 days after planting.

Ethylene quantification

Headspace volatiles were allowed to equilibrate in 100 ml KB or TSA broths contained in 150 ml sealed GIBCO reagent bottles (175 ml maximum capacity) for 6, 12, 24 and 48 h at ambient temperature on a laboratory bench. A gas chromatography (GC) syringe hole was drilled in each bottle cap, which was then lined with sterile Teflon-faced silicone rubber septa before closure. The latter ensures an air tight condition in each of the bottles.

Samples of the headspace (100 μl) from the inoculated broth and a non-inoculated control broth were analyzed by GC and gas chromatography–mass spectrometry (GC–MS). GC analyses were performed on a Hewlett–Packard (HP) 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a HP capillary column (Ultra 1 Cross-linked methyl silicone, 50 m × 0.2 mm ID × 0.33 μm film thickness) using nitrogen as the carrier gas at a flow rate of 0.35 ml/min. The oven temperature was initially isothermal at 40°C for 5 min, then programmed at 10°C/min to 150°C and held there for 8 min. Chromatographic peaks were integrated using a HP 3396 integrator. GC–MS analyses were carried out on a Fisons Instruments 8060 Series II chromatograph coupled to a Fisons Instruments VG Platform II MS (EI, 70 eV), employing the same chromatographic conditions as described. Compounds were identified by comparing their spectral data with those of the library (National Institute of Standards and Technology (NIST 1995) of the mass spectrometer. Authentication of the identity of the compounds was achieved by comparing the retention times of the headspace volatiles of the isolates with those of commercial samples of ethylene (Del Monte, Nairobi, Kenya) and 3-hydroxy-2-butanone (Aldrich, UK).

Experimental design and statistical analysis

Treatments were laid out in a randomized complete block design and replicated three times. Treatments consisted of three plant growth-promoting bacteria at three-cowpea varieties level and a control with no added bacteria also at three cowpea varieties levels. The experiment was repeated twice. Data were collected on a number of days of cowpea flowering, biomass, and

Table 1. Effects of *Enterobacter sakazakii* 8MR5, *Pseudomonas* 44MS8, and *Pseudomonas* 10M3 applied at planting on yield components of cowpea sown in *Striga hermonthica*-infested soil

Cowpea variety	Bacterial isolate[a]	Pod thickness (cm)	Pod length (cm)	Pod number	Pod weight (g)	Fresh biomass (g)	Dry biomass (g)	Days to flower
IT95K-286-4	8MR5	4.33	12.83	54.67	42.60	463.67	71.37	45.33
	44MS8	5.67	12.67	41.00	30.92	360.50	50.33	46.00
	10M3	3.67	11.93	47.33	22.22	399.66	60.03	46.50
	Control[b]	5.00	12.30	49.50	30.35	362.50	58.20	43.00
	SE	±0.59	±0.21	±4.07	±6.01	±34.52	±4.24	±0.80
IT94D-437-1	8MR5	5.00	12.63	23.00	24.40	420.00	72.20	47.00
	44MS8	6.33	14.67	19.67	26.90	413.50	68.63	48.00
	10M3	5.00	13.50	19.67	16.93	440.50	69.03	48.00
	Control[b]	4.00	12.95	15.50	11.40	536.00	88.90	48.50
	SE	±0.50	±0.61	±2.21	±2.91	±26.26	±3.74	±0.23
Rabuor (local)	8MR5	5.67	17.77	43.67	46.90	496.00	67.13	47.33
	44MS8	5.00	17.06	45.33	45.33	441.67	64.60	47.67
	10M3	5.00	17.55	46.50	51.10	424.00	53.70	46.50
	Control[b]	5.50	17.10	19.00	21.20	268.00	56.00	47.00
	SE	±0.47	±0.31	±4.50	±6.55	±39.18	±4.42	±0.29

[a] *8MR5 Ent. sakazakii* (1.68×10^6 c.f.u.), *44MS8 Pseudomonas* (1.33×10^6 c.f.u.), and *10M3 Pseudomonas* (1.10×10^6 c.f.u.)

[b] Check without bacterial seed treatment

pod characteristics. Pod wall thickness were characterized thus 3 = thin, 5 = intermediate, and 7 = thick. Pod length was measured in cm; samples were oven-dried at 80°C for 48 h to determine dry biomass. The data were subjected to analysis of variance and the treatment means were compared using Tukey's Studentized Range (HSD) Test (SAS 1998).

Results and discussion

Cowpea (variety, IT95K-286-4) treated with *Ent. sakazakii* 8MR5 and *Pseudomonas* 10M3 had significant and characteristically small pod wall thickness. The overall mean of the pod wall thickness of cowpea (variety, IT94D-437-1) sown under *Ent. sakazakii* 8MR5, *Pseudomonas* 44MS8, and *Pseudomonas* 10M3 was significantly higher than that of the non-inoculated control (Table 1).

Pod lengths for varieties, IT95K-286-4, IT94D-437-1, and Rabuor (local) were highly significant ($P < 0.05$) for *Ent. sakazakii* 8MR5, and *Pseudomonas* 44MS8 (Table 1). Hence the effect of variety on pod length was pronounced for *Ent. sakazakii* 8MR5 and *Pseudomonas* 44MS8. Average length of the pods from cowpea variety IT95K-286-4 sown to containers that receive *Pseudomonas* 10M3 was significantly shorter than the non-treated control. The difference obtained for the pod length may not only be genetically influenced but also be the resultant effect of bacterial treatment, as the non-inoculated controls showed no significant difference for pod length. Mean value of *Ent. sakazakii* 8MR5 was the highest for pod length across bacterial seed treatment in IT95K-286-4 and Rabuor, Kenya local (Table 1).

Among the yield components studied (pod wall thickness, pod length, pod weight, pod number, and biomass) number of pods per container was highly significant for cowpea variety. The number of pods produced by variety IT95K-286-4 ranks first followed by variety Rabuor (local). These pod numbers were significantly different from that produced by variety IT94D-437-1 (Table 1). This was also true for the pod weight at harvest. Although variety IT95K-286-4 has greater pod number than local Rabuor, the pod weight of IT95K-286-4 was lower. Rabuor local pods outweighed IT95K-286-4.

The pod weight per container was an indicator of the efficacy of the introduced bacteria. Overall, cowpea variety IT94D-437-1 has the lowest pod weight, even in the control experiment. Each of the three bacterial isolate improved the plant pod weight over the control (Table 1). Amongst the three cowpea varieties, Rabuor (local) produced the heaviest weight for all the three bacterial strains tested. However, in the control, variety IT95K-286-4 outweighs Rabuor (local) in pod weight. It is noteworthy that this difference in weight between IT95K-286-4 and Rabuor (local) was not significant. In general, pod weight of containers that received *Ent. sakazakii* 8MR5 were the best (Table 1) with 80.98% change over the non-inoculated control. The impact of variety was significant for pod weight. The introduced bacteria at planting affected the three tested cowpea varieties with better performance over the non-inoculated control.

All three bacterial treatments significantly increased Rabuor (local) fresh biomass over the non-treated control (Table 1). The *Ent. sakazakii* 8MR5 supported significantly greater biomass for IT95K-286-4 and Rabuor (local) variety. From the results presented, the average fresh biomass of IT94D-437-1 was highest in the absence of bacterial treatment. It is noteworthy that the coefficient of variation (CV) for the non-inoculated control was higher for fresh biomass weight than for the cowpea sown to bacterial-treated soils. The CV was more than double that of *Pseudomonas* 10M3 and *Ent. sakazakii* 8MR5 in particular. However, across the bacterial treatments, the overall mean of *Ent. sakazakii* 8MR5 (459.89 g/container), *Pseudomonas* 44MS8 (405.22 g/container), and *Pseudomonas* 10M3 (421.06 g/container) were much more than that of the control containers 388.83. Number of days to cowpea flowering was less consistent except for IT95K-286-4 and the differences were not significant.

We recorded biomass of the screenhouse container experiment at 61 days after planting. The major portion of mean square was from fresh biomass followed by the number of pods, then pod weight (Table 2).

From the data presented on the screening of three *S. hermonthica*-stimulating rhizosphere bacteria for phytotoxicity in cowpea (non-host), the two *Pseudomonas* strains and *Ent. sakazakii* 8MR5 have no deleterious effect on cowpea. The low performance of variety IT94D-437-1 was not due to the bacterial effect; such low pod characteristics were observed in the non-inoculated control, as evident in this study. However, it is of the utmost necessity to see how the maize host of *S. hermonthica* will respond during crop rotation. Rabuor local heaviest pod weight in comparison to the other varieties might be associated with a genetic trait of adaptation to the prevailing environmental condition in Kibos, Western Kenya where the experiment was conducted. Besides, varieties IT95K-286-4 and IT94D-437-1 are breeding lines—developed at IITA, Ibadan, Nigeria. These results also reveal that the farmers are very likely to buy the idea of augmenting

Table 2. Analysis of variance of pod characteristics and the number of days to flowering of three cowpea varieties evaluated at three bacterial seed treatment levels in soil under natural *Striga hermonthica*-infested conditions for 61 days

Source of variance	*df*	Mean square						
		Pod thickness	Pod length	Pod number	Pod weight	Fresh biomass	Dry biomass	Days to flower
Isolate	3	1.871	0.632	190.749	375.679	7350.224	183.012	1.321
var	2	1.038	67.813***	2202.534***	1146.595*	9308.607	738.136	14.118*
Error	20	3.591	2.317	141.575	331.706	11952.250	184.245	2.844
Total	25							

*, **, *** Significant at 0.05, 0.01 and 0.001 levels of probability

the stimulatory effect of some cowpea in the suicidal germination of *S. hermonthica*.

The results from these experiments clearly indicate that the effect of *Ent. sakazakii* 8MR5 is more pronounced on pod weight, biomass, and pod number while the effect of *Pseudomonas* 44MS8 was more pronounced in pod wall thickness and pod length. *Pseudomonas* 10M3 performed weakly. This study establishes that varieties have differential responses to bacterial inocula because of the microbial activity.

The inconsistency in flowering pattern may be affected by the environment or was not variety-specific. This varietal influence is similar to that reported by Burr et al. (1978) who worked on *Pseudomonas fluorescens* and *P. putida* using potato and the report of Howie and Echandi (1983) on rhizosphere bacteria on different cultivars of potatoes. In accordance with the findings in this study, neither Burr et al. (1978) nor Howie and Echandi (1983) obtained evidence of cultivar specificity in potato treated with PGPR. The difference in days to IT95K-286-4 flowering was significantly different from the two other varieties under the conditions tested. This is probably the reason why it supported the greatest number of pods at harvest.

The analytical results obtained showed repeatability of peak retention time. GC–MS analysis identified ethylene as the major component in the headspace volatiles of culture media of the three isolates after 48 h of incubation. Carbon dioxide (CO_2) was identified as a co-eluent with ethylene in all the analysis, while 3-hydroxy-2-butanone was identified as a trace component in the headspace volatiles of 8MR5. The combined concentration of ethylene with CO_2 was estimated for 100 μl headspace samples: *Pseudomonas* spp (trace), and *Ent. sakazakii* 8MR5 (210 nmoles/10^8 c. f. u./ml). GC-MS analysis showed relatively strong fragment ions for CO_2 in the headspace volatiles of *Pseudomonas* spp, with the weakest in the volatiles of *Ent. sakazakii* 8MR5. We did not detect ethylene in media incubated for less than 48 h, and in the control media.

Berner et al. (1999) reported that ethylene-producing bacteria are as effective in stimulating seed germination of *Striga* spp. as are soil injections of ethylene gas. The results of the present study revealed that the bacteria emit ethylene, but at varying concentrations and co-elutes with CO_2, as previously reported by Sato et al. (1987). The presence of CO_2 as a co-eluent with ethylene in the GC–MS analysis is in agreement with the previous observation of Odhiambo (1998) that CO_2 inhibits ethylene production under anaerobic conditions. The level of ethylene released by *Ent. sakazakii* 8MR5 was similar to the level required for stimulating suicidal germination in *Striga* seeds, suggesting the potential use of selected bacteria in the biological control of *Striga* spp.

Conclusions

Based on summary data presented in this work we concluded that the isolates are safe for introduction into the rhizosphere during cowpea planting, as they are not deleterious to plant health. Ethylene detected in the headspace of the three-bacterial isolates after 48 h suggests that they have a high potential for use in the biological control of *Striga* seeds.

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