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Effect of bacillus subtilis on bean nematodes in Kenya: A laboratory and green house experiment

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Abstract
Nematodes have been causing big losses in the yields and quality of crops worldwide. Bacteria are major biocontrol agents. Laboratory and green house experiments were conducted at the University of Nairobi College of Agriculture and Veterinary Sciences to determine the effect of *Bacillus subtilis* on bean parasitic and free living nematodes. *Bacillus subtilis* were isolated from soil using procedure by Racke and Sikora in 1992 in the plant pathology laboratory. Identification of *Bacillus subtilis* was done using biochemical tests outlined by Claus and Berkeley 1986. The green house experiment was arranged in a completely randomized design with 5 replications. It consisted of 5 pots with nematode infected soil and 5 with sterile sand all inoculated with 2ml of *Bacillus* subtilis. Un-inoculated soil and soil inoculated with *Bacillus subtilis* K194 were included as negative and positive controls respectively. *Bacillus subtilis* inoculum was placed in holes and radicals of germinated seeds inserted for the 12 pots and their 5 replications. 5 *Bacillus* isolates out of 10 were significantly abundant with colony forming units near those of *Bacillus subtilis* K194, the positive control. 2 isolates significantly increased the numbers of free living nematodes and three significantly reduced numbers of plant parasitic nematodes. Paratylenchus, Tylenchus and Meloydogyne were the most susceptible genera of nematodes. *Bacillus subtilis* inoculum can be readily applied in the soil to reduce infestation by plant parasitic nematodes while boosting beneficial free living nematodes.

Keywords: *Bacillus subtilis*, Nematodes, Bean, Biocontrol

1. Introduction
Plant parasitic nematodes cause an estimated US$100 billion in damage to Agricultural crops worldwide [1].
A broad specrum of parasitic genera of nematodes have been associated with leguminous crops [2]. Root-knot nematodes (*Meloidogyne* spp.), Lesion nematodes (*Pratylenchus* spp), Yam nematode (*Scutellonema spp.)*, spiral nematodes (*Helicotylenchus spp.)* stunt nematodes (*Tylenhorynchus)*, *Tylenchus* spp, *Aphelenhus* spp. sheath nematodes (*Hemicyclophora* spp*)*, stubby root nematodes (*Trichodorus* spp) and others are associated with beans. Members of the genera *Meloidogyne*, *Scutellonema* and *Helicotylenchus* are widely distributed in bean growing areas in Kenya [3].
The management of nematodes is more difficult than that of other pests because nematodes mostly inhabit the soil and usually attack the underground parts of the plants [4]. Long term environmental and management problems have been experienced with use of nematicides since the target molecules are scattered within the soil volume and their exact locations indefinite, therefore large volumes of chemicals are applied [5]
Nematodes in soil are subject to infections by bacteria and fungi. This creates the possibility of using such soil microorganisms to control plant-parasitic nematodes [6]. Biological control agents like *Paecilomyces lilacinus*, *Pochonia chlamydosporia*, *Trichoderma* spp., *Plectosphaerella cucumeria, Agrobacterium radiobacter*, *B. subtilis*, and *Pseudomonas* spp. have demonstrated potential as control agents for nematodes [7]. Biological Control is usually associated with different modes of action including parasitism, interfering with nematode host recognition processes, competition for nutrients in the roots and induction of systemic resistance [8]. Metabolites produced during bacterial fermentation have been shown to be nematicidal for instance the ones produced by B. amyloliquefacience combined with Paenobacillus macerans are commercial bionematicides that produce toxic compounds [9]. The most important enzyme commercially produced by bacterial fermentation are proteolytic ones from Bacillus Species [10].
B. subtilis, the model system for Gram-positive organisms, is able to produce more than two dozen antibiotics with a variety of structures [11]. Several bacteriocins have been reported such as lichenin produced by the B. licheniformis 26-103RA strain [12], megacin produced by strains of B. megaterium [13], antilisterial coagulin produced by B. coagulans [14], polyfermenticin SCD produced by B. polyfermenticus [15], and cerein produced by strains of B. cereus [16, 17].
Understanding nematophagous bacterial populations and their mechanisms of action against nematodes will provide a basis for improving the pathogenic activity of potential biocontrol strains, for developing novel biological control strategies, and for exploring their roles in an integrated nematode management system. In the present study a description of the known nematophagous bacteria is made paying specific attention to the action of Bacillus subtilis inoculums on different plant parasitic nematodes.

2. Materials and Methods
Soil sampling for isolation and characterization of *Bacillus spp.*
Soil samples were collected from the sites using the traversing method to ensure that it was representative. Using a soil auger, nine soil samples were collected at depths of 0-10cm and 10-

20cm. The soil was homogeneously mixed to constitute a composite sample from which 500g soil was taken placed in plastic bags and double sealed and then kept under shade. The samples were then delivered to the University of Nairobi, Department of Plant Science and Crop Protection's Plant Pathology Laboratory and kept at room temperature [18]. Isolation and identification of *Bacillus spp.*

Soil was collected from the University of Nairobi, College of Agriculture and Veterinary Sciences fields for extraction of *Bacillus spp.* Isolation of *Bacillus spp.* was done using the procedure described by Racke and Sikora [18] A plate of nutrient agar inoculated (by the spread-plate method) with 0.1 ml of a heated (80°C for 15 min) dilution of soil and incubated aerobically. The CFU (colony-forming units) are expected to be just endospores, as vegetative cells and reproductive spores of microorganisms would have been killed by the heat treatment. (An exception would be vegetative cells of thermophilic bacteria, but these organisms would not be growing and producing colonies under our 30°C incubation conditions). The heat treatment of the inoculum and the aerobic incubation of the plates make virtually all colonies on this plate to be those of various species of *Bacillus*. The isolates were then identified using cultural characteristics such as colour and shape of the bacteria cell as outlined [19]. Colonies resembling those of *Bacillus spp.* were purified before being stored in sterile soil in universal bottles. The bacterial colonies were inoculated in nutrient broth and incubated until they were turbid. Specific measurements were taken and inoculated in specific amounts of peat to make Bacillus inoculums.

Biochemical Tests

The tests included production of catalases, hydrolysis of gelatin, hydrolysis of starch, reduction of nitrate, growth in sodium chloride, acid production from carbohydrates and ability to form endospores.

Production of catalase

Bacillus isolates were grown on nutrient agar slant for 48 hours. The cultures were flooded with 0.5ml of 10% hydrogen peroxide. The lid was replaced immediately to avoid dispersal of aerosol and was observed for gas production.

Gelatin hydrolysis

Tubes containing nutrient agar supplemented with 0.4%gelatin were stab inoculated with each isolate by stabbing, incubated at 27^0C and observed for gelatin liquefaction at 3- day's intervals for a period of four weeks.

Hydrolysis of starch

Duplicate plates containing starch agar medium were inoculated with each test isolate by streaking and incubated at 27°C for 5 days. Three drops of iodine solution were added into the tubes followed by similar amounts of hydrochloric solutions. The resulting color changes were observed.

Growth in sodium chloride

Plates of nutrient agar containing 2, 5, 7, and 10% sodium chloride were inoculated with each of the test cultures. Cultures were then incubated at room temperature and were observed for bacterial growth after 5 days.

Utilization of carbohydrates

The isolates were evaluated for their ability to utilize different carbohydrates using the method by Hayward [20]. Nutrient broth was used as the basal medium supplement with 5% glucose, sucrose, maltose, arabinose, xylose, mannitol, cellobiose, dulcitol, or sorbitol. The pH of the medium was then adjusted to 7.0. Bromothymol blue was added as an indicator. The medium was then dispensed into universal bottles before being autoclaved at 121°C for 15 minutes.

A loopful of each test isolate was added to inoculate the media in universal bottles before incubation at 27°C and observed after 14 days. Utilization of a given carbohydrate was positive when the medium gradually turned yellow from the top downwards. Control bottles not stub inoculated with *Bacillus* isolates remained olivaceous green in color.

Heat test for endospores

The test was aimed at killing all the vegetative cells leaving the endospores which tolerate high temperatures (Tuitert *et al.*, 1998) [21]. Two to three ml of a turbid bacterial suspension was heated in a test tube at 80°C for 30 minutes, in a water bath. The bacterial suspension was streaked on nutrient agar and was incubated at 27°C. Observations were made 24-48hr later. Growth was monitored to see whether it indicated endospore formation since any growth would be due to endospores since the heat treatment killed all the vegetative cells.

Staining for endospores

A loopful of bacterial suspension was smeared onto a microscope slide and then air-dried. The smear was fixed by passing the slide through the flame 20 times.

The smear was stained for 10 minutes using saturated malachite green (10g/100ml). After gently washing with running tap water, the smear was counter stained with 0.25% safranin for 15-30 seconds. The slide was washed, blotted dry, and examined under a microscope. Endospores appear bright green under pale-pink cells.

Gram reaction

Twenty four hour old cultures of *Bacillus spp.* were grown on nutrient agar and used in this test. A diluted bacterial suspension was made in a drop of sterile distilled water. The suspension was spread thinly on a clean microscope slide and air-dried. The smear was fixed by passing the slide two to three times, through a flame. The smears were stained using procedures described

Greenhouse Experiment

This experiment was conducted to compare the effects of *Bacillus* isolates on plant parasitic nematodes under sterile and non-sterile soil conditions. Screening was done to identify the most effective *Bacillus* isolates. Soil sampling was done in a nematode infected field followed by potting in pots measuring 10cm in diameter and transferred into the greenhouse. Each pot was infected with 2ml of *Bacillus* suspension (ca 10^9 cfu /ml). Sand was autoclaved for three concecutive days and nutrients were added to support the growth of the beans. The extracted nematodes using modified Baermann's procedure

were used as inoculum for the sterile sand experiment. The jars were transported to the greenhouse and each of the 10 isolates with 10 replicates added by mixing with the sand. *B. subtilis* K194 and the uninoculated soil were included as controls to give a total of 12 replications. The greenhouse experiment was arranged in a completely randomized design.
A small amount of the inoculums was put in each hole and the radical of the germinated seed inserted for the 12 pots and their 5 replications. Watering of the greenhouse materials took place until 45 days. Effects of *Bacillus* isolates on plant parasitic nematodes were assessed by determining, nematode counts (initial and final) and indirectly by dry root, galling index and determination of colony forming units (CFU).

3. Data analysis
Data was analysed using General Statistics package (GENSTAT) for windows and means separated using LSD at 5% level of significance [22]. Correlations were done using Statistical Package for Social Scientists (SPSS) for windows at 5% levels of significance.

4. Results
In this section the findings of experiments conducted in the laboratory, on farm, on station and in the greenhouse are presented. Results include characterization of *Bacillus spp*, CFU and damage to bean roots by plant parasitic nematodes as well as the yield of the crop.

Cultural characteristics of *Bacillus spp.*
Bacillus isolates were heat stable and formed endospores with irregular margins and different colors. The five isolates had varying colony characteristics when grown on nutrient agar (Table 1). The colonies were dull or shiny in appearance. Some *Bacillus* isolates attached strongly on the media but some did not.

Table 1: Colony characteristics of *Bacillus* isolates from soils grown on nutrient agar

***Bacillus* Isolate**	**Colour**	**Colony appearance**	**Margin**	**Form or shape**	**Elevation**	**Surface**
A	Creamy	Dull	Undulate	Round	Flat	Spreading
B	White	Shiny	Undulate	Round	Flat	Spreading
C	White	Shiny	Undulate	Round	Raised	Spreading
D	Creamy	Dull	Undulate	Round	Raised	Spreading
E	White	Shiny	Undulate	Round	Raised	Spreading
F	Creamy	Shiny	Undulate	Round	Raised	Spreading
G	Creamy	Shiny	Undulate	Round	Flat	Spreading
H	Creamy	Shiny	Undulate	Round	Flat	Spreading
I	Creamy	Dull	Lobate	Round	Flat	Spreading
J	White	Shiny	Lobate	Round	Flat	Spreading

The colour of the colonies ranged from creamy to white. The bacterial cells were small rod-shaped and straight.

Biochemical test on Bacillus isolates
Based on their effectiveness in controlling plant parasitic nematodes, five Bacillus isolates tested positive to several biochemical tests except isolates B, C and G that tested negative to potassium hydroxide solubility test (Table 2). All the Bacillus isolates tested positive for Gram reaction, mobility, gelatin hydrolysis, catalase production, reduction of nitrates and NaCl tolerance. All the Bacillus isolates formed endospores with the exception of isolate D and F. All isolates hydrolysed starch except D.

Table 2: Characteristics of ten Bacillus isolates

***Bacillus* isolate**	**Gram stain**	**Mobility**	**Endospore formation**	**Levan production**	**Gelatin hydrolysis**	**Catalase production**	**Starch hydrolysis**	**Reduction of NO3**	**NaCl tolerance**	**Utilisation of CHOS aerobic**	**Utilization of CHOs (Anaerobic)**
A	1	1	1	1	1	1	1	1	1	0	1
B	0	1	1	1	1	1	1	1	1	0	1
C	0	1	1	1	1	1	1	1	1	0	1
D	1	1	0	1	1	1	0	1	1	1	1
E	1	1	1	1	1	1	1	1	1	0	1
F	1	1	0	1	1	1	1	1	1	0	1
G	1	1	1	1	1	1	1	1	1	0	1
H	0	1	1	1	1	1	1	1	1	0	1
I	0	1	1	1	1	1	1	1	1	0	1
J	0	1	1	1	1	1	1	1	1	0	1

0-negative reaction 1-Possitive reaction

Table 3: Plant parasitic nematodes Counts

Nematode genus	Nematode numbers in 200g soil	Composition (%)
Rotylenchus	460	20
Meloidogyne	335	15
Pratylenchus	330	14
Tylenchus	330	14
Paratylenchus	265	10
Helicotylenchus	155	7
Aphelenchus	105	5
Hemicronemata	85	4
Scutellonema	97	4
Hemicyclophora	70	3
Hoplolaimus	50	2
Ditylenchus	50	2
TOTAL	2332	100

Values are means of 12 samples; each sample consisted of 200cm^3 of soil.

Results from the Greenhouse experiment

The abundance of the ten *Bacillus* isolates was found to be significantly ($P \leq 0.05$) different in the sampled soil. The most abundant *Bacillus* isolates were A, D, H, F and B with Log_{10} transformed values of 5.16, 5.15, 4.89, 4.67 and 4.66 respectively (Fig 1). These were in the same range as K194, the positive control check. The least abundant *Bacillus* isolate was J with a CFU count of 2.38 and was in the same range as the negative control reading of 2.49. Isolates E and G were intermediate in abundance.

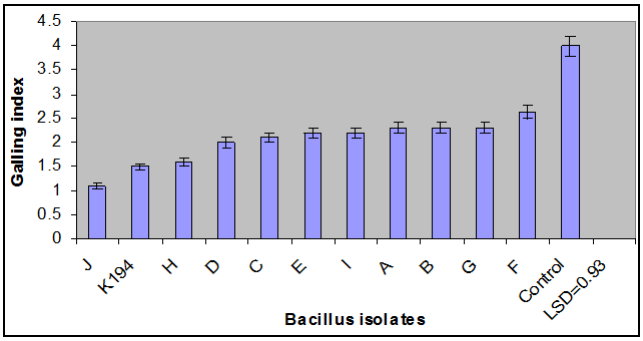

Fig 1: Abundance of ten Bacillus isolates found in soil samples.

The numbers of free living nematodes varied significantly ($P \leq 0.05$) after applying Bacillus isolates (Figure 1). The highest numbers were observed under isolates B and C while smallest number occurred when isolate I was applied 4.

Table 4: Effect of Bacillus isolates on numbers of plant parasitic nematodes in soils obtained.

***Bacillus* isolate**	**Mean No of nematodes**							**Mean**
	Helicotylenchus	***Meloidogyne***	***Paratylenchus***	***Rotylenchus***	***Scutellonema***	***Tylenchus***	***Pratylenchus***	
A	13	11	5	13	29	17	12	14
B	37	21	22	15	23	39	25	26
C	16	11	25	10	26	27	22	20
D	24	23	15	20	26	23	19	21
E	30	15	21	19	19	11	16	19
F	19	8	5	6	18	8	7	10
G	12	5	7	8	12	6	15	9
H	22	16	13	14	12	10	14	14
I	19	4	1	15	10	9	19	11
J	25	13	17	13	11	3	11	13
K194	14	11	0	3	9	2	3	6
Control	31	37	25	27	40	25	46	33
Mean	22	22	22	22	22	22	22	22
S.E	13	11	9	11	9	10	11	
LSD	11.4	9.9	7.7	10	8.3	8.5	9.8	

Values are means of 96 samples; each sample consisted of 200cm^3 of soil

The populations of the genera Helicotylenchus and Scutellonema were highest after application of Bacillus isolates, while the least numbers were those of Paratylenchus and Rotylenchus. Further observation showed that Meloidogyne and Tylenchus were also largely reduced by the isolates.

The effect of Bacillus isolates on galling in beans varied significantly ($P \leq 0.05$). The most extensive damage to bean roots occurred when Bacillus isolate F was applied, however, this was significantly less than damage score in control (Fig 2). Bacillus isolates A, B and G led to relatively higher GI scores of 2.30 followed by E and I where readings of 2.2 were recorded. The least damage was observed under Bacillus isolate J followed by H, these were comparable to the score of the isolate K 194. Since galling is primarily caused by Meloidogyne, it can safely be deduced that Bacillus isolates J and H are the most effective against the root-knot nematode.

Table 5: Effects of Bacillus isolates on bean dry weight in the greenhouse

Bacillus isolate	Shoot dry weight (grams)	
	Non sterile soil	Sterile soil
A	1.65	0.59
B	1.93	0.92
C	1.40	0.62
D	3.65	1.70
E	2.78	1.86
F	2.54	1.69
G	3.25	1.17
H	3.04	1.22
I	2.26	2.58
J	2.01	1.24
K194	5.01	2.26
Control	0.44	0.43
LSD	1.12	
LSD	1.12	

Values are means of 12 shoot samples each consisting of 3 shoots

There was a negative correlation between plant parasitic nematodes and nitrogen but a positive correlation was observed with carbon (Table 5). There were significant negative correlations between PPN and pH (r=-0.541, P=0.05). Free-living nematodes decreased with increase in pH, nitrogen and percent carbon in soil, but increased with increase in phosphorus.

Table 6: Correlation between soil fertility parameters and freeliving and plant parasitic nematodes

Soil fertility parameters	Correlation (r)	
	Free living nematodes	Plant parasitic nematodes
pH in water	-0.63	-0.51*
pH in $CaCl_2$	-0.34	-0.54*
P (ppm)	0.65	-0.22
% N	-0.48	0.36
K (C mol/kg)	-0.28	0.03
% C	-0.78	-0.47

*Significant at 0.05 level

There were significant positive correlation between soil amendments and the abundance of *B. subtilis* (Table 6). Manure had the highest correlation (r=0.579, P=0.01) on the abundance of *B. subtilis* followed by Farmers practice and Mavuno respectively.

5. Discussion

The findings where some of the *Bacillus* isolates (G, F, I) were found to lead to reductions in nematode numbers is consistent with others [23]. However, in this case a broader range of nematodes was included unlike in previous studies where the focus was mainly on *Meloidogyne.*

Efficacy of *Bacillus isolates.* in reducing damage to bean crop caused by plant parasitic nematodes and subsequent yields

Damage to plants in terms of galling is primarily due to nematodes belonging to *Meloidogyne* group. Thus assessing the efficacy of *Bacillus spp.s* in reducing damage caused by plant parasitic nematodes largely focuses on this genus. Least damage to bean roots was observed under isolates J and H. The ability of the isolate to reduce damage may be due to modification of root exudates (becoming toxic) which interferes with penetration of nematodes into roots [24].

Among the isolates, the highest bean yields were associated with *Bacillus* isolates D, G and H. while the least were linked with B, C, and J. It is interesting to note that although isolate J led to the least damage to beans, yields associated with it were low. In the end, the farmer is interested in yields and since it has been established that *Bacillus spp*. alone leads to lower yields.

6. Conclusion and recommendations

The soils in College of Agriculture and Veterinary Sciences of the University of Nairobi harbour a wide array of *Bacillus isoates* with the dominant ones being A, D, H, F and B. These soils are infested with a wide diversity of plant parasitic nematodes associated with beans, the most abundant of which are *Rotylenchus* and *Meloidogyne*.

Bacillus isolates G, F and I was suppressive to a wide range of plant parasitic nematodes associated with beans. The least damage to bean roots was observed under isolates J and H. However, the highest bean yields were associated with isolates D, G and H. Application of *B.subtilis* alone resulted to low yields in the greenhouse.

7. References

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