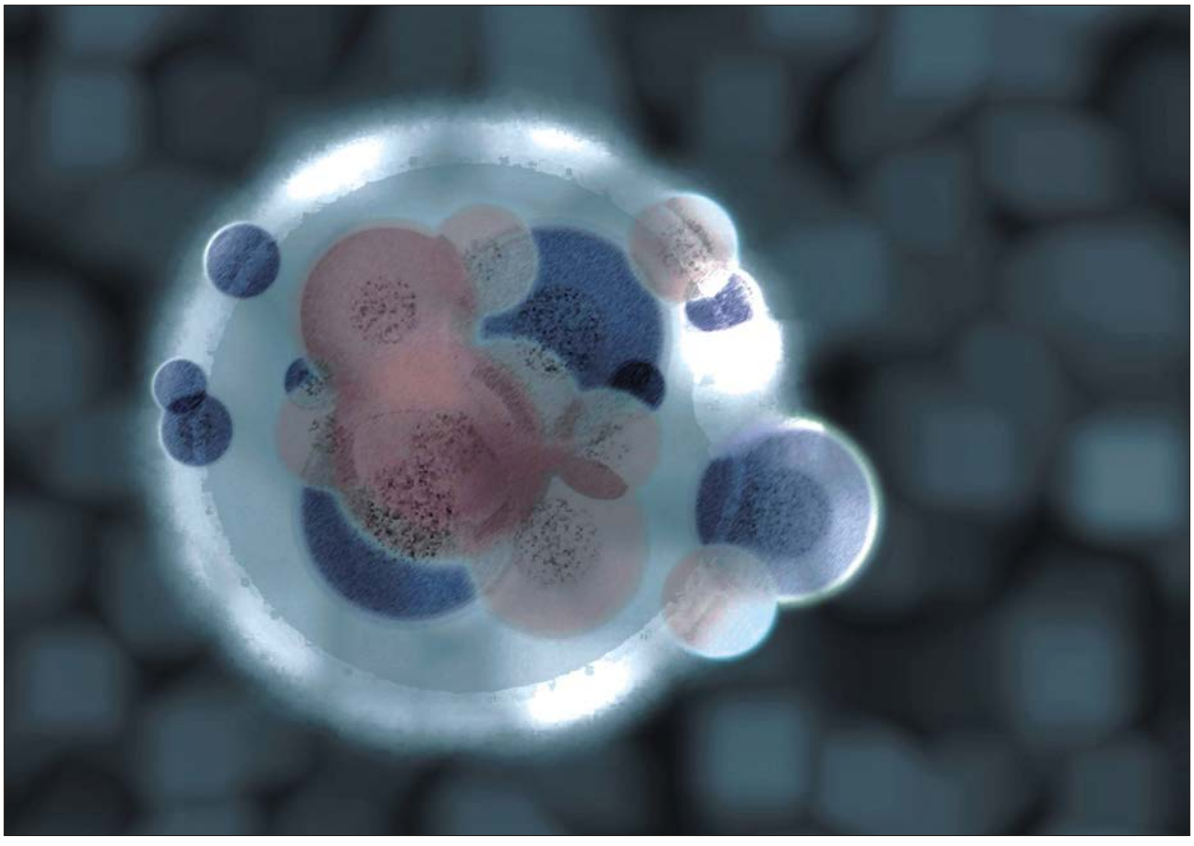

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Symbiotic Nitrogen Fixation of Two Soybean Genotypes as Affected by Root-Knot Nematode and Microsymbionts

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Abstract: The ability of soybean to symbiotically fix Nitrogen (N) was found to be substantially reduced ($p \leq 0.05$), after inoculation with root-knot nematode (*Meloidogyne incognita*). Galling, which is a commonly used indicator of root-knot crop damage, may provide less accurate information about reductions in the symbiotic N fixation of soybean. The experiments were undertaken in Nigeria in pots, using two soybean genotypes (TGx 1448-2E and TGx 1485-1D) with differential susceptibility to nematode. The microsymbionts *Glomus mosseae* (200 spores), *Bradyrhizobium japonicum* (10^6 cells mL^{-1}) and *Trichoderma pseudokoningii* (6.8×10^7 spores) were applied $plant^{-1}$. Treatments involving nematode inoculation had 1000 second-stage juveniles $plant^{-1}$. Ureide N (fixed N) in stems and petioles, nodulation score and activity, nematode infection and arbuscular mycorrhizal colonization were assessed at harvest using standard methods. Regression and correlation analyses demonstrated that root nematode densities assessed at harvest were highly correlated negatively ($p \leq 0.001$) with Symbiotic N Fixation (SNF) as measured from the ureide N. Results confirmed that inoculation of *M. incognita* reduced SNF, nodulation score, nodule activity and plant growth parameters. However, fixed N was not necessarily related to galling damage. Results indicated that nodulation score and galling may not be the most suitable parameters, by which levels of productivity or damage could be determined in the presence of *Meloidogyne* sp., but rather ureide N is a more accurate assessment of the effect of *M. incognita* on soybean.

Key words: Arbuscular mycorrhiza, N fixation, N dependence, plant-parasitic nematodes, rhizobial nodulation, ureide N

INTRODUCTION

Soybean (*Glycine max* L. Merr.) can obtain Nitrogen (N) from the soil or from the atmosphere through symbiosis with *Bradyrhizobium japonicum*. Estimates of the amount of N_2 fixed range between 25 and 85% of the total N present in soybean shoots (Jefing *et al*., 1992; Vasilas *et al*., 1995). The N fixed by the nodulating bacteria when in mutualistic association with legumes, known as Symbiotic N Fixation (SNF) and the atmospherically fixed N are important alternative sources of usable N for legumes and other crops. However, SNF is dependent on numerous factors, including host crop, genotype, microsymbiont(s) and pests, especially those that reduce effective nodulation (Graham, 1992).

Numerous species of plant-parasitic nematodes, representing several genera, are associated with soybean (Sikora *et al*., 2005). Of these, *Meloidogyne* sp. (root-knot nematodes) are regarded among the most damaging to soybean (Fourie *et al*., 2001). In addition to deformation of the root system and causing the formation of galls, nutrient and water uptake are affected (Sikora *et al*., 2005). Infection by *Meloidogyne* sp. can also lead to reduced nodulation or inhibit nodulation by symbiotic N fixing bacteria (Musarrat and Haseeb, 2000). Depending on the crop and indeed the species of *Meloidogyne*, the effects on the crop can vary. Ibewiro *et al*. (2000) in the derived savanna of West Africa found that the presence of *Meloidogyne* sp. increased nodulation on *Lablab purpureus* L. (Sweet) but not on *Mucuna pruriens* (L.) DC. var. *utilis* (Wright) Bruc. The same study, however, demonstrated that N derived from symbiotic fixation in *M. pruriens* was not affected by *Meloidogyne* sp. but it was reduced in *L. purpureus*, even though nodulation was increased. Observations, therefore, on the extent of nodulation in relation to nematodes could be misleading about the overall functional capacity of the nodules. However, there is little

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information relating the reduction of N in SNF due to nematode activity. Based on the fact that between 25 and 85% of nitrogen required by soybean comes from SNF (Jefing, *et al*., 1992), which reduces cost of fertilizer input into soybean production; it is worthwhile to investigate means through which SNF could be improved and factors that could lead to its reduction. The current study was undertaken to examine the relationships between SNF, nodulation of two soybean genotypes and infection by *M. incognita* in the presence of some microsymbionts, using data from a study established to study improvement of root-knot nematode management with inoculation of microsymbionts (Oyekanmi *et al*., 2007).

MATERIALS AND METHODS

Site and experimental details: The experiments were undertaken at IITA, Ibadan, Nigeria (7°30′N; 3°5′E) in 2004. Two promising soybean genotypes were used: TGx 1448-2E (medium maturing) and TGx 1485-1D (early maturing), supplied from IITA breeders' stocks. The experimental design was a randomised complete block design with four replications in pots. Four seeds were planted in 3 kg (dry weight) of non-sterilized sandy loam soil, then thinned to 1 seedling pot^{-1} at 7 days after emergence. Pots were maintained in a plastic-covered screenhouse and irrigated daily by hand.

Treatments: The microsymbionts *Bradyrhizobium japonicum* (Jordan) and *Glomus mosseae* (Nicol and Gerd.) were obtained from IITA's soil microbiology unit. *B. japonicum* originated from promiscuous soybean in the northern guinea savanna in Nigeria. *G. mosseae* was isolated from field soil from the derived savanna zone of Nigeria and the antagonistic fungus *Trichoderma pseudokoningii* (Rifai) was isolated from a maize field in IITA Ibadan.

Treatments were *B. japonicum*, *T. pseudokoningii* and *G. mosseae*, applied to soybean seeds in single, dual and triple combinations, with *M. incognita* inoculation. There were two controls, untreated (no microorganisms and no nematode application) and a nematicide control (Furadan® 3G (carbofuran) applied at the equivalent of 100 kg ha^{-1} with *M. incognita* application).

Spores of *G. mosseae* from soil were extracted, multiplied and quantified using the method of Walker *et al*. (1991). Spores were incorporated into pots at a rate of approximately 200 spores pot^{-1} by spreading (layering) 60 g of soil spore mixture into the centre of the pot at a depth of 3 cm prior to planting.

Two strains of *B. japonicum* were used IRJ 2180A and R 25B (combined), which were multiplied on Yeast Mannitol Agar (YMA) with bromothymol blue stain in 90 mm diameter glass Petri dishes in the dark and incubated at 28°C for 7 days. Colonies of *B. japonicum* were scraped off the YMA, quantified using a haemocytometer and diluted with Sterile Distilled Water (SDW) to approximately 10^6 cells mL^{-1}. Soybean seeds were surface sterilized (95% ethanol for 3 min, rinsed seven times with SDW) and inoculated with 5 mL of the *B. japonicum* suspension 60 g $seed^{-1}$, mixed in 15 mL of starch solution as a sticking agent (Somasegaran and Hoben, 1994).

The suspension of *T. pseudokoningii* spores was obtained by scraping the mycelial growth from the culture and mixing with 20 mL of 0.1% Tween 80 solution, which enhances the even distribution of spores (Adekunle *et al*., 2001) and passing the mixture through sterile muslin cloth. Spores were applied to sterilized seeds (described above) at the rate of approximately 6.8×10^7 spores 60 g $seed^{-1}$.

Raising and inoculation of nematodes: Meloidogyne incognita was isolated originally from soybean at IITA, in 2001 and multiplied on tomato. Infected tomato roots were finely chopped and rinsed in 1.02% NaOCl to help release eggs from the nematode egg mass matrix (Hussey and Barker, 1973). Eggs were caught on a 20 μm aperture sieve after passing through nested sieves, rinsed in 5 changes of tap water and maintained at room temperature in tap water for 10 days. Hatched J2 were inoculated in water suspension after being adjusted to 500 J2 mL^{-1}. At 7 days after planting, the soil around each plant was moistened with 50 mL of water and the seedlings, except untreated control pots without nematodes were inoculated with 1000 *M. incognita* eggs and J2 in 1 mL water suspension. A micropipette was used to drop the nematode suspension to the base of the seedling in the rhizosphere, via two holes made using a pencil and covered with soil after inoculation.

Nodulation and nematode parameters measurement: Infection by *B. japonicum* was assessed at harvest by determining nodulation activity and score. Nodulation activity was determined by dissecting four nodules $root^{-1}$ transversely with a razor. The internal nodule mass colouration was assessed on a scale of 0-5 (Somasegaran and Hoben, 1994); 0 = whitish colour (no activity), 1 = ash colour, 2 = dark ash colour, 3 = fairly pink, 4 = pink, 5 = very pink (high level of activity). Nodulation score was measured using a scale of 0-5: 0 = no nodule, 1 = 1-9 nodules: 2 =10-20, 3 = 21-30, 4 = 3-40, 5 = 41 nodules and above per root system (Somasegaran and Hoben, 1994).

Nematode densities (J2 and adult females) were assessed from a 2 g subsample of each root after staining

in hot lactoglycerol with acid Fuchsin stain (Bridge *et al*., 1982). Root galling index was assessed on a scale of 1-5 where, 1 = no galling, 2 = 1-25% of roots with galls, 3 = 26-50% with galls, 4 = 51-75% with galls and 5 = >75% root galling (Benjamin and Grover, 1987).

Harvesting, ureide N analysis and mycorrhizal assessment: At 8 weeks after planting, harvesting of plants was carried out so that roots were in a suitable condition, without much deterioration, to enable galling and nodulation assessments. At harvest, plant stems and petioles were oven-dried to constant weight, milled using a hammer mill grinding machine and sieved with 150 μm aperture sieve (Endecotts). Afterwards, 0.5 g ground plant tissue plant^{-1} was measured and poured into 100 mL of SDW in a 150 mL conical flask and boiled for 5 min. to extract sap, using filtration apparatus and suction pump (Peoples *et al*., 1989). The concentration of ureide N in the processed sap was determined using a spectrophotometer. Calibration to determine the quantitative relationship between the composition of N solutes in xylem exudates and proportional plant dependence upon N fixation, was carried out according to Peoples *et al*. (1989).

Mycorrhizal colonization of roots was assessed from a 5 g plant^{-1} root subsample. Roots were cleared with 10% KOH, in an autoclave liquid cycle at 121°C for 15 min. Roots were then rinsed with 5 changes of tap water, bleached with 3% H_2O_2 rinsed as described above and stained with chlorazol black E stain composition (Brundrett *et al*., 1994). Colonization of roots was estimated by the grid-line-intersect method (Giovannetti and Mosse, 1980).

Statistical treatment of data: Differences between treatments and genotypes were assessed with ANOVA using the General Linear Model (GLM) procedure on Statistical Analysis Systems (SAS, 1999). Single linear regression analysis and Spearman Rank Order Correlation were used to assess the relation between root nodulation, N fixation, mycorrhization and *M. incognita* related variables at harvest across treatments for individual and combined soybean genotypes using SigmaStat 3.0 for Windows and Microcal Origin for Windows version 3.

RESULTS

Across treatments, SNF was higher in cv TGx 1485-1D than in cv. TGx 1448-2E; it was significantly lower in soybean plants in the nematode-only treatment for both cultivars than in all other treatments, except for the *G. mosseae* + *T. pseudokoningii* in cv. TGx1448-2E plants (Table 1). It was also higher in the untreated (no nematode, no microorganism) control treatment than in all other treatments for both genotypes, except for the *G. mosseae* only treatment for cv. TGx1485-1D. The SNF capacity was, therefore, consistently reduced in the presence of *M. incognita*, whether in combination with antagonistic microorganisms or not. In the nematicide (carbofuran) treatment, SNF was higher by 128% for cv. 1 and 25% for cv. 2, when compared with the *M. incognita* control. However, SNF values in the nematode-only treatment compared with the untreated control was 444% lower in cv. 1 and 133% lower in cv. 2.

Percentage mycorrhizal colonization was recorded in all treatments. In treatments with *G. mosseae* application, the highest root colonization was obtained in the triple

Table 1: Mean percentage SNF, percentage mycorrhizal colonization, nodule activity and nodulation score of two soybean genotypes, TGx 1448-2E (cv. 1) and TGx 1485-1D (cv. 2) at harvest at 8 weeks after planting

	Percentage SNF[b]		Percentage mycorrhizal colonization		Nodule activity[c]		Nodulation score[d]	
Treatments[a]	cv. 1	cv. 2	cv. 1	cv. 2	cv. 1	cv. 2	cv. 1	cv. 2
B+G+T	9.8	39.4	37.0	19.4	2.0	2.7	3.5	3.5
B+G	12.1	28.8	33.1	29.3	2.0	1.7	2.5	2.0
G+T	8.3	23.4	25.8	14.1	2.2	1.7	3.2	2.0
B+T	13.9	41.0	34.5	18.9	2.0	2.5	3.5	3.0
G	19.9	50.4	31.7	30.5	2.0	3.2	4.0	3.2
T	19.2	35.5	27.5	20.8	2.0	2.5	3.2	2.5
B	28.5	30.3	23.5	24.5	2.0	2.5	2.5	2.5
Nematode only	7.8	20.8	22.0	17.5	2.0	1.7	2.5	1.7
Carbofuran	17.8	25.4	30.3	20.1	2.0	2.5	3.5	3.2
Untreated	42.5	48.6	20.2	17.3	1.2	3.2	3.7	4.7
Mean	18.0	34.4	28.5	21.2	1.9	2.4	3.2	2.8
SE (p≤0.05)	0.02	0.04	9.1	7.9	0.3	1.4	2.0	1.8
Trt×cv. interaction[e]	**		**		*		*	

[a]G = *Glomus mosseae* + *Meloidogyne incognita*; T = *Trichoderma pseudokoningii* + *M. incognita*; B = *Bradyrhizobium japonicum*, + *M. incognita*; Nematode only = *M. incognita* inoculated only; Carbofuran = Carbofuran + *M. incognita*; Untreated = Un-inoculated control (no nematode, no microbe); SE = Standard error; [b]Values were obtained from the calibration curve equation on ureide extracts from shoot (stems plus petioles) of plants: $X = 1.4 + 0.31P + 0.0057P^2$, where, p≤ the proportion of plant N from N_2 fixation and X = Relative abundance of ureides in extracts of the shoot. Equation linearized as: Log X = Log 1.4 + P log 0.31P + 2P Log 0.0057; c: Nodule score based on a score of 0-5 where, 0 = No nodule and 5 = 41 nodules or more per root system; [d]: Nodule activity based on a colouration scale of 0-5 where, 0 = White (no activity) and 5 = Very pink (high level of activity); [e]: Interaction statistically significant at *p≤0.05, **p≤0.01

Table 2: Correlation coefficient matrix from Spearman Rank Order for nematode-related variables, mycorrhizal colonization, SNF, nodulation score and activity at harvest 8 weeks after planting, following inoculation with *Meloidogyne incognita* for two soybean genotypes combined across treatments. N = 80

Variables	Galling index	Percentage mycorrhizal colonization	Percentage SNF	Nodule score	Nodule activity[e]
Nematode density[a]	-0.025	0.546***	-0.756***	-0.077	-0.217*
Galling index[b]		-0.037	0.09	0.099	0.31**
Percentage mycorrhizal colonisation			-0.389***	0.052	-0.044
Percent SNF[c]				0.101	0.286**
Nodule score[d]					0.16

[a]: Nematode root density per 2 g root; analysis undertaken using log (x+1) transformed data; *correlation significant at p≤0.05, **p≤0.01 ***p≤0.001; [b]: galling index based on a scale of 1-5 where, 1 = no galling and 5 = severe galling; [c]: Percentage ureide N calculated with: X = 1.4 + 0.31P + 0.0057P^2. Where, P≤the proportion of plant N from N_2 fixation and X = relative abundance of ureides in extracts of the shoot axis. Equation linearized as: Log X = Log 1.4 + P log 0.31P + 2P Log 0.0057; d: Nodule score based on a score of 0-5 where, 0 = no nodule and 5 = 41 nodules or more per root system and [e]: Nodule activity based on a colouration scale of 0-5 where, 0 = white (no activity) and 5 = very pink (high level of activity)

combination of microorganisms for genotype TGx 1448-2E. The genotype TGx 1485-1D had lower responsiveness to *G. mosseae*; this was evident in the lower level of root colonization obtained across the treatments (Table 1).

Nodule activity was not significant across treatments except for the untreated, which had a significantly lower nodule activity compared to other treatments in TGx 1448-2E. Likewise, a significant difference was not evident in TGx 1485-1D except for the nematode-only treatment, when compared with *Glomus* + nematode and untreated control (Table 1). Nodulation score was not significant in TGx 1448-2E when treatments were compared with one another. However, there were significant differences in TGx 1485-1D when the *Bradyrhizobium* + *Glomus* + *Trichoderma* treatment and untreated control were compared with the nematode-only control.

Across treatments and cultivars, no correlation was observed between nodulation score (number of nodules) and *M. incognita* root density at harvest (Table 2). However, nodulation activity (p≤0.05) and SNF (p≤0.001) were negatively correlated with *M. incognita* root density at harvest, but not with galling. SNF was negatively correlated with *M. incognita* density across treatments for both genotypes combined (Fig. 1). The genotype TGx 1448-2E, had a higher negative relationship when correlation was conducted separately on a genotype basis, TGx1448-2E (r = 0.60; p≤0.001) than cv TGx1485-1D (r = 0.21; p≤0.01). Interestingly, mycorrhizal colonization was positively correlated with nematode density (treatments and genotypes combined), but significant only for cv TGx1448-2E (r = 0.17; p≤0.01) when analysed for each genotype (Table 2). Mycorrhizal

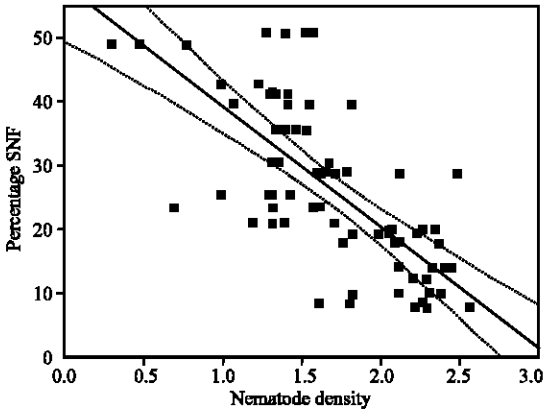

Fig. 1: Relationship between root nematode (juvenile) density (log [x+1]) and percentage ureide N content (symbiotic nitrogen fixation) in soybean stems and petioles at harvest following inoculation with *Meloidogyne incognita* in pots for two soybean genotypes combined across treatments, with 95% confidence limits. N = 80. Percentage ureide = 58.04-18.8× nematode density; r = -0.737; p≤0.001

colonization was correlated negatively with SNF for treatments and genotypes combined but when analysed separately, significantly only for cv. TGx1448-2E (r = 0.15; p≤0.01). Galling index was not correlated with mycorrhizal colonization. Galling was also not correlated with root nematode density at harvest. No correlation was observed between galling and SNF (p≤0.05), but nodule activity was highly positively correlated with SNF.

DISCUSSION

The SNF of soybean is undoubtedly severely affected as a result of *M. incognita* infection. The presence of the beneficial microorganisms led to higher SNF than in *M. incognita* alone, but only in the presence of *G. mosseae* alone was SNF higher than in untreated plants. The presence of the microorganisms also provided improved SNF better than the application of carbofuran for some, but not all treatments. Application of the microorganisms, in general therefore, suppressed nematode densities and damage and (in the presence of *M. incognita*) improved the growth of soybean (Oyekanmi *et al*., 2007). However, the two genotypes reacted differently to both the presence of *M. incognita* and the application of microorganisms. The early maturing genotype TGx 1485-1D supported lower densities of *M. incognita* and therefore is more resistant. The same genotype, however, had consistently higher SNF (mean of 34.4%) than cv. TGx 1448-2E (mean of 18.0%) that

appeared to be affected by the application of micoorganisms. However, Meyer *et al.* (2001) established in a study involving different microorganisms that, antagonism may occur when microorganisms are combined together in the presence of nematodes. This could have been responsible for SNF reduction observed in some treatments involving combination of microorganisms. It is also apparent from the current study that *M. incognita* affected SNF differently, depending on the genotype, while the effect on SNF is also known to depend on the specific nematode species (Carneiro *et al.*, 2002).

From the current study, it is not clear what effect the application of the beneficial microorganisms had on SNF, without *M. incognita* inoculation; as they were not applied and assessed in isolation of *M. incognita*. However, under natural conditions, crops and germinating seedlings are exposed to myriad soil-borne microorganisms, which will each interact with one another and the developing crop. The plant roots will therefore inevitably be exposed to similar organisms in the field, for instance, root-knot nematodes are omnipresent in tropical farming systems (Sikora *et al.*, 2005).

In this study, there was no correlation between nematode density and nodulation score (although there was a negative association with nodule activity). This indicated that the nodulation score, as mostly measured, may not be the most suitable parameter to measure levels of productivity or damage, in the presence of *Meloidogyne* sp., but rather that SNF determination is essential to ascertain the effect of *M. incognita* on soybean. Such information will enhance the development of management strategies for *M. incognita* control and soybean SNF improvement in *M. incognita* infested soil.

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