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Symbiotic N nutrition, bradyrhizobial biodiversity and photosynthetic functioning of six inoculated promiscuous-nodulating soybean genotypes

Flora Pule-Meulenberg[a], Cynthia Gyogluu[b], Jesse Naab[c], Felix D. Dakora[d,*]

[a] Department of Biotechnology, Tshwane University of Technology, 175 Nelson Mandela Drive, Private Bag X680, Pretoria 0001, South Africa
[b] Department of Crop Science, 1 State Artillery Road, Tshwane University of Technology, Private Bag X680, Pretoria 0001, South Africa
[c] Savanna Agricultural Research Institute, Wa Experiment Station, Box 494, Wa, Ghana
[d] Department of Chemistry, Tshwane University of Technology, 175 Nelson Mandela Drive, Arcadia Campus, Private Bag X680, Pretoria 0001, South Africa

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ABSTRACT

Six promiscuous soybean genotypes were assessed for their ability to nodulate with indigenous root-nodule bacteria in Ghana, with *Bradyrhizobium japonicum* WB74 serving as positive control. Although the results revealed free nodulation of all six genotypes in both inoculated and uninoculated plots, there was a marked effect of inoculation on photosynthetic rates and whole-plant C. Inoculation also increased stomatal conductance in TGx1485-1D, TGx1448-2E, TGx1740-2F and TGx1445-3E, leading to significantly elevated transpiration rates in the last two genotypes, and a decrease in TGx1485-1D, TGx1440-1E and Salintuya-1, resulting in reduced leaf transpiration and decreased C accumulation. Nodulation, total plant biomass, plant N concentration and content also increased and ∂^{15}N of the six genotypes, except for TGx1448-2E decreased. Significantly higher %Ndfa resulted in all the soybean genotypes tested (except for TGx1485-1D), and the symbiotic N yield in TGx1740-2F and TGx1448-2E doubled. PCR-RFLP revealed 18 distinct IGS types present in root nodules of the six promiscuous soybean genotypes, with IGS type II being isolated from all six genotypes, followed by IGS types X and XI from five out of the six genotypes. Marked differences in strain IGS type symbiotic efficiency were revealed. For example, as sole nodule occupant, IGS type XI produced high symbiotic N in TGx1445-3E, but low amounts in TGx1448-2E. Inoculated Salintuya-1, which trapped nine strain IGS types in its root nodules, was the most promiscuous genotype, but produced less symbiotic N compared to genotypes with fewer strains in their root nodules.

Introduction

Soybean (*Glycine max* L. (Merrill)) is a major grain legume grown worldwide. It is an important source of protein and oil for human consumption (Vasilia and Boerma, 2009), and supplies high protein levels for livestock (Robaina et al., 1995). Soybean plants are capable of effective root nodules with different bacterial symbionts, including *Bradyrhizobium japonicum* (Jordan, 1982), *Bradyrhizobium elkanii* (Kuykendall et al., 1992), *Bradyrhizobium liaoningense* (Xu et al., 1995) and *Sinorhizobium fredii* (Chen et al., 1988). As a result, it can contribute to the N economy of nutrient poor soils. In Brazil, where soybean is grown without N fertilizers, the mean yield obtained is about 2.4 t ha^{-1} (Alves et al., 2004). In Africa, soybean cultivation is relatively limited due to a range of causes, which include poor knowledge of the crop, low understanding of inoculation techniques with root nodule bacteria, and little knowledge of its utilization as a food legume. Additionally, many factors appear to affect soybean cultivation as a food crop. These range from competition for nodule occupancy to soil type, frequency of nodulation, to P supply and liming (Botha et al., 2004; Okogun and Sanginga, 2003; Mpepereki et al., 2000; Wasike et al., 2009).

In soils that have not been previously cropped to soybean, the absence of compatible populations of root-nodule bacteria can pose a challenge to increased yields and farmer adoption. Under these conditions, inoculation with infective strains is needed to achieve effective nodulation. To overcome the difficulties associated with inoculation as experienced by African farmers, the International Institute of Tropical Agriculture (IITA) in Nigeria developed promiscuous soybean varieties (the so-called TGx or glycine tropical cross) that nodulate effectively with indigenous *Bradyrhizobium* spp. (Abaidoo et al., 2007). But even before the development of these TGx lines, there were earlier reports of effective nodulation of soybean in Tanzania, Malawi, South Africa, Zambia and Zimbabwe without inoculation (Mpepereki et al., 2000), occurring in soils without any known history of previous inoculation with soybean microsymbionts.

Since the development of the TGx lines at IITA, few studies (Abaidoo et al., 2007) have evaluated these soybean genotypes

* Corresponding author. Tel.: +27 123826120; fax: +27 123826286.
E-mail address: dakorafd@tut.ac.za (F.D. Dakora).

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for their inbred trait of promiscuous nodulation. We therefore do not know whether these soybean lines can indeed nodulate freely with indigenous root-nodule bacteria in African soils. Furthermore, there is little information on their growth and symbiotic N yield. The aim of this study was to (i) assess plant growth and nodulation with native soil bacteria, (ii) determine the level of N_2 fixation, (iii) assess the extent of nodule occupancy by introduced *versus* native strains, and (iv) measure IGS type symbiotic efficiency in root nodules of six promiscuous soybean genotypes.

Materials and methods

Field design and planting

The experiment was conducted at Wa, Ghana, using a two factorial design, with inoculation (i.e. uninoculated and inoculated with *Bradyrhizobium japonicum* strain WB74) serving as the main treatment, and six promiscuous-nodulating soybean genotypes as sub-treatments. The study was done at the Savanna Agricultural Research Institute (SARI) site at Bamahu, Wa, in 2006. The site is located in the Guinea savanna (latitude 10°03′N, longitude 2°30′W, and altitude 370 m) and has a unimodal rainfall (1100 mm annual mean) that starts in May and ends in September/October. The soils are classified as Ferric Luvisols (FAO, 2001). Prior to experimentation, the site had been fallowed for about three years.

The six soybean genotypes used were TGx1485-1D, TGx1740-2F, TGx1448-2E, TGx1440-1E and Salintuya-1. Four replicate plots were planted for each soybean genotype. Before planting, some soybean seed was inoculated with a peat-based inoculant of *B. japonicum* strain WB74 to attain a cell number of about 10^8 cells per seed (Vincent, 1970). To reduce cross-contamination, uninoculated plots were planted first, followed by inoculated treatments. Each field plot measured 5 m × 3 m, with inter-row spacing of 75 cm and intra-row spacing of 5 cm. Weeds were controlled using a hand hoe, and uninoculated plots were always weeded first, before inoculated, in order to reduce contamination.

Gas-exchange studies

At 67 DAP, photosynthetic rates, stomatal conductance, intercellular CO_2 concentration and transpiration rates were measured in four young leaves (flag leaves) per plot for each treatment using a portable infrared gas analyzer (CIRAS-2, PP System, Hitchin, UK). Measurements were made from 8 to 11 a.m. and from 2 to 4 p.m. for each replicate plot per day. Leaves were allowed 4–5 min to acclimate to the light environment in the chamber. Without troubleshooting, each measurement took approximately 2 min, which was the minimum time allowed for the readings to stabilize before they were recorded. Measurements were taken with the following conditions in the leaf chamber: light intensity of 1200 μmol photons m^{-2} s^{-1}, CO_2 concentration of 400 ppm, flow rate of 400 μmol s^{-1}, leaf temperature of 25 °C and relative humidity of 44%.

Plant harvest

At flowering, 8 plants per genotype were excavated from the inner row of each plot, and separated into shoots, roots and nodules. The shoots and roots were dried at 60 °C over 48 h and milled to a fine powder (0.85 mm) for stable isotope analysis of N and C. The root nodules were counted, oven-dried at 45 °C over 48 h, weighed, and stored prior to bacterial DNA extraction for PCR-RFLP analysis.

$^{15}N/^{14}N$ isotopic analysis

About 2.0 mg of each milled plant sample was weighed into a tin capsule (Elemental Microanalysis Ltd., Okehampton, UK) and run on a Thermo Finnigan Delta Plus XP stable light isotope mass spectrometer coupled via a Conflo III device to Thermo 1112 Flash elemental analyzer against an internal reference plant material (*Nasturtium* sp.) The *Nasturtium* sp. had been calibrated against air, which is an IAEA standard for N, and the results expressed relative to air.

The isotopic composition of ^{15}N was measured as the difference in the number of atoms of ^{15}N to ^{14}N in atmospheric (atm) N_2 (Junk and Svec, 1958; Mariotti, 1983):

$$\delta^{15}N(‰) = \frac{[^{15}N/^{14}N]_{sample} - [^{15}N/^{14}N]_{standard}}{[^{15}N/^{14}N]_{standard}} \times 1000$$

Whole-plant ^{15}N natural abundance was calculated as an average of the ^{15}N natural abundance values of all plant parts weighted by their respective total N (Robinson, 2001).

The N content of each organ was determined as the product of %N and sample weight (Pausch et al., 1996):

$$\text{N amount (mg plant}^{-1}) = \%N_{organ} \times \text{dry weight}_{organ}$$

%Ndfa and N-fixed values

The proportion of N derived from biological N_2 fixation in the plant was calculated as (Shearer and Kohl, 1986):

$$\%Ndfa = \frac{\left[\delta^{15}N_{ref} - \delta^{15}N_{leg}\right]}{\left[\delta^{15}N_{ref} - B\,\text{value}\right]} \times 100$$

where $\delta^{15}N_{ref}$ is the mean ^{15}N natural abundance of shoots of non-N_2-fixing reference plants, $\delta^{15}N_{leg}$ is the mean ^{15}N natural abundance of the legume (soybean) shoots, and the *B* value is the ^{15}N natural abundance of soybean shoots which were dependent solely on symbiotic N_2 fixation for their N nutrition. *B* values of −0.72 and 0.60 for soybean shoots and roots, respectively, were used in calculating %Ndfa (F.D. Dakora and S.B. Chimphango, unpubl. data). A range of reference plant species, including sorghum (*Sorghum bicolor* L.), maize (*Zea mais* L.), millet (*Pennisetum glaucum* L.), *Hyptis* sp, *Andropogon* sp and *Rottboelia* sp., were sampled and analyzed, and their combined mean $\delta^{15}N$ value used in estimating %Ndfa.

The amount of N-fixed was calculated as (Maskey et al., 2001):

$$\text{N-fixed} = \left(\frac{\%Ndfa}{100}\right) \times \text{legume N}$$

Measurement of ^{13}C and %C in shoots

The shoot samples (2 mg for soybean and 2.5 mg for sorghum) that were weighed into tin capsules (Elemental Microanalysis Ltd., Okehamptom, UK), and fed into a Thermo Flash Elemental Analyzer 1112 (Fisions Instruments SpA, Strada Rivoltana, Italy) via a Thermo Conflo III devise coupled to a Thermo Finnigan Delta Plus XP Stable Light Isotope Mass Spectrometer (Finnigan MAT GmbH, Bremen, Germany) for ^{15}N analysis, concurrently provided the %C and ^{13}C values following combustion. The ratio of $^{13}C/^{12}C$ in each shoot sample was used to calculate the ^{13}C natural abundance, or $\delta^{13}C$, as (Stout and Rafter, 1978):

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}} \times 1000$$

Table 1
Effect of inoculation with *Bradyrhizobium japonicum* strain WB74 on the photosynthetic assimilation rate (*A*), stomatal conductance (gs), transpiration rate (*E*) and internal CO_2 to ambient CO_2 concentration (ci/ca) in six soybean cultivars grown at Wa in Ghana in 2006. The treatment means were computed by grouping means of all genotypes. Values (means ± SE) with dissimilar letters in the same columns are significant at $p \leq 0.01$(**); $p \leq 0.001$(***).

Treatment	*A* (μmol m^{-2} s^{-1})	gs (mol m^{-2} s^{-1})	*E* (mmol H_2O m^{-2} s^{-1})	Ci/Ca
Inoculation				
Uninoculated	17.7 ± 0.4b	0.39 ± 0.01a	4.1 ± 0.1a	0.57 ± 0.02a
Inoculated	18.8 ± 0.3a	0.37 ± 0.01a	3.9 ± 0.1a	0.55 ± 0.02a
Genotype				
TGx1485-1D	19.3 ± 0.6a	0.35 ± 0.03c	3.8 ± 0.0c	0.51 ± 0.03b
TGx1740-2F	19.1 ± 0.7a	0.40 ± 0.01ab	4.1 ± 0.1ab	0.51 ± 0.03b
TGx1448-2E	17.7 ± 0.5a	0.37 ± 0.02bc	3.9 ± 0.1bc	0.61 ± 0.01a
TGx1440-1E	17.9 ± 0.6a	0.34 ± 0.02c	3.9 ± 0.2bc	0.56 ± 0.03ab
TGx1445-3E	17.9 ± 0.5a	0.43 ± 0.01a	4.3 ± 0.0a	0.60 ± 0.02a
Salintuya-1	17.4 ± 0.6a	0.39 ± 0.02ab	4.1 ± 0.2ab	0.57 ± 0.03ab
2-Way ANOVA (*F* statistics)				
Inoculation	4.4**	1.3	2.6	0.49
Variety	1.7	4.3***	4.4***	2.6**
Inoculation × variety	1.1	4.6***	5.9***	0.6

where $(^{13}C/^{12}C)_{sample}$ is the isotopic ratio of the sample, and $(^{13}C/^{12}C)_{standard}$ is the isotopic ratio of PDB, a universally accepted standard from belemnite Pee Dee limestone formation (Craig, 1957). Shoot C content per plant (g $plant^{-1}$) was calculated as: %C x shoot dry matter per plant.

DNA extraction from root nodules

Total DNA (plant and microbial) was extracted from 30 nodules per genotype from each of the inoculated or uninoculated treatments (i.e. total of 360 nodules), using the method described by Krasova-Wade and Neyra (2007) and Pule-Meulenberg et al. (2010). The purity and quantity of the DNA was controlled by horizontal electrophoresis in 0.8% Sigma II agarose gel, using a molecular weight marker (Smart Ladder) for gel calibration. Electrophoresis was performed at 100 V for 30 min. The gel was stained in an aqueous solution of ethidium bromide (1 μg/mL) for 30 min, rinsed with sterile distilled water for 15 min and photographed under UV light with Gel Doc (Bio-Rad) software.

PCR-RFLP analysis

Depending on its concentration and the amount of impurities present, each DNA sample was diluted with sterile MilliQ water and PCR performed in a PerkinElmer 2400 Thermal cycler in a total volume of 25 μL reaction mixture using Ready-to-go Taq DNA polymerase (Pharmacia Biotech). A negative control with water (no DNA) was included in all the PCR runs. The 16S-23S IGS rDNA PCR amplification was carried out using two primers, FGPL132-38 and FGPS1490-72 (Normand et al., 1996). The protocol used included initial denaturation at 94 °C for 15 min; 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), extension (72 °C for 1 min) and final extension at 72 °C for 7 min. Amplified DNA products were separated by horizontal gel electrophoresis in 0.8% agarose gel. RFLP was carried out as described in Pule-Meulenberg et al. (2010). Electrophoregrams with similar migratory patterns were grouped together and assigned to the different IGS groups (IGS types I to XVIII).

Assessing strain IGS type symbiotic efficiency

Following studies of nodule occupancy, the identification of strain IGS types found inside root nodules, as well as the amounts of N-fixed per genotype, it was possible to assign the symbiotic N yield of any soybean genotype to a single IGS type, or to a group of IGS types, isolated from root nodules of that particular genotype. That way, the N_2-fixing efficiency of IGS types could be determined according to whether they were sole or multiple occupants of host-plant nodules.

Statistical analysis

All data followed normal distribution, and were subjected to 2-Way ANOVA using the STATISTICA 2007 package (StaSoft Inc., Tulsa, OK, USA) to determine the effect of inoculation on photosynthetic rates, growth and symbiotic performance. Means were separated using the Duncan Multiple Range Test where the genotype × inoculation interaction was significant, and specific effects obtained.

Results

Photosynthetic rates, stomatal conductance and transpiration

A 2-Way ANOVA analysis of gas-exchange parameters revealed significantly greater photosynthetic rates in inoculated relative to uninoculated plants (Table 1). However, stomatal conductance, transpiration rate, and internal CO_2 concentration were not affected by inoculation. There were also no significant differences in photosynthetic rates among the six genotypes tested (Table 1). But, the stomatal conductance, transpiration and internal CO_2 concentration differed among and between the genotypes, with TGx1445-3E showing the highest values for these three parameters (Table 1). The genotype × inoculation interaction showed that plant inoculation significantly increased stomatal conductance in TGx1740-2F, TGx1448-2E and TGx1445-3E, but decreased it in TGx1440-1E and Salintuya-1 (Fig. 1A). As a result, transpiration was also lower in inoculated Salintuya-1 plants but higher in TGx1740-2F and TGx1445-3E, which showed much greater stomatal conductance with inoculation (Fig. 1B).

Effect of inoculation on $\delta^{13}C$ and C level in soybean genotypes

Inoculating six soybean genotypes with *B. japonicum* strain WB74 showed no effect on the $\delta^{13}C$ of shoots and roots, as well as on the C concentration of shoots and roots (Table 2). However, C accumulation in whole plants was significantly greater in inoculated relative to uninoculated control plants (Table 2). Shoot $\delta^{13}C$ also differed among and between the six soybean genotypes (Table 2), with TGx1445-3E and Salintuya-1 showing higher ^{13}C discrimination (i.e. more negative) while TGx1448-2E exhibited much lower ^{13}C discrimination (i.e. less negative). There were, however, no differences in root $\delta^{13}C$ values. The concentration

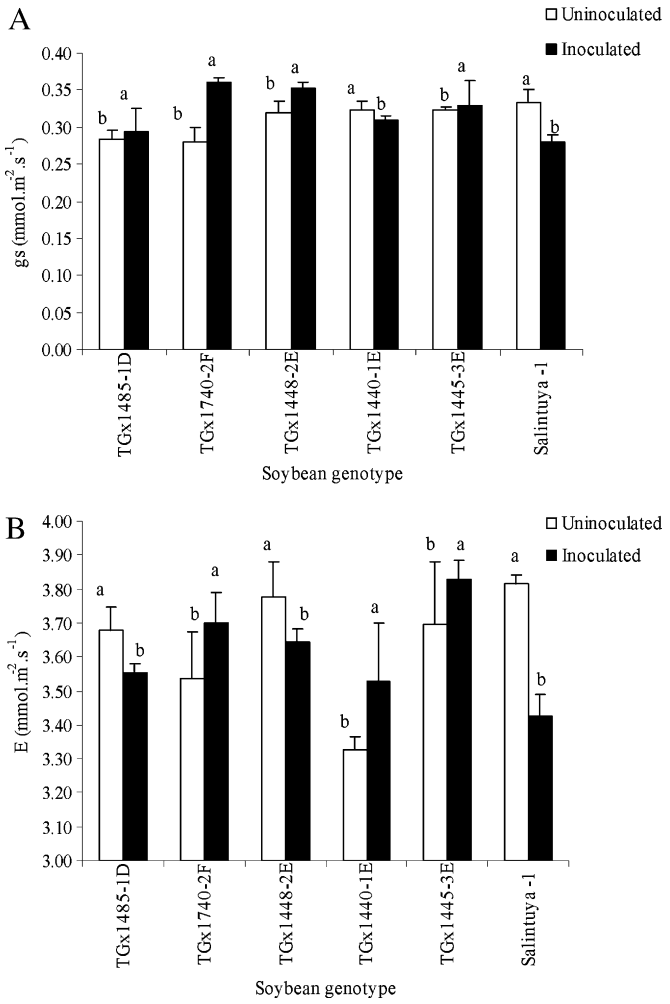

Fig. 1. Interactive effects of inoculation with *Bradyrhizobium japonicum* strain WB74 and soybean genotype on (A) stomatal conductance and (B) transpiration rate. Vertical lines on bars represent SE ($n = 4$). Bars followed by dissimilar letters are significantly different at $p \leq 0.001$.

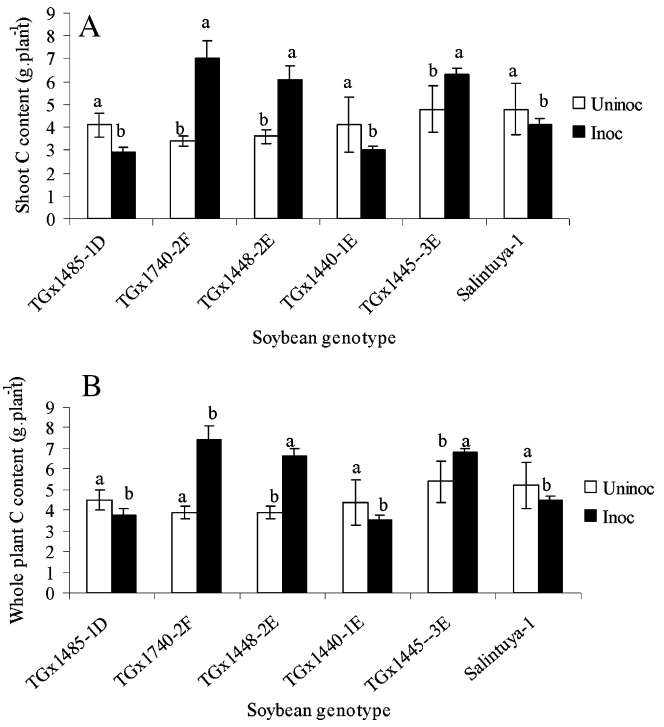

Fig. 2. Interactive effects of inoculation with *Bradyrhizobium japonicum* strain WB74 and soybean genotype on C content of (A) shoots (B) whole plant. Vertical lines on bars represent SE ($n = 4$). Bars followed by dissimilar letters are significantly different at $p \leq 0.01$.

of C in shoots, roots and whole plants was also similar in all six soybean genotypes. However, C accumulation was higher in shoots and whole plants of TGx1445-3E, followed by TGx1740-2F, when compared to the other genotypes (Table 2). Genotype x inoculation interaction was significant for C content in shoots and whole plants, and a greater C accumulation was revealed in shoots and whole plants of TGx1740-2F, TGx1448-2E and TGx1445-3E (Fig. 2).

Effect of inoculation on nodulation, plant growth, and N level

A 2-Way ANOVA analysis showed that inoculation with *B. japonicum* strain WB74 significantly increased nodule number and nodule mass relative to uninoculated control plants (Table 3). Compared to the other five genotypes, TGx1740-2F exhibited the highest nodulation, with about 517 nodules per plant (Table 3). Nodule dry matter, however, did not differ among the six genotypes.

The 2-Way ANOVA analysis also showed that inoculation of soybean plants with *B. japonicum* strain WB74 significantly increased shoot and whole-plant dry matter yield in the soybean genotypes tested (Table 3). Of the six genotypes, TGx1445-3E, TGx1448-2E

Table 2
Effect of inoculation with *Bradyrhizobium japonicum* strain WB74 on $\delta^{13}C$, %C and C content of six soybean genotypes planted at Wa, Ghana in 2006. The treatment means were computed by grouping means of all genotypes. Values (mean ± SE) with dissimilar letters in a column are significant at $p \leq 0.01$(**); $p \leq 0.001$(***).

Treatment	$\delta^{13}C$ (‰)		%C			C content (g plant^{-1})		
	Shoots	Roots	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant
Inoculation								
Uninoculated	−28.4 ± 0.1a	−28.5 ± 0.1a	44.0 ± 0.2a	37.0 ± 0.7a	40.5 ± 0.4a	4.1 ± 0.3a	0.3 ± 0.0a	4.6 ± 0.3b
Inoculated	−28.4 ± 0.0a	−28.5 ± 0.0a	42.7 ± 0.7a	38.6 ± 1.5a	40.7 ± 0.8a	4.9 ± 0.4a	0.3 ± 0.0a	5.4 ± 0.4a
Genotype								
TGx1485-1D	−28.4 ± 0.1ab	−27.3 ± 0.2a	43.8 ± 0.3a	41.2 ± 4.1a	42.5 ± 2.0a	3.5 ± 0.3b	0.3 ± 0.0a	4.2 ± 0.3bc
TGx1740-2F	−28.5 ± 0.1ab	−27.4 ± 0.1a	43.8 ± 0.3a	38.4 ± 1.3a	41.1 ± 0.7a	5.2 ± 0.8a	0.3 ± 0.0a	5.7 ± 0.8a
TGx1448-2E	−28.3 ± 0.1a	−27.3 ± 0.1a	41.2 ± 2.1a	35.7 ± 1.3a	38.4 ± 1.3a	4.9 ± 0.6ab	0.3 ± 0.0a	5.3 ± 0.5ab
TGx1440-1E	−28.5 ± 0.0ab	−27.3 ± 0.1a	44.4 ± 0.2a	37.0 ± 1.4a	40.7 ± 0.7a	3.6 ± 0.6b	0.3 ± 0.0a	3.9 ± 0.5c
TGx1445-3E	−28.6 ± 0.1b	−27.3 ± 0.2a	43.2 ± 0.3a	37.2 ± 1.0a	40.2 ± 0.6a	5.6 ± 0.6a	0.3 ± 0.0a	6.1 ± 0.6a
Salintuya-1	−28.6 ± 0.1b	−27.4 ± 0.1a	43.7 ± 0.4a	37.5 ± 1.2a	40.6 ± 0.5a	4.5 ± 0.6ab	0.3 ± 0.0a	4.9 ± 0.6abc
F-Statistics (2-Way ANOVA)								
Inoculation	2.6	2.3	4.2	10.0	0.03	6.9	0.2	5.6**
Genotype	2.7**	0.1	1.9	0.9	1.6	3.2**	0.6	3.4**
Inoculation × genotype	1.9	0.8	2.6	0.9	1.9	4.6***	1.8	4.5**

Table 3
Effect of inoculation with *Bradyrhizobium japonicum* strain WB74 on dry matter yield, %N, and N content of six soybean genotypes planted at Wa, Ghana, in 2006. The treatment means were computed by grouping means of all genotypes. Values (mean ± SE) with dissimilar letters in a column are significantly different at $p \leq 0.01$(**); $p \leq 0.001$(***).

Treatment	Nodulation		Dry matter (g plant^{-1})			%N		N content (g plant^{-1})		
	Nod no.	Nod mass	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant
Inoculation											
Uninoculated	141 ± 27b	1.5 ± 0.3b	9.4 ± 0.8 b	1.9 ± 0.1a	11.3 ± 0.8b	4.4 ± 0.1 a	1.6 ± 0.1 b	3.0 ± 0.1b	0.42 ± 0.04b	0.03 ± 0.00a	0.46 ± 0.04b
Inoculated	382 ± 50a	4.0 ± 0.6a	11.6 ± 0.9a	2.0 ± 0.10a	13.6 ± 1.0a	4.6 ± 0.1a	1.9 ± 0.0a	3.2 ± 0.1a	0.53 ± 0.05a	0.04 ± 0.00a	0.57 ± 0.05a
Genotype											
TGx1485-1D	133 ± 27b	1.0 ± 0.2a	8.0 ± 0.8b	1.9 ± 0.1a	9.9 ± 0.8b	4.4 ± 0.1a	1.7 ± 0.2a	3.1 ± 0.1a	0.35 ± 0.03b	0.04 ± 0.01a	0.38 ± 0.03c
TGx1740-2F	517±112a	3.9 ± 1.2a	11.9 ± 1.8a	1.9 ± 0.1a	13.8 ± 1.8a	4.6 ± 0.1a	1.9 ± 0.1a	3.2 ± 0.1a	0.55 ± 0.10a	0.04 ± 0.00a	0.59 ± 0.10ab
TGx1448-2E	210 ± 45b	2.0 ± 0.7a	12.1 ± 1.5a	1.9 ± 0.2a	13.9 ± 1.7a	4.4 ± 0.2a	1.8 ± 0.1a	3.1 ± 0.1a	0.55 ± 0.08a	0.03 ± 0.00a	0.58 ± 0.08ab
TGx1440-1E	148 ± 29b	2.5 ± 0.6a	8.0 ± 1.3b	1.8 ± 0.2a	9.8 ± 1.5b	4.4 ± 0.2a	1.8 ± 0.1a	3.1 ± 0.1a	0.36 ± 0.07b	0.03 ± 0.00a	0.39 ± 0.07c
TGx1445-3E	250 ± 85b	3.9 ± 1.0a	13.0 ± 1.4a	2.2 ± 0.1a	15.2 ± 1.5a	4.7 ± 0.1a	1.7 ± 0.1a	3.2 ± 0.1a	0.62 ± 0.07a	0.04 ± 0.00a	0.66 ± 0.08a
Salintuya-1	313 ± 83b	3.3 ± 1.2a	10.2 ± 1.3ab	1.8 ± 0.2a	12.0 ± 1.5ab	4.4 ± 0.2a	1.7 ± 0.1a	3.0 ± 0.1a	0.45 ± 0.07ab	0.03 ± 0.01a	0.48 ± 0.07bc
2-Way ANOVA (*F* statistics)											
Inoculation	32.8***	17.8***	6.2**	0.2	5.5**	1.63	13.5***	7.4***	5.40**	3.77	5.67**
Variety	7.5***	2.3	4.0***	0.7	3.6***	0.83	1.0	1.0	3.66***	0.51	3.52**
Inoculation × variety	2.3	1.6	5.6***	1.8	5.3***	1.66	2.6**	0.7	4.75***	1.63	4.73***

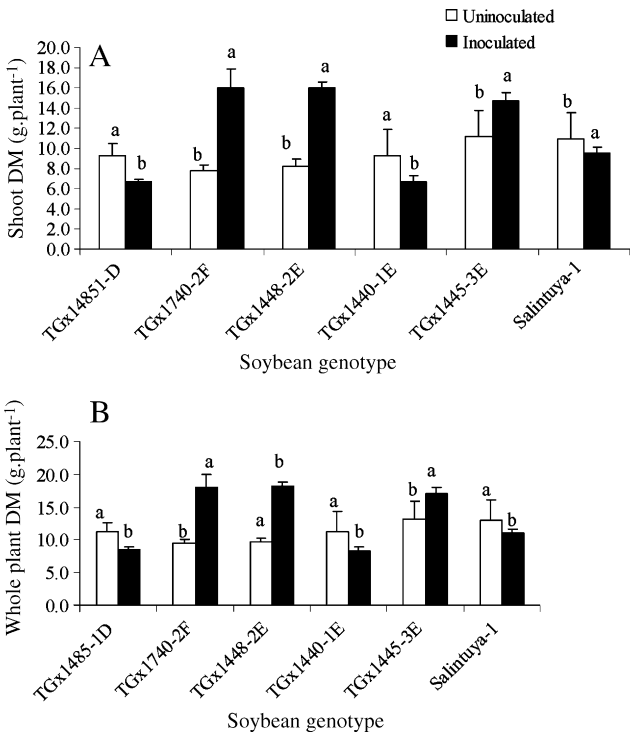

Fig. 3. Interactive effects of inoculation with *Bradyrhizobium japonicum* strain WB74 and soybean genotype on dry matter of (A) shoot, and (B) whole plant. Vertical lines on bars represent SE ($n = 4$). Bars followed by dissimilar letters are significantly different at $p \leq 0.001$.

and TGx1740-2F accumulated more biomass in shoots and whole plants compared with the other 3 genotypes (Table 3). Analysis of genotype × inoculation interaction confirmed a marked effect of inoculation on the shoot and whole-plant dry matter yield of TGx1740-2F, TGx1448-2E and TGx1445-3E (Fig. 3A and B).

The N concentration in roots and whole plants were also much higher than those of uninoculated controls (Table 3). As a result, the N content of shoots and whole plants was also greater than that of controls. As with plant biomass, genotype x inoculation analysis again revealed greater N concentrations and N content in TGx1740-2F, TGx1448-2E and TGx1445-3E with inoculated relative to uninoculated plants (Table 3).

δ^{15}N, %Ndfa and amounts of N-fixed

A 2-Way ANOVA analysis of symbiotic parameters revealed lower shoot δ^{15}N values with *Bradyrhizobium* inoculation. The %Ndfa values of shoots, roots and whole plants, as well as N-fixed in these organs, were significantly higher in inoculated relative to uninoculated plants (Table 4). There were significant differences in symbiotic performance between and among the six promiscuous soybean genotypes. For example, genotypes such as TGx1448-2E and TGx1740-2F showed lower shoot δ^{15}N values, high shoot %Ndfa and greater amounts of N-fixed, whether measured per plant or per hectare (Table 4). It was only TGx1445-3E, which exhibited greater ∂^{15}N in shoots, roots and whole plants, and therefore lower %Ndfa (Table 4), that still showed increased symbiotic N yield because of its greater biomass and higher N content (Table 4).

As shown in Table 4, the genotype x inoculation interaction was significant for all the symbiotic parameters. Significantly lower δ^{15}N values with *Bradyrhizobium* inoculation of all six soybean genotypes were revealed, except for TGx1448-2E (Fig. 4A). As a result, %Ndfa values were also higher in all genotypes inoculated

Table 4
Effect of inoculation with *Bradyrhizobium japonicum* strain WB74 on the symbiotic performance of six soybean genotypes planted at Wa, Ghana, in 2006. The treatment means were computed by grouping means of all genotypes. Values (mean ± SE) with dissimilar letters in a column are significant $p \leq 0.01$(**); $p \leq 0.001$(***).

Treatment	δ^{15}N(‰)			%Ndfa			N-fixed (mg plant^{-1})			N-fixed (kg ha^{-1})		
	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant	Shoots	Roots	Whole plant
Inoculation												
Uninoculated	1.3 ± 0.11a	1.8 ± 0.10a	1.6 ± 0.07a	78.3 ± 1.2b	34.0 ± 2.6b	56.1 ± 1.1b	330 ± 30b	10 ± 0b	340 ± 30b	87.6 ± 7.9b	2.9 ± 0.4b	90.5 ± 8.1b
Inoculated	1.0 ± 0.07b	2.0 ± 0.16a	1.5 ± 0.09a	81.3 ± 0.8a	41.3 ± 1.7a	61.3 ± 0.8a	430 ± 40a	20 ± 0a	450 ± 0.03a	114.4 ± 9.4a	4.2 ± 0.3a	118.5 ± 9.6a
Genotype												
TGx1485-1D	1.0 ± 0.06bc	2.0 ± 0.27bc	1.5 ± 0.16c	81.5 ± 0.6ab	41.5 ± 1.9b	61.5 ± 0.7b	280 ± 20b	10 ± 0b	290 ± 20bc	75.3 ± 6.1b	4.0 ± 0.7a	79.3 ± 6.5b
TGx1740-2F	1.1 ± 0.03b	1.8 ± 0.13c	1.4 ± 0.07cd	80.2 ± 0.3b	40.5 ± 5.7b	60.4 ± 2.9b	440 ± 80a	20 ± 0a	460± 80a	117.7 ± 20.6a	4.1 ± 0.8a	121.8 ± 21.3a
TGx1448-2E	0.9 ± 0.09c	1.7 ± 0.06c	1.3 ± 0.04de	82.7 ± 1.0a	25.1 ± 3.1d	53.9 ± 1.1e	450 ± 60a	10 ± 0b	460 ± 50ab	119.5 ± 16.5a	2.5 ± 0.6b	122.0 ± 7.0a
TGx1440-1E	0.9 ± 0.13c	2.5 ± 0.23a	1.7 ± 0.07b	82.7 ± 1.4a	28.9 ± 2.2c	55.8 ± 1.8d	290 ± 50b	10 ± 0b	300 ± 40c	78.1 ± 4.3b	2.5 ± 0.3b	80.6 ± 4.4b
TGx1445-3E	2.0 ± 0.15a	2.2 ± 0.23b	2.1 ± 0.08a	70.8 ± 1.6c	44.9 ± 0.9a	57.9 ± 0.8c	440 ± 50a	20 ± 0a	460 ± 40a	117.6 ± 14.5a	4.6 ± 0.4a	122.1 ± 4.7a
Salintuya-1	1.0 ± 0.10b	1.3 ± 0.07d	1.2 ± 0.06e	80.8 ± 1.1b	45.0 ± 0.6a	62.9 ± 0.5a	307 ± 50ab	10 ± 0b	317 ± 50abc	97.6 ± 4.3ab	3.7 ± 0.7ab	101.3 ± 4.8ab
2-Way ANOVA (*F* statistics)												
Inoculation	42.24***	2.53	1.71	42.2***	167.66***	205.5***	7.58**	11.19***	10.19***	7.58***	11.19***	8.01***
Variety	65.51***	16.99***	34.43***	65.5***	152.55***	62.3***	2.95**	3.6***	3.55***	2.95**	3.6***	2.91**
Inoculation × variety	16.25***	17.44***	12.56***	55.7***	111.54***	55.7***	4.67***	4.78***	6.33***	4.67***	4.78***	4.81***

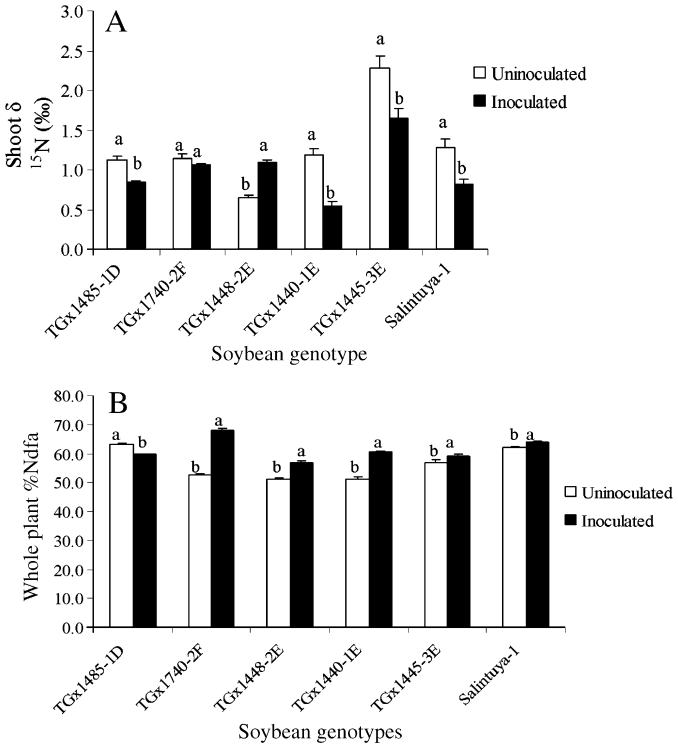

Fig. 4. Interactive effects of inoculation with *Bradyrhizobium japonicum* strain WB74 and soybean genotype on (A) shoot δ^{15}N, and (B) whole plant %Ndfa. Vertical lines on bars represent SE ($n = 4$). Bars followed by dissimilar letters are significantly different at $p \leq 0.001$.

with *B. japonicum* strain WB74 (Fig. 4B). Because plant biomass was increased with inoculation of TGx1740-2F, TGx1448-2E and TGx1445-3E (Fig. 3A and B), the greater %Ndfa values also resulted in markedly larger amounts of N-fixed in the three genotypes (Fig. 5).

Nodule occupancy and strain IGS types

Eighteen IGS types (including the inoculant strain, *B. japonicum* WB74), were isolated from root nodules of both inoculated and uninoculated soybean plants (Fig. 6, Tables 5 and 6). IGS type II

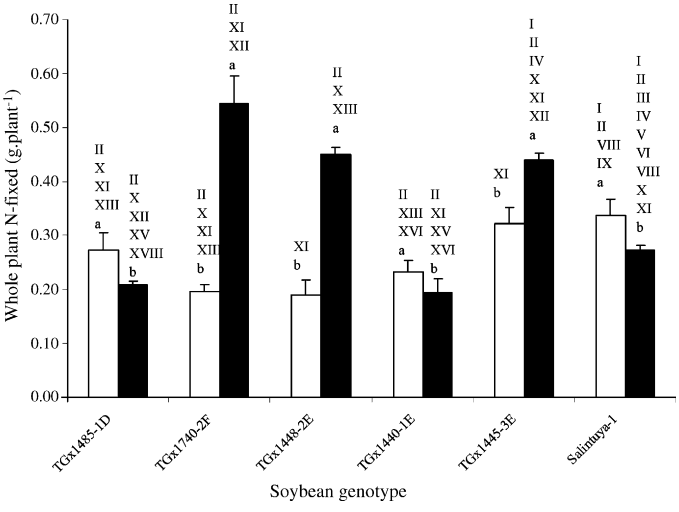

Fig. 5. Interactive effects of inoculation with *Bradyrhizobium japonicum* strain WB74 and soybean genotype on whole plant N-fixed. Vertical lines on bars represent SE ($n = 4$). Bars followed by dissimilar letters are significantly different at $p \leq 0.001$.

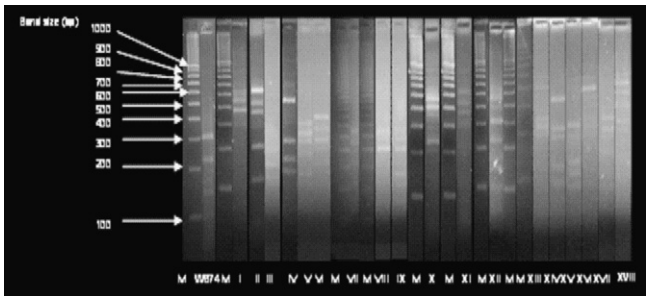

Fig. 6. IGS types from digested products of MspI of indigenous root nodule bacteria isolated from six soybean genotypes grown in Wa, Ghana. M: molecular marker; I–XVIII: strain IGS type.

was isolated from all six soybean genotypes with mean nodule occupancy of 27%, followed by IGS types X and XI which were isolated from 5 of the 6 genotypes. However, IGS types VI, VII, XVII and XVIII were each isolated from single (but different) genotypes (Table 5). Ironically, the naturalized local genotype, Salintuya-1, showed the highest level of promiscuity in hosting 9 of the 18 IGS types (Table 5). This was followed by TGx1445-3E and TGx1485-1D, which were each nodulated by 6 IGS types (Table 5).

Strain IGS type symbiotic efficiency

Relating nodule functioning (measured here as N-fixed per plant) to the IGS types found inside root nodules of the six promiscuous soybean genotypes revealed significant differences in the N_2-fixing efficiency of these IGS types (Fig. 6). It was noteworthy that sole nodule occupancy by IGS type XI in uninoculated plants of TGx1445-3E resulted in significantly high N yield compared to its performance as a sole occupant of nodules from uninoculated plants of TGx1448-2E (Fig. 6). Similar differences in symbiotic functioning were obtained for combinations of strain IGS types found in root nodules of inoculated and uninoculated soybean genotypes (Fig. 6). In this study, inoculated plants of TGx1740-2F exhibited the highest symbiotic N yield, even though the genotype hosted only three IGS types. This was in contrast to Salintuya-1, which had a high nodule occupancy of nine different IGS types, but produced a relatively lower amount of N-fixed (Fig. 6). Soybean genotype TGx1740-2F produced the highest symbiotic N and harbored three IGS types, including the inoculant strain (i.e. IGS type XII).

Discussion

Promiscuous-nodulating soybean genotypes (TGx lines) developed for easy use by African farmers without inoculation requirement were evaluated under field conditions in Ghana to assess whether these materials indeed nodulate freely with indigenous root-nodule bacteria in African soils, and to measure plant growth and N_2 fixation. The field testing was done against a background of uninoculated and inoculated treatments with a commercial soybean inoculant (*B. japonicum* strain WB74). Although the results revealed free nodulation of all six genotypes in both inoculated and uninoculated plots, there was a marked effect of inoculation on photosynthetic rates, whole-plant C, nodule number, nodule mass, total plant biomass, as well as N content and N concentration per plant (Tables 1–3). As a result, the percent N derived from fixation and actual amounts of N-fixed were increased by inoculation of the promiscuous soybean genotypes relative to uninoculated controls (Table 4), a finding consistent with the data by Koutroubas et al. (1998). However, in another study involving soybean, the introduced strain was outcompeted by indigenous soil bradyrhizobia, and resulted in no effect of inoculation on N-fixed and grain yield (Okogun and Sanginga, 2003).

Significant interactions also revealed marked differences in specific effects caused by the introduced strain on individual soybean genotypes. For example, plant inoculation with *B. japonicum* strain WB74 increased shoot and whole-plant dry matter in TGx1740-2F, TGx1448-2E and TGx1445-3E, as a consequence of greater C accumulation in shoots and whole plants (Figs. 2 and 3). In general, inoculation caused a decrease in $\partial^{15}N$ values of the six genotypes (except for TGx1448-2E), leading to significantly higher %Ndfa values in all the soybean genotypes, except for TGx1485-1D (Fig. 4). Perhaps as a result of the increased C supply from photosynthate (Fig. 2), N derived from fixation (especially N-fixed) was markedly

Table 5
Percent nodule occupancy of the strain IGS types for inoculated and uninoculated soybean genotypes.

IGS type	TGx1740-2F		TGx1448-2E		TGx1445-3E		Salintuya-1		TGx1440-1E		TGx1485-1D	
	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc	Inoc	Uninoc
I	0	0	0	0	21.4	0	48.2	26.7	0	0	0	0
II	10.5	50	25	0	28.6	0	11.1	13.3	66.7	66.6	21.4	44.4
III	0	0	0	0	0	0	11.1	0	0	0	0	0
IV	0	0	0	0	7.1	0	3.7	0	0	0	0	22.2
V	0	0	0	0	0	0	3.7	0	0	0	0	0
VI	0	0	0	0	0	0	7.4	0	0	0	0	0
VII	0	0	0	0	0	0	7.4	0	0	0	0	0
VIII	0	0	0	0	0	0	0	53.3	0	0	0	0
IX	0	0	0	0	0	0	0	6.7	0	0	0	0
X	0	25	25	0	14.3	0	3.7	0	0	0	7.1	11.2
XI	5.3	12.5	0	100	14.3	100	3.7	0	22.2	0	0	0
XII	78.9	0	0	0	14.3	0	0	0	0	0	35.7	0
XIII	5.3	12.5	50	0	0	0	0	0	0	16.7	0	0
XIV	0	0	0	0	0	0	0	0	0	16.7	0	22.2
XV	0	0	0	0	0	0	0	0	5.6	0	7.1	0
XVI	0	0	0	0	0	0	0	0	5.6	0	0	0
XVII	0	0	0	0	0	0	0	0	0	0	3.8	0
XVIII	0	0	0	0	0	0	0	0	0	0	24.9	0

Table 6
Overall percent nodule occupancy of strain IGS type in the root nodules of six soybean genotypes.

IGS type	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
Overall % nodule occupancy of all analyzed nodules	12.9	27.2	2.0	3.4	0.7	1.4	1.4	4.8	0.7	4.8	12.2	15.0	4.8	2.0	1.4	0.7	0.7	3.4

increased in TGx1740-2F, TGx1448-2E and TGx1445-3E (Fig. 5). Relative to uninoculated controls, symbiotic N yield was more than doubled by *B. japonicum* inoculation of TGx1740-2F and TGx1448-2E (Fig. 5). In addition to the inoculation effects, there were also marked variations in symbiotic performance of the six soybean genotypes. Although photosynthetic rates were similar (Table 1), TGx1740-2F produced the highest nodule count per plant (Table 3), and together with TGx1448-2E and TGx1445-3E, also produced the highest plant biomass and N content (Table 3) from significantly increased C accumulation (Table 2). As a result, these three genotypes exhibited the highest overall %Ndfa values, and thus the greatest amounts of N-fixed relative to the other genotypes (Table 4).

To ascertain the nodulation performance of the introduced strain, PCR-RFLP analysis was performed on 360 pink effective nodules harvested from both inoculated and uninoculated soybean plants. The results showed 18 distinct IGS types present in root nodules of the six promiscuous-nodulating soybean genotypes grown in Ghana. Strain IGS type II was isolated from all six soybean genotypes with mean nodule occupancy of 27.2%, which indicated a high level of promiscuity and perhaps distribution in the soil. This was followed by IGS type XII with overall nodule occupancy of 15%, isolated from 3 out of 6 genotypes, then IGS types X and XI, which were isolated from 5 out of the 6 genotypes (Fig. 6, Table 5). This result was unexpected with IGS type XII, as it was identified as the introduced strain WB74. An assessment of strain IGS type symbiotic efficiency revealed marked differences in symbiotic N yield by both single and multiple occupants of root nodules (Fig. 5). It was interesting that, even with the same IGS type as sole occupant of root nodules on a host plant, the level of symbiotic efficiency differed with soybean genotype (Fig. 5; see also Pule-Meulenberg et al., 2010). As shown in Fig. 5, IGS type XI produced high symbiotic N yield in TGx1445-3E but less so in TGx1448-2E. Furthermore, inoculated plants of Salintuya-1 trapped as many as 9 strain IGS types in their root nodules, and this host plant was therefore the most promiscuous of the six genotypes. However, its symbiotic N yield was less than (or equal to) that of other genotypes with fewer IGS types in their root nodules. As observed with cowpea (Pule-Meulenberg et al., 2010), the presence of multiple IGS types in root nodules not only made assessment of IGS type symbiotic efficiency difficult, but also appeared to indicate that their interaction can reduce symbiotic N yield (Fig. 5). Although it has been demonstrated that the symbiotic performance of a double strain inoculant of *Rhizobium leguminosarum* was 2.5 times more superior to its sole counterpart in subterranean clover (Rambaugh et al., 1990), it is not clear whether the IGS types of those strains were the same or different. More studies are therefore needed to assess the positive or negative effects of strain IGS types on nodule functioning, especially when they are present as sole or multiple occupants in the same nodule and/or on the same host plant.

Nitrogen fixation in symbiotic legumes is driven by recently produced photosynthate (Rogers, 2009), just as adequate N nutrition is needed for biosynthesis of enzymes and macromolecules involved in CO_2 reduction in plants (e.g. chlorophyll, see Makoi et al., 2010). Thus, where inoculation enhances nodule formation and functioning, photosynthetic rates, and thus C accumulation, should be expected to rise from improved symbiotic N nutrition, as found in this study (Fig. 2). Although photosynthetic rates were similar for all six soybean genotypes, stomatal conductance was greater in TGx1740-2F and TGx1445-3E, and resulted in higher transpiration rates (Table 1). However, there were also specific effects of *Bradyrhizobium* inoculation on the water relations of some soybean genotypes. For example, bacterial inoculation induced an increase in stomatal conductance of TGx1485-1D, TGx1448-2E, TGx1740-2F and TGx1445-3E, which resulted in significantly elevated transpiration rates in the last two genotypes (Fig. 1). This *Bradyrhizobium*-induced increase in stomatal conductance (i.e. wider opening of stomata) appeared to have enhanced CO_2 capture and reduction, as indicated by the greater C accumulation in TGx1740-2F and TGx1445-3E (Fig. 2). However, *Bradyrhizobium* inoculation was also found to decrease stomatal conductance in two of the six soybean genotypes (namely, TGx1440-1E and Salintuya-1), leading to reduced leaf water loss from transpiration (Fig. 1B) and decreased C accumulation (Fig. 2), possibly due to a decline in CO_2 influx and reduction by Rubisco, and the net result was lower ^{13}C discrimination in TGx1485-1D and TGx1440-1E, but not in Salintuya-1 (Table 2).

Based on the effects of inoculation on stomatal conductance and transpiration obtained in this study (Fig. 1) and elsewhere (Figueiredo et al., 1999), bradyrhizobial presence in the rhizosphere is likely to play a major role in alleviating the effect of water stress in symbiotic legumes. Mechanistically, this can happen through the action of rhizobial products such as abscisic acid (Phillips and Torey, 1970) or lumichrome (Phillips et al., 1999) in decreasing leaf stomatal conductance and reducing water loss via transpiration. A recent study has, in fact, shown that the application to roots of 10 nM lumichrome, 10 nM abscisic acid, or 10 mL of cultured infective cells of *B. japonicum* strain WB74 resulted in significantly decreased leaf stomatal conductance and reduced water loss via transpiration in soybean leaves (Matiru and Dakora, 2005). Taken together, our data suggest that bacterial presence in the rhizosphere can trigger plant response to drought, especially in arid environments where water can be a limiting factor for plant growth.

In conclusion, the six promiscuous-nodulating soybean genotypes tested in this study could nodulate freely with indigenous root-nodule bacteria in a Ghanaian soil. Of these, we identified TGx1445-3E and Salintuya-1 as good candidates for use by African farmers without inoculation, as they could derive 60% or more of their N from symbiotic fixation, as well as produce about 100 kg N ha^{-1} in uninoculated plots.

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