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Symbiotic Effectiveness of *Rhizobium* (*Agrobacterium*) Compared to *Ensifer* (*Sinorhizobium*) and *Bradyrhizobium* Genera for Soybean Inoculation under Field Conditions

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ABSTRACT

The symbiotic potential of twenty Egyptian strains of soybean-nodulating rhizobia related to *Agrobcaterium* (*Rhizobium*), *Ensifer* (*Sinorhizobium*) and *Bradyrhizobium* genera has been evaluated under greenhouse and sandy loam-field soil conditions. Greenhouse-screening results showed positive symbiotic interactions between all tested rhizobia and soybean cultivar, *Giza* 22. The growth parameters and N-shoot content of the inoculated soybean plants were clearly affected and appeared depending upon rhizobial strain type. Four promising rhizobial strains (*Ensifer* NGB-SR3, *Bradyrhizobium* NGB-SR4, *Agrobcaterium* NGB-SR7, *Bradyrhizobium* NGB-SR14) were tested for soybean inoculation under field conditions. During flowering stage, there was a prolific nodulation pattern with all tested inoculants resulted in nodule masses ranged from 265-361 mg plant^{-1}, compared to the un-inoculated control (15-31 mg plant^{-1}). At harvest, the superiority of *Bradyrhizobium* NGB-SR4 and *Agrobcaterium* NGB-SR7 over other tested rhizobia was evident regarding soybean seed yield, seed N-yield and crude protein content. We confirm the nodulating machinery stability of *Agrobacterium* under greenhouse and sandy loam field soil conditions and their potential use as efficient soybean inoculants along with other traditional soybean micro-symbionts; *Bradyrhizobium* and *Sinorhizobium* genera.

Key words: *Rhizobium*, *Agrobacterium*, symbiotic effectiveness, field trial, inoculants, soybean

INTRODUCTION

A hallmark trait of legumes is their ability to develop root nodules and to fix N_2in symbiosis with compatible rhizobia (Graham and Vance, 2003). In semi-arid lands including Egypt, Nitrogen (N) deficiency is frequently one of the major factors limiting the yield of legume crops which makes the contribution of Symbiotic Nitrogen Fixation (SNF) of great importance, especially when legumes are involved in the cropping systems. SNF agents are among the most powerful alternative solutions which play an important role in reducing the consumption of chemical N-fertilizers, increasing soil fertility, decreasing the production cost and eliminating the undesirable pollution impact of chemical fertilizers in the environment. Worldwide, about 44-66 Million Metric Tons (MMT) of nitrogen is biologically fixed annually, providing nearly half of all nitrogen requirements used in agriculture (Alberton *et al*., 2006).

Soybean (*Glycine max* (L.) Merrill) is the world's foremost provider of vegetable protein and oil (Lee *et al*., 2007). In 2007, soybean emerged as the dominant oilseed in the world with the share of 57% (223 MMT) of the total production of global major oilseeds (393 million metric tons) (FAO, 2009; USDA, 2009). The protein content in soybean seed is approximately 40% and the oil content is approximately 20% (Qiu and Chang, 2010). In addition to its importance as a source of protein for human nutrition and fodder, soybean has now become an important world commodity because of its wide range of geographical adaptation, unique chemical composition, functional health benefits and industrial applications (Ali, 2010).

Soybean is currently known to be nodulated naturally under field conditions by *Bradyrhizobium japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall *et al*., 1992), *B. liaoningense* (Xu *et al*., 1995), *B. yuanmingense* (Appunu *et al*., 2009), *B. diazoefficiens* (Delamuta *et al*., 2013), *Ensifer* (*Sinorhizobium*) *fredii* (Scholla and Elkan, 1984) and *E. xinjiangense* (Chen *et al*., 1988). In addition to traditional soybean micro-symbionts, few strains of the genus *Agrobacterium* (now, rather controversially, included in the genus *Rhizobium* (Young *et al*., 2001; Farrand *et al*., 2003), could establish efficient symbioses with soybean plants (Chen *et al*., 2000).

During the 20th century, inoculation of legumes with root-nodule bacteria developed in many parts of the world became one of the most cost effective of all agricultural practices (Herridge *et al*., 2008). Soybean seeds contain a large amount of nitrogen and the total amount of nitrogen assimilated in a plant is highly correlated with the soybean seed yield (Ohyama *et al*., 2009). One ton of soybean grains require about 70-90 kg N, which is about four times more than in the case of rice (Hoshi *et al*., 1982). The amount of fixed N through biological nitrogen fixation process has been substantially varied from zero to 98% (Keyser and Li, 1992). Through field conditions, on an average, 50-60% of soybean N demands (111 kg ha^{-1}) is met by biological N_2 fixation (Salvagiotti *et al*., 2008). The variable extent of nitrogen fixation by soybean cultivars is probably due to differences in plant genotypes, symbiotic effectiveness of rhizobial strains and their compatibility (Appunu *et al*., 2008).

Through a preliminary plant nodulation assay, we previously confirmed the nodulation of soybean cultivar *Giza* 22 by eleven *Agrobacterium* strains isolated from Egyptian soils (Youseif *et al*., 2014). In this study, we evaluated the symbiotic performance and potential use of these *Agrobacterium* strains as soybean inoculants under greenhouse and field conditions, compared to other soybean-nodulating rhizobial genera; *Bradyrhizobium* and *Sinorhizobium*.

MATERIALS AND METHODS

Bacterial strains: Twenty Egyptian strains of soybean nodulating rhizobia including eleven *Rhizobium* spp. (Syn. *Agrobacterium*), four *Ensifer* spp. (Syn. *Sinorhizobium*) and five *Bradyrhizobium* spp. (Youseif *et al*., 2014), were screened for their symbiotic effectiveness with soybean plants under greenhouse experiments and field trials. Two reference strains (*B. japonicum* USDA110 and *S. fredii* HH303) were used in this study and were supplied from Biological Nitrogen Fixation Unit, Agricultural Research Center (ARC), Giza, Egypt.

Soybean cultivar: Seeds of soybean (*Glycine max* L. Merrill) variety *Giza* 22 were used in this study. The seeds were provided from Legumes Research Department, Field Crops Research Institute, ARC, Giza, Egypt.

Symbiotic effectiveness experimental design

Greenhouse experiment: Pot experiments were conducted at the controlled greenhouse of Biological Nitrogen Fixation Unit, ARC, Giza to study the effect of twenty local rhizobial strains in comparison with two reference strains (*B. japonicum* 110 and *S. fredii* HH303) on growth, nodulation, nitrogen fixation and nitrogen uptake of soybean plants. Two un-inoculated controls; Control 1+ starter N dose (48 kg N ha^{-1}) and Control 2 + full N dose (180 kg N ha^{-1}) were included. Plastic pots (30 cm diameter) were filled with 10 kg of sandy soil and arranged in a complete randomized block design with three replicates. Six seeds were planted in each pot. Each seed was inoculated with 1 ml of a log phase rhizobial culture (10^9 cells mL^{-1}). After complete germination, plants were thinned to four plants/pot. Growth conditions of soybean plants were 22-32°C (night/day), a relative humidity of 70-80% and a photoperiod of 14 h. After 60 days of planting, plants were uprooted and assayed for number and dry weight of nodules, nitrogenase activity, shoots and roots dry weight and total nitrogen uptake by soybean plants.

Field trials: The field experiment was carried out in a sandy loam soil at Nubaria region Al-Behira governorate (latitude: 30°57' 28.1" N and longitude: 29° 51' 7.3" E) during the summer-growing season of 2009. The experimental plots were arranged in a randomized complete block design with four replicates. Plot area was 4.2 m^2 and consisted of four rows, spaced 0.6 m apart. After 60 days of planting, plants were uprooted and assayed for number and dry weight of nodules, shoots and roots dry weight and total nitrogen uptake by soybean plants. At harvest, the yield, yield components, N-yield and crude protein of soybean plants were estimated.

Fertilization: Phosphorus; all treatments received the recommended dose of super phosphate (15.5% P_2O_5) at the rate of 480 kg ha^{-1}. Potassium; all treatments received the recommended dose of potassium sulfate (48.5% K_2O) at the rate of 240 kg ha^{-1}. Nitrogen; all rhizobial treatments received ammonium sulfate (20.5% N) at a rate of 48 kg N ha^{-1} as a starter dose of nitrogen, while the un-inoculated controls were fertilized at a rate of 48 and 180 kg N ha^{-1} as a starter dose and recommended full dose of chemical N-fertilizers, respectively.

Determinations: Nitrogenase enzyme activity in fresh root nodules was measured according to the acetylene reduction assay (Hardy *et al.*, 1973), using DANI 1000 (ColognoMonzese (MI) -Italy) FID gas chromatography. Total nitrogen content in soybean plant materials was determined by the wet digestion using micro-Kjeldahl procedures (Jackson, 1973). The crude protein percentage was determined by multiplying the measured nitrogen percentage by 6.25 factor according to AOAC (1960).

Soil analysis: The soil used in pot experiments and field trials were analyzed according to Page *et al.* (1982). The main physical and chemical properties of soils used in this study are presented in Table 1.

Inoculant preparation: Vermiculite supplemented with 10% peat was used as a powder carrier (Saleh *et al.*, 2001), packed in polyethylene bags (300 g carrier per bag), sealed and sterilized by gamma irradiation (2.5×10^6 rads). Rhizobial strains were grown in YEM medium (Vincent, 1970) and cultures of (1×10^9 CFU ml^{-1}) were injected into the carrier to satisfy 60% of water holding capacity. At sowing, soybean seeds were coated with rhizobial inoculants at a rate of 300 g of inoculant/40 kg seeds. Arabic gum solution (16%) was used as adhesive agent for seed coating (Saleh *et al.*, 2001).

Table 1: Physical and chemical properties of the soil used in greenhouse experiment and field trials

Property	Greenhouse exp.	Field trials
Particle size distribution		
Sand (%)	90.00	44.18
Silt (%)	3.13	36.2
Clay (%)	6.87	19.62
Texture grade	Sandy	Sandy loam
$CaCO_3$ (%)	1.62	10.85
Saturation percent S.P (%)	21.9	24.5
pH	7.42	8.5
E.C. (dS m^{-1} at 25 °C)	0.31	1.05
Soluble cations (meq L^{-1})		
Ca^{2+}	0.48	2.28
Mg^{2+}	0.28	1.78
Na^+	1.62	3.75
K^+	0.58	2.35
Soluble anions (meq L^{-1})		
CO_3^{2-}	0.00	0.00
HCO_3^-	0.81	3.31
Cl^-	0.61	3.45
SO_4^{2-}	1.54	3.4
Total N (%)	0.02	0.018
Total soluble-N (ppm)	19.87	16.3
Available-P (ppm)	6.73	5.8
Available-K (ppm)	168.3	132
Organic matter (%)	0.24	0.28
DTPA extractable (ppm)		
Fe	0.98	0.69
Mn	0.32	0.58
Zn	0.5	0.46
Cu	0.26	0.07

Statistical analysis: Data was analyzed for variance using the MSTATC analysis software (Snedecor and Cochran, 1980).

RESULTS

Evaluation of the symbiotic effectiveness under greenhouse conditions: The effect of inoculation witht wenty local rhizobial strains related to *Agrobcaterium* (*Rhizobium*), *Ensifer* (*Sinorhizobium*) and *Bradyrhizobium* genera compared with reference strains (*B. japonicum* 110 and *S. fredii* HH303) on nodulation, nitrogen fixation, growth parameters and nitrogen uptake of soybean plants is shown in Table 2 and 3. All local rhizobia successfully nodulated soybean cultivar (*Giza* 22) and resulted in different nodulation patterns compared with tested reference strains (Table 2). *Agrobacterium* strains gave nodules dry weight ranged from 315-422 mg plant^{-1} and recorded N_2-ase activity of root nodules with a range of 16.9-59.5 µmol C_2H_4/g dry nodules/h (Table 2). However, *Bradyrhizobium* or *Sinorhizobium* strains gave nodules dry weight ranged from 302-468 mg plant^{-1} and recorded N_2-ase activity of root nodules with a range of 26.7-65.8 µmol C_2H_4/g dry nodules/h. On the other hand, the un-inoculated controls did not form any nodules. Shoot and root dry weight, as an indirect measure of the nitrogen fixation benefit, varied considerably among all tested strains (Table 3). Roots dry weight of soybean plants

Table 2: Effect of different rhizobial strains on nodulation status and nitrogenase activity of soybean root nodules under greenhouse conditions

Treatments	Homology to reference strains¥	Nodulation status*		N_2-ase activity**
		No. of nodules/plant	Dry wt. of nodules (mg $plant^{-1}$)	µmole C_2H_4/g dry nodules/h
NGB-SR 1	*Agrobacterium*	116^{cdef}	371_{ab}	34.1±8.210
NGB-SR 2	*Sinorhizobium*	133^{bcdef}	409^{ab}	52.6±7.450
NGB-SR 3	*Sinorhizobium*	153^{bcde}	468^{a}	60.8±13.91
NGB-SR 4	*Bradyrhizobium*	123^{cdef}	413^{ab}	65.8±3.030
NGB-SR 5	*Bradyrhizobium*	185^{b}	426^{ab}	52.6±7.450
NGB-SR 6	*Agrobacterium*	124^{cdef}	422^{ab}	41.2±9.190
NGB-SR 7	*Agrobacterium*	124^{cdef}	406^{ab}	59.5±14.22
NGB-SR 8	*Sinorhizobium*	229^{a}	408^{ab}	53.5±16.55
NGB-SR 9	*Sinorhizobium*	165^{bc}	432^{ab}	49.1±11.47
NGB-SR 10	*Agrobacterium*	106^{def}	315^{b}	32.6±4.820
NGB-SR 11	*Bradyrhizobium*	81^{f}	302^{b}	26.7±8.250
NGB-SR 12	*Agrobacterium*	82^{f}	345^{b}	27.9±10.03
NGB-SR 13	*Agrobacterium*	84^{f}	370^{ab}	17.8±4.050
NGB-SR 14	*Bradyrhizobium*	128^{cdef}	430^{ab}	58.7±15.80
NGB-SR 15	*Bradyrhizobium*	98^{ef}	402^{ab}	40.1±13.21
NGB-SR 16	*Agrobacterium*	110^{cdef}	392^{ab}	25.5±8.410
NGB-SR 17	*Agrobacterium*	93^{f}	392^{ab}	28.9±6.350
NGB-SR 18	*Agrobacterium*	104^{def}	370^{ab}	16.9±4.350
NGB-SR 19	*Agrobacterium*	120^{cdef}	378^{ab}	26.4±4.980
NGB-SR 20	*Agrobacterium*	108^{def}	378^{ab}	39.1±11.47
B. japonicum USDA110		158^{bcd}	422^{ab}	42.5±8.340
S. fredii USDA HH303		126^{cdef}	403^{ab}	49.1±11.47
Un-inoculated control 1 (48 kg N ha^{-1})		0^{g}	0^{c}	0
Un-inoculated control 2 (180 kg N ha^{-1})		0^{g}	0^{c}	0

¥Different rhizobial homology according to 16S rDNA sequencing (Youseif *et al.*, 2014), *Means followed by the same letter are not significantly different at 5% level, **Means followed by the Standard Deviation (SD)

Table 3: Effect of different rhizobial strains on plant growth and shoot nitrogen content of soybean under greenhouse conditions*

Treatments	Dry wt. of root (g $plant^{-1}$)	Dry wt. of shoot (g $plant^{-1}$)	Shoot N-content (mg N $plant^{-1}$)
NGB-SR 1	1.23^{de}	3.59^{bcde}	57.60^{gh}
NGB-SR 2	1.47^{bcd}	4.11^{bc}	76.00^{bcde}
NGB-SR 3	1.73^{abc}	4.29^{ab}	81.53^{ab}
NGB-SR 4	1.72^{abc}	4.15^{bc}	82.30^{ab}
NGB-SR 5	1.43^{bcd}	3.92^{bcd}	76.23^{bcde}
NGB-SR 6	1.33^{cde}	3.45^{cde}	58.37^{gh}
NGB-SR 7	1.83^{ab}	4.07^{bc}	77.47^{abc}
NGB-SR 8	1.33^{cde}	3.89^{bcd}	68.10^{def}
NGB-SR 9	1.33^{cde}	3.84^{bcd}	70.47^{cdef}
NGB-SR 10	1.10^{de}	3.42^{cde}	57.50^{gh}
NGB-SR 11	1.00^{e}	3.05^{efg}	51.20^{hi}
NGB-SR 12	1.10^{de}	3.47^{cde}	53.73^{ghi}
NGB-SR 13	0.97^{e}	2.62^{fg}	41.73^{jk}
NGB-SR 14	1.50^{bcd}	4.26^{ab}	76.63^{bcd}
NGB-SR 15	1.13^{de}	3.42^{cde}	54.13^{ghi}
NGB-SR 16	1.27^{de}	3.54^{bcde}	50.13^{hij}
NGB-SR 17	1.10^{de}	3.66^{bcde}	52.40^{hi}

Table 3: Continue

Treatments	Dry wt. of root (g $plant^{-1}$)	Dry wt. of shoot (g $plant^{-1}$)	Shoot N-content (mg N $plant^{-1}$)
NGB-SR 18	1.27^{de}	3.64^{bcde}	58.00^{gh}
NGB-SR 19	1.27^{de}	3.19^{defg}	47.57^{ij}
NGB-SR 20	1.30^{de}	3.39^{cdef}	61.87^{fg}
B. japonicum 110	1.37^{cde}	3.84^{bcd}	67.57^{ef}
S. fredii HH303	1.45^{bcd}	3.92^{bcd}	71.80^{cde}
Un-inoc. control 1 (48 kg N ha^{-1})	0.97^{e}	2.53^{g}	37.37^{k}
Un-inoc. control 2 (180 kg N ha^{-1})	2.01^{a}	5.01^{a}	86.40^{a}

*Means followed by the same letter are not significantly different at 5% level

inoculated with *Agrobacterium* strains ranged from 0.97-1.83 g $plant^{-1}$. While, soybean plants inoculated with *Bradyrhizobium* or *Sinorhizobium* strains gave root dry weight ranged from 1.00-1.73 g $plant^{-1}$, respectively. The shoot dry weight of soybean plants inoculated with *Agrobacterium* strains ranged from 2.62-4.07 g $plant^{-1}$ and most of them were significantly higher than the un-inoculated control + starter N dose (2.53 g $plant^{-1}$), indicating that all plants benefited from forming symbiosis with the rhizobial strains. Whereas, soybean plants inoculated with *Bradyrhizobium* or *Sinorhizobium* strains gave shoot dry weight ranged from 3.05-4.29 g $plant^{-1}$, respectively. The maximum nitrogen uptake was exhibited in soybean plants inoculated with *Bradyrhizobium* spp. NGB-SR4, *Sinorhizobium* spp. NGB-SR3 and *Agrobacterium* spp. NGB-SR7 strains by 82.3, 81.5 and 77.5 mg N $plant^{-1}$, respectively (Table 3) with no significant differences compared with the full N-fertilized control (86.4 mg N $plant^{-1}$).

Evaluation of soybean inoculation under field trials: Based on greenhouse screening experiment, the symbiotic efficiency of the highest efficient local rhizobial strains (*Sinorhizobium* spp. NGB-SR3, *Bradyrhizobium* spp. NGB-SR4, *Agrobacterium* spp. NGB-SR7, *Bradyrhizobium* spp. NGB-SR14) were evaluated under sandy loam field conditions at Nubaria region, Al-Behira governorate during the summer-growing season of 2009 (Table 4-6). The results showed that, all tested strains were able to nodulate field-grown soybean (Table 4). *Agrobacterium* spp. NGB-SR7 and *Bradyrhizobium* spp. NGB-SR4 strains showed the highest nodules dry weight by 361 and 354 mg $plant^{-1}$, respectively. While, reference strains *B. japonicum* USDA 110 and *S. fredii* HH303 gave 265 and 295 mg $plant^{-1}$, respectively. On the other hand, the un-inoculated starter N-fertilized (48 kg N ha^{-1}) and full N-fertilized (180 kg N ha^{-1}) controls; exhibited dry nodules masses by 31 and 15 mg $plant^{-1}$, respectively. The growth parameters and N-shoot content of the inoculated soybean plants were clearly affected by different rhizobial inoculants (Table 4). The highest roots, shoots dry weight and N-shoot uptake were expressed in soybean plants inoculated by *Bradyrhizobium* spp. NGB-SR4 and *Agrobacterium* spp. NGB-SR7 strains and with no significant differences as compared to the full N-fertilized un-inoculated control. At harvest, this general phenomenon was evident in respect to different yield components of soybean crop including plant height, number of pods/plant and seed index (Table 5). Seed and straw yields, the uppermost important crop parameters, were obviously influenced and appeared variable depending upon the inoculant strains. Soybean plants inoculated by *Bradyrhizobium* spp. NGB-SR4 and *Agrobacterium* spp. NGB-SR7 strains resulted in the highest seed yield by 3.64 and 3.55 ton ha^{-1}, respectively with other tested inoculants (Table 5). Whereas, the full

Table 4: Effect of different rhizobial inoculants on nodulation status, plant growth and total N-content of field-grown soybean, 60 days of cultivation*

Treatment	Nodules/plant		Dry wt. (g plant^{-1})		Shoot N content (mg plant^{-1})
	No. of nodules	Dry wt. (mg plant^{-1})	Root	Shoot	
Sinorhizobium NGB-SR 3	70^{a}	308^{ab}	4.50^{b}	26.4^{bc}	574^{b}
Bradyrhizobium NGB-SR 4	61^{a}	354^{a}	5.19^{a}	29.3^{ab}	628^{ab}
Agrobacterium NGB-SR 7	66^{a}	361^{a}	5.22^{a}	30.8^{a}	618^{ab}
Bradyrhizobium NGB-SR 14	71^{a}	268^{b}	4.33^{b}	28.7^{ab}	595^{b}
S. fredii USDA HH303	70^{a}	295^{ab}	4.57^{b}	25.3^{bcd}	567^{b}
B. japonicum USDA110	59^{a}	265^{b}	3.60^{c}	22.8^{cd}	483^{c}
Un-inoculated control 1 (48 kg N ha^{-1})	6^{b}	31^{c}	3.34^{c}	21.5^{d}	443^{c}
Un-inoculated control 2 (180 kg N ha^{-1})	3^{b}	15^{c}	5.62^{a}	32.2^{a}	684^{a}

*Means followed by the same letter are not significantly different at 5% level

Table 5: Effect of different rhizobial inoculants on yield and yield components of field-grown soybean*

Treatment	Plant height (cm)	No. of branches plant^{-1}	No. of pods plant^{-1}	Seed index (g)	Seed yield (ton ha^{-1})	Straw yield (ton ha^{-1})
Sinorhizobium NGB-SR 3	74.0^{bc}	2.88^{a}	70.4^{ab}	16.5^{abc}	3.36^{cd}	4.63^{bc}
Bradyrhizobium NGB-SR 4	80.1^{ab}	2.85^{a}	77.0^{a}	17.0^{ab}	3.64^{ab}	5.17^{a}
Agrobacterium NGB-SR 7	80.5^{ab}	3.00^{a}	69.5^{ab}	16.7^{ab}	3.55^{bc}	5.04^{ab}
B*radyrhizobium* NGB-SR 14	72.4^{bc}	2.73^{ab}	60.9^{bc}	16.3^{bc}	3.07^{ef}	4.30^{cd}
S. fredii USDA HH303	72.5^{bc}	2.70^{ab}	67.2^{ab}	16.4^{abc}	3.27^{de}	4.58^{bc}
B. japonicum USDA110	61.9^{cd}	2.25^{b}	49.4^{c}	15.3^{c}	2.90^{f}	3.93^{de}
Un-inoculated Control 1 (48 kg N ha^{-1})	55.4^{d}	2.15^{b}	48.9^{c}	15.2^{c}	2.63^{g}	3.57^{e}
Un-inoculated Control 2 (180 kg N ha^{-1})	87.1^{a}	3.25^{a}	77.1^{a}	17.7^{a}	3.86^{a}	5.50^{a}

*Means followed by the same letter are not significantly different at 5% level

Table 6: Effect of different rhizobial inoculants on N- yield and crud protein percentage of field-grown soybean*

Treatment	N-yield (kg N ha^{-1})		Crude protein (%)	
	Straw	Seed	Straw	Seed
Sinorhizobium NGB-SR 3	73.2^{bc}	187^{bc}	9.9^{ab}	34.9^{ab}
Bradyrhizobium NGB-SR 4	87.0^{ab}	207^{ab}	10.5^{a}	35.5^{ab}
Agrobacterium NGB-SR 7	84.2^{ab}	201^{ab}	10.4^{a}	35.3^{ab}
Bradyrhizobium NGB-SR 14	66.7^{cd}	169^{cd}	9.7^{ab}	34.4^{b}
S. fredii USDA HH303	72.8^{bc}	181^{c}	9.9^{ab}	34.5^{b}
B. japonicum USDA110	58.8^{de}	154^{de}	9.4^{b}	33.9^{c}
Un-inoculated control 1 (48 kg N ha^{-1})	52.4^{e}	138^{e}	9.1^{b}	32.7^{c}
Un-inoculated control 2 (180 kg N ha^{-1})	93.7^{a}	220^{a}	10.6^{a}	35.7^{a}

*Means followed by the same letter are not significantly different at 5% level

N-fertilized control (180 kg N ha^{-1}) gave a seed yield of 3.86 ton ha^{-1}. Similarly, the maximum seed N-yield was expressed in soybean plants inoculated with *Bradyrhizobium* spp. NGB-SR4 and *Agrobacterium* spp. NGB-SR7 by 207 and 201 kg N ha^{-1}, respectively (Table 6) with no significant differences as compared to the full N-fertilized un-inoculated control (220 kg N ha^{-1}). Regarding seed crude protein, soybean plants inoculated by *Bradyrhizobium* spp. NGB-SR4 and

Agrobacterium spp. NGB-SR7 strains showed the highest seed crude protein by 35.5 and 35.3%, respectively (Table 6) with no significant differences, compared to the full N-fertilized plants (35.7%).

DISCUSSION

The positive response of soybean yield to rhizobial inoculants as well as the importance of rhizobial symbiosis for the sustainability of soybean cultivation received considerable coverage in the scientific literatures (Appunu *et al.*, 2008; Albareda *et al.*, 2009; Pauferro *et al.*, 2010). The primary objectives of this study were (1) To confirm the nodulating machinery stability of *Agrobacterium* strains to can effectively nodulate their original host, soybean and (2) To evaluate the symbiotic effectiveness of *Agrobacterium* strains compared to *Bradyrhizobium* and *Sinorhizobium* strains under greenhouse conditions and field trials. Strains under investigation were isolated from soybean root nodules and identified as *Agrobacterium* (*Rhizobium*), *Sinorhizobium* or *Bradyrhizobium* spp. based on nodulation phenotypes and sequences of full length of 16S rDNA (Youseif *et al.*, 2014). *Agrobacterium* strains have been shown to possess *nif*H and *nod*A genes similar to those in other fast growing soybean symbionts (Youseif *et al.*, 2014). Under greenhouse conditions, there were significant increases in shoot dry weight and shoot N-content of soybean-inoculated plants over the starter N (48 kg N ha^{-1}) un-inoculated control (Table 3). Soybean plants inoculated by *Agrobacterium* strains showed 4-61 and 12-107% increases in shoot dry weight and shoot N-content, respectively over starter N un-inoculated plants. However, soybean plants inoculated by *Sinorhizobium* or *Bradyrhizobium* strains showed 21-70% and 37-120% increases in shoot dry weight and shoot N-content, respectively over starter N un-inoculated plants. Four promising rhizobial strains (*Sinorhizobium* spp. NGB-SR3, *Bradyrhizobium* spp. NGB-SR4, *Agrobacterium* spp. NGB-SR7 and *Bradyrhizobium* spp. NGB-SR14) were selected for further evaluation as soybean inoculants under sand loamy field conditions. During flowering stage, there were significant increases in shoot dry weight and shoot N-content of soybean plants inoculated with *Agrobacterium* spp. NGB-SR7 by 43 and 40%, respectively over the starter N un-inoculated plants (Table 4). On the other hand, soybean plants inoculated by *Sinorhizobium* or *Bradyrhizobium* strains resulted in 6-36 and 9-42% increases in shoot dry weight and shoot N-content, respectively over starter N un-inoculated plants. At harvest, *Bradyrhizobium* spp. NGB-SR4 and *Agrobacterium* spp. NGB-SR7 strains resulted in the highest significant increases in soybean seed yield and seed N-yield by 35-38 and 46-50%, respectively over the starter N un-inoculated plants (Table 5 and 6). The positive nodulation of soybean by micro-symbionts was previously confirmed, including *S. fredii* (Albareda *et al.*, 2009); *B. japonicum* (Appunu *et al.*, 2008; Meghvansi *et al.*, 2010) and *B. yuanmingense* (Appunu *et al.*, 2009). However, little information has been published regarding to nodulation of soybean by *Agrobacterium* spp. (Chen *et al.*, 2000). The first confirmed legume-nodulating symbiont from the *Rhizobium* (*Agrobacterium*) clade was strain IRBG74 (Cummings *et al.*, 2009). IRBG74 effectively nodulated *S. cannabina* and seven other *Sesbania* spp. that nodulated with *Ensifer* (*Sinorhizobium*)/*Rhizobium* strains (Cummings *et al.*, 2009). To our knowledge, this is the first confirmed naturally occurring agrobacterial symbiont of soybean to be evaluated under field conditions. The ability of *Agrobacterium* spp. to nodulate legume roots may be attributed to its own a transferred *Sym* plasmid, thus they acquired the ability to form root nodules and fix nitrogen symbiotically (Sawada *et al.*, 2003; Cummings *et al.*, 2009). Using *gus* gene labeling, Mhamdi *et al.* (2005) gave the evidence that *Agrobacterium* isolates were indeed recovered from

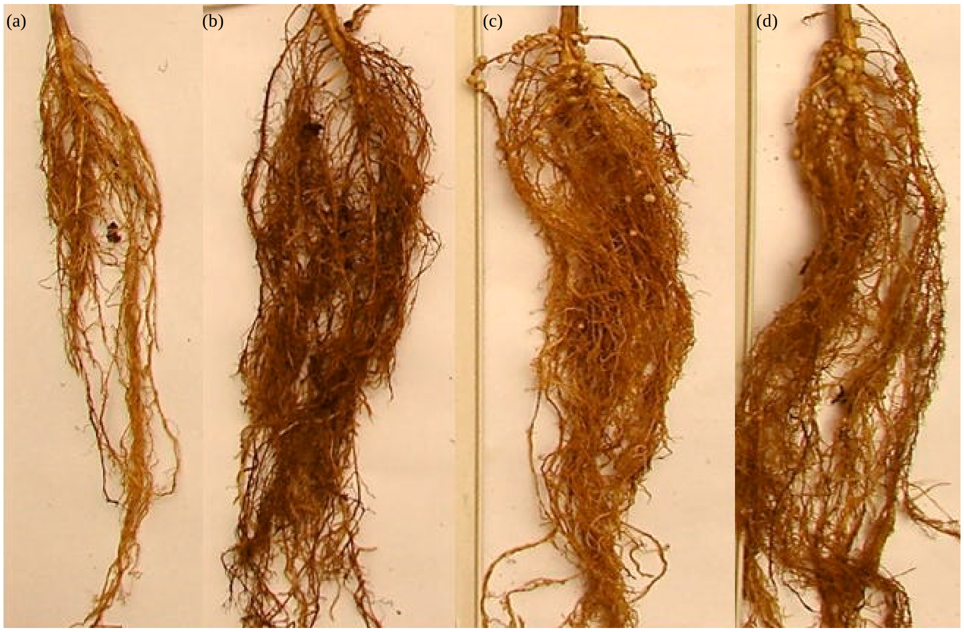

Fig. 1(a-d): Nodulated roots of soybean inoculated by *Agrobacterium* NGB-SR7 strain compared to *S. fredii* HH303 inoculated treatment and the un-inoculated chemical N-fertilized controls (48 and 180 kg N ha^{-1}) under greenhouse conditions, (a) C+starter N dose (48 kg N ha^{-1}), (b) C+full N dose (180 kg N ha^{-1}), (c) *S. Fredii* USDA HH303 and (d) *Agrobacterium* strain NGB-SR7

the inside nodules of inoculated common bean. However, many *Agrobacterium* strains isolated from root nodules failed to nodulate their original hosts (Wang *et al*., 2006). The symbiotic instability of *Agrobacterium* strains has been reported (De Lajudie *et al*., 1999). A transient acquisition of a symbiotic plasmid was supposed to be an adequate explanation (Mrabet *et al*., 2006) and this makes them a poor choice for legume inoculation (Shamseldeen *et al*., 2005). On the contrary to previous reports, the present study revealed the symbiotic efficiency and nodulation stability of *Agrobacterium* NGB-SR 7 strain to nodulate soybean roots and fix N_2 under greenhouse conditions (Fig. 1) and field experiments to the same degree as conventional rhizobial genera. These results agreed with those obtained by Chen *et al*. (2002) who reported the ability of two isolates of *A. tumefaciens* in Paraguay to re-nodulate soybean roots in sterilized modified Leonard jars. Our study confirmed the symbiotic effectiveness stability of *Agrobacterium* spp. and clearly showed the possibility of employing these strains to elaborate commercial soybean inoculants under sand loamy field conditions to the same degree as 'conventional' rhizobia.

The dominant effect of rhizobial inoculants over the full N-fertilized treatment was previously reported (Tahir *et al*., 2009). Field experiments conducted in two types of soils with alkaline and moderately acid pH have demonstrated that,uninoculated full N-fertilized (200 kg N ha^{-1}) control does not improve the soybean yields in comparison with the best rhizobia inoculants (Albareda *et al*., 2009). In the same way, the present study showed that, there is no significant differences in soybean yield between inoculated treatments (*Bradyrhizobium* NGB-SR4 and

Agrobacterium NGB-SR7) and the full N dose (180 kg N ha^{-1}) un-inoculated control under sand loamy soil conditions, which makes the rhizobial inoculants are successful alternatives to chemical N-fertilizers.

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