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In Ethiopia, inoculation of soybean with rhizobial inoculants is not common practice, but could provide an option to increase grain yields in the low nitrogen (N) acidic soils. In these acid soils, the selection of acid tolerant rhizobia is one strategy that may increase the performance of soybean. In this study, rhizobial strains isolated from Ethiopian soils were evaluated for their acid tolerance and symbiotic N fixation efficiency with soybean, in controlled environments. Following this, four isolated rhizobial strains were evaluated in six field experiments in major soybean growing areas of Ethiopia. Inoculation with the commercial strain or with one of two locally-sourced isolates, that were developed as inoculants, improved soybean yield. The yield increase due to inoculation with the commercial strain was consistent and greater than other treatments, while the increase due to the two locally-sourced strains was comparable to, or greater than, application of 46 kg N/ha in soils, where the resident rhizobial population was ≤ 1.4×10^3 cfu/g soil. For soils with high background rhizobial populations, there was no response to inoculation. In one of the experimental sites (Bako), the percentage of N fixed (%Ndfa) was 55 for the commercial strain and 35 for a local strain, ES3. This study demonstrated that field validation is a necessary step in the selection of acid tolerant strains of rhizobia to increase soybean production for Ethiopia.

Key words: Soybean, rhizobia, acid tolerance, nodulation, symbiotic effectiveness

Introduction

Soybean, *Glycine max* (L.). Merr. has the largest worldwide production of any crop legume (Sinclair et al. 2014) and contributes greater symbiotic N fixation than any other crop legume (Herridge et al. 2008). Soybean production is increasing in sub-Saharan Africa, driven by its high value for food and feed, and its ability to improve soil fertility (Sinclair et al., 2014). In Ethiopia, soybean is cultivated mainly in the southern and western regions, and production has increased from 1,620 t in 2002 to 61,000 t in 2014 (CSA 2012, Bekabil 2015). Although the average yield of soybean has recently increased in Ethiopia to 2 t/ha, it is still below the world average of 2.35 t/ha (Abate et al. 2012) which may be related to soil constraints and management of the crop.

The cultivation of soybean in sub-Saharan Africa is often affected by low soil fertility, soil acidity, soil salinity and organic matter depletion (Bromfield & Ayanaba 1980, Abaidoo et al. 2007, Amissah-Arthur & Jagtap 2007, Thuita et al. 2012). In Ethiopia, about 40% of the cultivated land is sufficiently acidic that it reduces crop production (Abdenna et al. 2007). Production of soybean in acid soils can be constrained by toxic concentrations of H^+, Al and Mn, and deficiencies in Ca, Co, Cu, Mg, P, Fe and Mo (O'Hara 2001, Indrasumunar et al. 2011). Although soybean yields can be increased in acid soils by application of N fertiliser, this is uneconomical when compared with the costs of inoculation with rhizobia (Ronner et al. 2016), and is unsuitable for smallholder farmers.

Symbiotic N fixation in acidic soils is itself constrained by several acidity-related problems affecting the symbiosis, viz. the persistence of rhizobia, nodule formation, and the function of the symbiosis (Date & Halliday 1979, Howieson & Ewing 1986, Peoples et al. 2009). It was previously demonstrated that a commercial strain of *Bradyrhizobium japonicum* (strain 532c) increased soybean yield in Ethiopia, Kenya and Nigeria (Jefwa et al. 2014), although the effectiveness of the strain was inconsistent across Ethiopia, and performed poorly in Bako and Assossa regions (Asrat, personal communication). Hence, despite the potential to improve yield and soil fertility, biological N fixation by soybean in areas with acid soil is greatly constrained. The inconsistent results of inoculation with strain 532c (Aserse et al. 2012), may therefore be related to soil acidity. Improving the symbiosis through amelioration of soil acidity by the addition of lime is expensive (Foy 1988), and a more cost-effective approach has been the selection of acid-tolerant crops (Taylor 1991) combined with selection of suitable acid-tolerant microorganisms (White 1966, Howieson & Ewing 1986, Howieson et al. 1988).

In this study, soybean-nodulating rhizobia were isolated from Ethiopian soils and then tested for their acid tolerance. These strains were then evaluated for their symbiotic effectiveness in controlled conditions. Four selected isolated strains were then used to produce inoculants, and the ability of the inoculants to increase grain yield was investigated in six field experiments in soybean- growing regions of Ethiopia. We hypothesised that the strains isolated would be adapted to acid soils, and that those found to be symbiotically efficient may provide effective inoculants for use by farmers.

Material and Methods

Soil sampling

Soils were collected from 154 sites in the major soybean production areas of Ethiopia,

including Jimma and Illubabur zones of the Oromia regional state, Southern Ethiopia (from Hawassa to Amaro), Bako area of Oromia, and Assossa area in the Benishangul Gumuz regional state. Soil samples (0-20 cm) were taken from four points in each field and mixed, and used for isolation of rhizobia after storing at 4 ° C for 20 d.

Isolation of rhizobia

Soybean cv. Clark, a widely grown cultivar preferred by Ethiopian farmers for its short maturity, was grown (3 seeds/pot) in 1 kg pots containing field soil in a greenhouse at Holleta Agricultural Research Centre in January and February 2014. After 8 weeks of growth, nodules were collected from the roots and rhizobia were isolated from nodules. The strains isolated were maintained on yeast mannitol agar (YMA) slants, as described by Somasegaran and Hoben (1985).

Assessment of acid tolerance

Cultures of strains from Ethiopian soils and the Australian commercial soybean strain (CB1809) were grown in yeast extract mannitol broth (YMB) medium (Somasegaran & Hoben 1985). After 7 d of growth, 100 µl of culture were streaked on duplicate plates of pH 4.5 medium containing high Mn and Al, and low P and Ca (Gemell et al. 1993). The inoculated plates were incubated at 28 ^{o}C for 12 d, and the presence or absence of colonies was monitored visually to assess growth on the acidic media.

Assessment of nodulation and symbiotic effectiveness

River sand was washed with sulphuric acid (38%; 5 L/20 kg sand) to reduce organic matter that could be an N source for the plants (Lupwayi & Haque 1994). The sand was then rinsed with tap water until its pH was neutral. The washed sand was autoclaved and placed in 1 L pots that had been washed, sprayed with 95% alcohol and dried. Before sowing, the soybean seeds were surface sterilized with 95% ethanol for 10 s and then with 2.5% sodium hypochlorite for 3 min, followed by rinsing with seven changes of sterile distilled water. Each pot was sown with five seeds and after emergence, the seedlings were thinned to three per pot before inoculation. Each treatment was applied to three replicate pots.

Reference and isolated strains that grew on acid-stressed media were streaked on standard yeast mannitol agar (YMA) plates and incubated at 28 ^{o}C for 7 d. The reference strains used were 532c, a commercial strain used in Ethiopia (Legume Technology Ltd, Notts, UK) and CB1809 isolated from EasyRhiz® freeze dried inoculant (New Edge Microbials Pty Ltd, Albury, Australia). Single colonies were inoculated into YMB and grown on a shaker incubator at 28 ^{o}C, 120 rpm for 5 d. The plants were inoculated 7 days after sowing (DAS) with 1 mL of bacterial suspension (10^{9} cfu/mL) per plant. The replicates were arranged in a completely randomised design in a greenhouse at Holleta Agricultural Research Centre where the average day and night temperatures were 26 °C and 15 °C, respectively, with a 12 h photoperiod.

The plants were supplied with N-free nutrient solution twice a week (Broughton & Dilworth 1970). In addition, an N-supplied control treatment received 20 mL of ammonium nitrate solution (5 mM) per pot, once per week. Plants were grown in April and May 2014, and harvested 60 DAS.

Field experiments

Six soybean inoculation field experiments were conducted at sites with nitisol soils (FAO-Unesco 1974) at Jimma (three sites, one at the Jimma Agricultural Research Centre and two on farms in Ababiya and Seifu farms), Bako on a farm and Assossa (two sites, one at the Agricultural Research Centre and the other on a farm) from June to November 2014). Details of soils at these sites are provided in Table 1.

Inoculant preparation

Based on symbiotic effectiveness in controlled conditions, four Ethiopian strains (designated as ES1 to ES4 for the purpose of this report) with the greatest measured symbiotic effectiveness, plus the commercial strain 532c were prepared as inoculants for use in the field. The carrier was filter mud, a by-product of sugar cane processing, that has previously been used as a carrier (Philpotts 1976). The filter mud was ground and passed through a 200 mesh sieve and neutralised by the addition of $CaCO_3$, sealed in polyethylene bags (125 g per bag) and autoclaved. The strains were grown in 25 ml YMB broth in 50 ml flasks. After 5 d of growth, the broths were transferred to 1 L YMB broth and grown for 5 days to achieve 10^9 cfu/ml, before being used to inoculate the carrier. Rhizobial numbers in the final inoculant were determined by plate count.

Experimental design

Each field experiment consisted of eight treatments with four replicates arranged in individually randomised complete block designs. Treatments included five rhizobial treatments (four isolated strains and a commercial strain, strain 532c), N-supplied treatments with either 46 or 23 kg N/ha and one control treatment receiving neither rhizobia nor N fertiliser. The Bako experiment had an additional treatment of non-nodulating haricot bean as a reference treatment sown in one plot for each of the four replicate blocks. Phosphorus was applied at a rate of 20 kg P/ha as triple superphosphate, to ensure that P availability did not limit the expression of N fixation. Both P and N fertilisers, where applicable, were applied in rows to a depth of 5 cm and incorporated into the soil in a separate operation before seeding, according to farmer practice. Seeds were inoculated with the carrier material under shade immediately before sowing. The carriers containing the test strains (10 g containing 10^9 cfu/g) were mixed with seeds that were moistened with sugar solution (2 g in 20 ml water, as a sticker) to enhance inoculant contact with the seed. Treatments were applied taking care to avoid cross contamination of the test strains. Seeds were hand sown in rows 60 cm apart and seeds in rows were 5 cm apart from each other; rows were 4 m long and there were 4 rows per plot. Blocks and plots were separated by 1 m wide buffers, to assist in maintaining hygiene and to limit the chance of contamination from one plot to another.

Soil sample collection and analysis

The soils collected from the field were air-dried, ground, passed through a 2-mm sieve. Samples were analysed for texture using a hydrometer (Gee & Bauder 1986), pH (1:2.5, H_2O), soil phosphorus (Bray II), cation exchange capacity (Chapman 1965), organic carbon (Walkley & Black 1934), and exchangeable acidity (Van Reeuwijk 2002) at Holleta Agricultural Research Soil Laboratory, using the protocols outlined by Sertsu and Bekele (2000). The most probable number of soybean-nodulating rhizobia per gram of soil was

166 determined for each sample using the methods as outlined by Somasegaran and Hoben
167 (1985).

168 ***Data collection***

169 In the greenhouse experiment, plants were harvested at 60 DAS. The shoots were dried for
170 48 h at 70 ^{o}C, weighed and the total shoot N was analysed in Holleta Agricultural Research
171 Laboratory using the Kjeldahl method (Bremner & Mulvaney 1982). Nodule numbers were
172 recorded for each plant. For the field experiments, nodules were counted on the roots of five
173 plants removed at 80 cm intervals along the outer rows at 60 DAS. At the same time, shoots
174 were harvested and oven dried at 70 $^{\circ}$C for 48 h and dry weight was recorded. Grain yield
175 and total above-ground biomass (AGB, dried for 48 h at 70 $^{\circ}$C) including both shoots and
176 grain were recorded during harvest.

177 ***Assessment of N fixation***

178 The ^{15}N natural abundance technique (Unkovich et al. 2008) was used to estimate the N
179 fixation in the treatments at the Bako experimental site. Dried shoot samples were finely
180 ground and analysed for total N concentration (µg N/g) and ^{15}N composition using a PDZ
181 Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass
182 spectrometer (Sercon Ltd., Cheshire, UK). The percentage of soybean N derived from N
183 fixation ($\%N_{dfa}$) was calculated by comparing the ^{15}N in soybean plants (δ^{15}N legume) with
184 the δ^{15}N of the non-nodulating haricot bean reference (δ^{15}N haricot) which was assumed to
185 reflect the δ^{15}N of the plant-available soil N. A δ^{15}N value for soybean grown under N-free
186 media entirely reliant upon N fixation for its N (B value; -1.83‰) was used (Unkovich et al.
187 2008). The following formula was used for calculating $\%N_{dfa}$:

188 $$\%N_{dfa} = 100 (\delta^{15}N \text{ haricot} - \delta^{15}N \text{ legume}) / (\delta^{15}N \text{ haricot} - B)$$

189 The shoot N and total shoot N derived from fixation were calculated using the following
190 formulae:

191 $$\text{Legume shoot N} = (\%N / 100) \times (\text{legume shoot weight}) \text{ and}$$

192 $$\text{Amount shoot N fixed} = (\%N_{dfa} / 100) \times (\text{Legume shoot N})$$

193 ***Statistical analysis***

194 The data from the greenhouse experiment were subjected to analysis of variance (ANOVA)
195 using the General Linear Model procedure of GenStat (VSN International 2014). Means of
196 all the treatments were calculated and the differences were tested and considered significant
197 when $p < 0.05$. The means of the treatments were differentiated using the LSD. The
198 symbiotic effectiveness percentage was calculated by the method described in Purcino et al.
199 (2000) as:

200 Effectiveness (%) = (Shoot Dry Weight of inoculated plant / Shoot Dry Weight of N-fertilised
201 treatment) × 100.

202 Based on this effectiveness scale, isolates were considered highly effective (HE) when

203 percentage of effectiveness > 80%, effective, (E) between 50% and 80%, and of low
204 effectiveness (LE) between 50% and 35%. Isolates were considered ineffective when the
205 percentage effectiveness was less than 35.

206 Data collected from each field experiment were combined and subjected to Bartlett's
207 test for homogeneity of variance. Data from each site were also independently subjected to
208 ANOVA and LSD was used to separate the treatments, using GenStat software (VSN
209 International 2014).

210 **Results**

211 ***Soil collection, rhizobial isolation and acid tolerance screening***

212 From the initial 154 soils collected from the major soybean growing regions, 80 strains were
213 isolated from nodules (Fig.1 and Table 2). Of the 80 rhizobial strains, 50 grew on acid-
214 stressed media at pH 4.5 and these strains were further evaluated for symbiotic
215 effectiveness in the greenhouse.

216 ***Evaluation of symbiotic effectiveness under controlled conditions***

217 The evaluation of symbiotic N fixation of soybean in the greenhouse indicated variation in N
218 fixation among the 50 isolates and the two reference strains (Fig. 2). Shoot dry weight
219 (SDW) among treatments varied from 4.13 g/plant and 3.67 g/plant when inoculated with the
220 commercial strains 532c and CB1809, to only 1.37 g/plant in the un-inoculated treatment.
221 The N-supplied treatment weighed 3.67 g/plant and four strains were identified that had
222 higher SDW than the remaining local strains; these are listed as ES1, ES2, ES3 and ES4
223 (Fig.2). Results for selected strains that were the most effective are shown in Table 3. The
224 shoot N content of the plants ranged from 0.8% (negative control) to 3.3% (strain 532c),
225 while the shoot dry weight ranged from 1.4 g/plant (negative control) to 4.1 g/plant (strain
226 532c). The N-fertilised plants had a shoot N content of 2.9%. Seedlings inoculated with the
227 four isolates ES1 to ES4 (Table 2) and reference strains had significantly greater shoot N
228 concentrations and shoot dry weights than the negative control and other isolates. No
229 relationship was observed between the shoot dry matter of the plants and their nodule
230 number ($R^2 = 0.0061$, data not shown). The selected strains were categorised as either
231 effective or highly effective. The four Ethiopian isolates with the greatest symbiotic
232 effectiveness (ES1 to ES4) were selected for field evaluation. Reference strain, 532c, was
233 superior to CB1809 in the above three measures and was used in the field as a reference
234 strain.

235 ***Rainfall at field sites***

236 The Jimma sites had received less precipitation prior to sowing (June; 144 mm) compared
237 with other sites, but that site received more rainfall over the June to November growing
238 season (total 1009 mm) (Fig. 3). The Bako site received more precipitation in the first two
239 months of the growing season (483 mm), but received the least over the rest of the growing
240 season (348 mm). The Assossa sites received the least total precipitation (790 mm),
241 although the amount of rainfall received during the last four months (493 mm) was greater
242 than that at Bako.

Soil analysis

Soil physical, chemical and biological analytical results are shown in Table 1. The soils at the experimental sites were either extremely acidic (Sites 1, 2 and 4) or strongly acidic (Sites 3, 5 and 6) with pH (H_2O) ranging between 4.26 and 4.81. The total soil N and P status at the experimental sites were low.

Soybean-nodulating rhizobia were not detected in the Bako soil, while soils at Ababiya farm and Seifu farm contained low resident soybean-nodulating rhizobial populations (16 and 250 cfu/g soil, respectively) compared with the other sites. The soil at the Assossa Agricultural Research Centre site contained 1.4×10^3 cfu/g soil while the remaining two sites had abundant background rhizobial populations of $>10^6$ cfu/g soil (Table 1).

Field experiments

Combined data analysis was initially conducted for all sites to determine if there were overall trends. A combined analysis of soybean yield for the six sites indicated variance heterogeneity (Bartlett's test for homogeneity of variances, $\chi = 108.4$ with 5 degree of freedom and $p < 0.001$). This indicated the likelihood of high variation among sites due to factors such as soil properties or rainfall (Table 1, Fig. 3). Therefore, general treatment effects could not be compared across sites and results were subsequently analysed independently for each location.

At Ababiya, inoculation with 532c produced the largest number of nodules per plant, followed by ES4 and ES3, respectively, while the other treatments were not significantly different from the negative control (Fig. 4). All the treatments resulted in higher yield than the control (Fig. 5). Strain 532c produced the highest yield followed by ES3 and ES4 that resulted in a similar yield with application of 46 kg/ha N. ES1 and ES2 resulted in a yield similar to that obtained with the application of 23 kg/ha N (Fig. 5).

At the Seifu site, inoculation with 532c resulted in significantly greater nodule numbers than the negative control, followed by ES4, whereas other treatments were not different from the negative control in nodule numbers. Inoculation with 532c also resulted in the highest grain yield, followed by ES4, ES3 and application of 46 kg N/ha. Inoculation with ES1, ES2 and application of 23 kg/ha N gave no significant yield increase over the control.

At Bako, inoculation with 532c and ES3 resulted in greater nodule numbers than the other treatments (Fig. 4). The experiment conducted at Bako had lower grain yields in its negative control compared with the negative control of other sites (13.7% to 150% decrease compared with Assossa Research Centre and Assossa farm (Fig. 5). Yield increased following inoculation with 532c and ES3, but not more than with the application of 46 kg N/ha.

At Bako, the use of a sown reference species allowed ^{15}N natural abundance N fixation measurements to be made on soybean. Total shoot N at harvest ranged from 50 kg/ha (un-inoculated control) to 226 kg/ha (532c) and the three strains (532c, ES2 and ES3) had significantly higher shoot N than the control (Table 4). The percentage of N derived from the atmosphere was greater in plants inoculated with 532c (55%) and ES3 (35%) than for other treatments. Accordingly, the amount of fixed N in the shoots of plants inoculated with 532c and ES3, (126 and 60kg/ha respectively) were significantly greater than the other treatments (Table 4).

At the Jimma Agricultural Research Centre experimental site, only strain 532c, ES3 and application of 46 kg/ha N resulted a higher yield over the negative control, while nodule numbers did not differ, and were universally high in all treatments.

At the Assossa Agricultural Research Centre site, there were no differences observed among treatments for nodule numbers (Fig 4). Treatments other than ES3 had greater grain yield than the negative control. Yield increase resulting from inoculation with ES4 was comparable to those obtained for N applications, while strain 532c significantly improved yield over the control but did not achieve the largest yield at this site. Treatments ES1 and ES2, that did not lead to high yields at other sites, produced a higher yield than the control.

At the Assossa farm site, nodule numbers were universally high (Fig.4) and did not differ among treatments. Yields did not show significant difference among inoculant treatments, however, strain 532c and ES2 increased yield significantly relative to the control.

Overall, the responses of soybean to the application of 23 and 46 kg N/ha, and inoculation with strain 532c increased grain yield, relative to the uninoculated control treatments (Fig. 6). The association between yield and nodule number per plant at the six experimental sites was examined by linear regression, which revealed that in three of the six experiments (Fig 7: a, b, d) there were positive correlations between nodule number and grain yield, while for the other three sites (Fig 7, c, e, f) correlations did not exist.

Discussion

Field response to inoculation

We hypothesized that locally adapted strains that were evaluated for acid tolerance and symbiotic effectiveness would provide greater acid tolerance and prove to be robust inoculants across different soybean-growing regions of Ethiopia. In contrast to our expectations, the commercial strain proved to be a more effective inoculant than locally isolated strains used as inoculants, when assessed at six experimental sites. At two of the experimental sites, inoculation with field isolates from Ethiopian soils (ES3 and ES4) resulted in yields similar to, or greater than, the application of 46 kg N/ha, indicating the value that they could provide to soybean crops grown in Ethiopian soils with low mineral N. In contrast, ES1 and ES2 performed poorly except in the Assossa area, and are unsuited to field application in other areas, potentially due to poor acid tolerance. This study confirmed the importance of field evaluation of isolates, in addition to evaluation in the laboratory, before suitable strains can be identified (Howieson et al. 1988).

Application of 46 kg N/ha improved yield at five of the six sites, indicating the extent of soil N limitation in grain production in Ethiopia (Atnaf et al. 2015). Application of 23 kg N/ha, however, improved yield at only two sites, indicating that the N demand of the soybean crop exceeded this level of N application, which agrees with previous research that demonstrated the high N demand of soybean (Herridge 2002). In Ethiopia, farmers typically use N fertiliser where they have access, rather than inoculating their soybeans. However, fertiliser costs are high for smallholder farmers in developing countries. Soybean requires 120 kg N to produce a tonne of grain (Herridge 2002) and urea was 60 USD per 100 kg in Ethiopia in 2015 (Ayele et al. 2016) so that N sufficient for a 2 t/ha crop would cost at least $144/ha, assuming limited soil mineral N supply and a 100% use efficiency of the fertiliser. In contrast, a commercial inoculant to treat 1 ha of soybean (320 g / 80 kg) costs 13.3 USD

(Jefwa et al. 2014), while local inoculants (500 g) applied to 1 ha cost less than 7.5 USD in Ethiopia. Thus, in using inoculants instead of N fertilisers, there is a cost savings of $130 USD/ha, based on recent data. It is, however, unlikely that Ethiopian farmers would provide these quantities of N fertilizer to their crops, further highlighting the importance of inoculant use. Similarly, on-farm research on soybean responses to inoculation and P application in Northern Nigeria indicated that 95% of the farmers achieved an economic benefit by using soybean inoculant (Ronner et al. 2016).

Plants inoculated with strain 532c and ES3 fixed 105 and 55 kg N/ha at the Bako experimental site, respectively. Strain 532c fixed 55% N, while ES3 fixed 35% of the N in the crop. Previous estimates of $\%N_{dfa}$ for soybean were 68% in experimental sites and 58% in farmers' fields (Herridge et al. 2008). The lower $\%N_{dfa}$ for some of the strains in the current study might be due to low precipitation during pod-filling in 2014, when N fixation is known to be important (Zapata et al. 1987, Bergersen *et al.* 1992). N fixation is known to be limited by factors that affect biomass accumulation of the host plant (Giller 2001), including environmental and agronomic factors, and cultivar (Herridge et al. 2008).

Two types of responses were observed in the relationship between nodule number per plant and grain yield: three sites had strong and positive associations between nodule numbers and grain yield, and three sites had no associations. Inoculation with a commercial strain in Ethiopia previously improved soybean yield and nodulation in some parts of Ethiopia, although the responses were not universal (Jefwa et al. 2014). The relationships observed in our study could be explained by the background population densities of soil rhizobia. At sites with strong correlations between nodule number and yield, the initial soil rhizobial numbers were low and ranged from non-detectable to 250 cells/g soil. Sites with weak or negative correlations between nodule number and yield, i.e., Jimma and the Assossa sites, had high soil rhizobial populations ranging from $1.4x10^3$ to $>10^6$ cells/g soil. The inverse relationship between yield response to inoculation and resident rhizobial population has been observed previously (Thies et al. 1991, Brockwell et al. 1995, Denton et al. 2000).

Screening acid-tolerant rhizobia in the laboratory

The sites chosen for field experiments had soil pH (H_2O) as low as 4.26. Rhizobia were screened on agar media of pH 4.5, since low pH reduces rhizobial survival to a greater extent on plates than in soil of the same pH (Appunu & Dhar 2006). This might be due to the presence of microsites in soils with a higher pH, or the association of the microorganisms with cations, anions or organic molecules in the soil (Howieson et al. 1988, Appunu & Dhar 2006). Growth of the freshly isolated rhizobial strains on acidic agar media ranged from none to profuse. Such differences in tolerance to acidity among strains of the same species have been reported previously for various *Rhizobium* and *Bradyrhizobium* (Howieson et al. 1988, Graham et al. 1994, Appunu & Dhar 2006). Hence, strains that showed profuse growth on low pH agar media, with high concentrations of Al and Mn with low concentrations of Ca and P, coupled with high symbiotic potential, were selected for testing effectiveness in the field. The commercial strain, 532c, was also able to grow on the same acid-stressed media. Among the four strains selected, three demonstrated symbiotic effectiveness under field conditions (ES3 and ES4 at Sites 1 and 2; ES3 at Site 4; and ES1 and ES4 at Site 5), supporting the need for a rapid screening of rhizobia for acid tolerance to assist in selection of strains in the field (Indrasumunar et al. 2011).

Assessment of N fixation effectiveness in the greenhouse

Assessment of N fixation effectiveness in the greenhouse indicated that 40% of the strains that tolerated pH 4.5 on agar media had low effectiveness and 56% were considered effective in fixing N, but only 4% of the strains were found to be highly effective. However, since strains that grew on acid-stressed media were evaluated for N fixation efficiency in the greenhouse in soils of neutral pH, it is possible that the results of the effective strains could have differed, had the greenhouse screening been performed at an acidic pH. The yield responses to inoculation with the commercial strain 532c in the field were generally better than with the locally-sourced strains, although inoculation with ES3 and ES4 were similar in yield to the commercial strain at one field site each. Assessment of symbiotic effectiveness on acidic media should be considered as an option to identify an acid-tolerant strain that is symbiotically effective, and may assist in developing locally adapted strains, well-suited to soil constraints (Alexandre et al. 2009).

Conclusion

Soils of Ethiopia are typically low in N status and acidic. Inoculation of soybean with effective rhizobial isolates increased yield, although locally adapted isolates from acidic Ethiopian soils were not as effective as the commercial strain when tested at multiple sites. The use of effective strains was demonstrated to bring economic benefits to resource-limited smallholder farmers over the current practice of using N fertilisers. Screening of strains with high acid-tolerance *in vitro* and symbiotic effectiveness in the greenhouse was a rapid way to identify acid-tolerant strains that may increase grain yield in acid soils, but field testing is a necessary step in demonstrating this value for soybean production.

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Reference

Abaidoo R, Keyser H, Singleton P, Dashiell K & Sanginga N 2007. Population size, distribution, and symbiotic characteristics of indigenous *Bradyrhizobium* spp. that nodulate TGx soybean genotypes in Africa. *Appl. Soil Ecol.,* **35,** 57-67.

Abate T, Alene AD, Bergvinson D, Shiferaw B, Silim S, Orr A & Asfaw S. 2012. *Tropical Grain Legumes in Africa and South Asia: Knowledge and Opportunities.,* ICRISAT, Nairobi, Kenya.

Abdenna D, Negassa C & Tilahun G 2007. Inventory of soil acidity status in crop lands of Central and Western Ethiopia. *Utilisation of diversity in land use systems: Sustainable and organic approaches to meet human needs*. Tropentag Witzenhausen.

Alexandre A, Brígido C, Laranjo M, Rodrigues S & Oliveira S 2009. Survey of chickpea rhizobia diversity in Portugal reveals the predominance of species distinct from *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*. *Microb. Ecol.,* **58,** 930-941.

Amissah-Arthur A & Jagtap S 2007. Soybeans in Sub-Saharan Africa: modelling possibilities and potential. *IER,* **9,** 44-54.

Appunu C & Dhar B 2006. Symbiotic effectiveness of acid-tolerant *Bradyrhizobium* strains with soybean in low pH soil. *Afr. J. Biotechnol.,* **5,** 842-845.

Aserse A, Räsänen LA, Aseffa F, Hailemariam A & Lindström K 2012. Phylogenetically diverse groups of *Bradyrhizobium* isolated from nodules of *Crotalaria* spp., *Indigofera* spp., *Erythrina brucei* and *Glycine max* growing in Ethiopia. *Mol. Phylogenet. Evol.,* **65,** 595-609.

Atnaf M, Tesfaye K & Kifle D 2015. The importance of legumes in the Ethiopian farming system and overall economy: An overview. *Am. J. Exp. Agric.,* **7,** 347-358.

Ayele GK, Gessess AA, Addisie MB, Tilahun SA, Tebebu TY, Tenessa DB, Langendoen EJ, Nicholson CF & Steenhuis TS 2016. A biophysical and economic assessment of a community-based rehabilitated gully in the Ethiopian Highlands. *Land Degrad. Dev.,* **27,** 270-280.

Bekabil UT 2015. Empirical review of production, productivity and marketability of soya bean in Ethiopia. *IJUNESST,* **8,** 61-66.

Bremner JM & Mulvaney C 1982. Nitrogen total. In: Page AL, Miller RH & Keeney DR (eds) *Methods of soil analysis. Part 2. Chemical and microbiological properties,* pp 595-624. Amercan Society of Agronomy, Madison.

Brockwell J, Bottomley PJ & Thies JE 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. *Plant Soil***,** 143-180.

Bromfield E & Ayanaba A 1980. The efficacy of soybean inoculation on acid soil in tropical Africa. *Plant Soil,* **54,** 95-106.

Broughton WJ & Dilworth MJ 1970. Plant nutrient solutions. In: Somasegaran P & Hoben H (eds) *Methods in legume-rhizobium technology: Handbook for rhizobia,* pp 245-249. University of Hawii Press, Maui, Hawii.

Chapman H 1965. Cation-exchange capacity. In: Black CA, et al (ed) *Methods of soil analysis. Part 2. Chemical and microbiological properties,* pp 891-901. American Society of Agronomy, Madison, WI. .

CSA 2012. Agricultural Sample Survey 2011-2012. *Annual Bulletin*. CSA, Addis Ababa, Ethiopia.

Date RA & Halliday J 1979: Selecting *Rhizobium* for acid, infertile soils of the tropics. *Nature,* **277,** 62-64.

448 Denton MD, Coventry DR, Bellotti WD & Howieson JG 2000. Distribution, abundance and
449 symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* from alkaline pasture soils in
450 South Australia. *Aust. J. Exp. Agric.,* **40,** 25-35.
451 FAO-Unesco 1974. FAO/UNESCO Soil Map of the World 1: 5,000,000. UNESCO, Paris.
452 Foy CD 1988: Plant adaptation to acid, aluminum-toxic soils. *Commun. Soil Sci. Plant Anal.,*
453 **19,** 959-987.
454 Gee GW & Bauder JW 1986. Particle-size analysis. In: Klute A (ed) *Methods of soil analysis.*
455 *Part 1. Physical and mineralogical methods.,* pp 383-411. American Society of Agronomy,
456 Inc. and Soil Science Society of America, Inc., Madison, WI.
457 Gemell L, Roughley R, Reed M & Hartley EJ 1993. Screening of *Rhizobium leguminosarum*
458 bv. *Trifolii* for adaptation to acid and neutral soils using a selective agar medium. *Soil Biol.*
459 *Biochem.,* **25,** 1463-1464.
460 Giller KE 2001. *Nitrogen fixation in tropical cropping systems,* CAB International,
461 Wallingford, UK.
462 Graham PH, Draeger KJ, Ferrey ML, Conroy MJ, Hammer BE, Martinez E, Aarons SR &
463 Quinto C 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial
464 studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. *Can. J. Microbiol.,* **40,**
465 198-207.
466 Herridge D (ed) 2002. *Legume N and Inoculants: Global and Vietnamese Perspectives,*
467 ACIAR, Brisbane, Australia.
468 Herridge DF, Peoples MB & Boddey RM 2008. Global inputs of biological nitrogen fixation in
469 agricultural systems. *Plant Soil,* **311,** 1-18.
470 Howieson J & Ewing M 1986. Acid tolerance in the *Rhizobium meliloti–Medicago* symbiosis.
471 *Aust. J. Agric Res.,* **37,** 55-64.
472 Howieson J, Ewing M & D'antuono M 1988. Selection for acid tolerance in *Rhizobium*
473 *meliloti*. *Plant Soil,* **105,** 179-188.
474 Indrasumunar A, Dart PJ & Menzies NW 2011. Symbiotic effectiveness of *Bradyrhizobium*
475 *japonicum* in acid soils can be predicted from their sensitivity to acid soil stress factors in
476 acidic agar media. *Soil Biol. Biochem.,* **43,** 2046-2052.
477 Jefwa J, Pypers P, Jemo M, Thuita M, Mutegi E, Laditi M, Faye A, Kavoo A, Munyahali W &
478 Herrmann L 2014. Do commercial biological and chemical products increase crop yields and
479 economic returns under smallholder farmer conditions? In: Vanlauwe B & VanAsten PaB, G.
480 (eds) *Challenges and opportunities for agricultural intensification of the humid highland*
481 *systems of sub-Saharan Africa.,* pp 81-96. Springer International Publishing, Cham,
482 Switzerland.
483 Lupwayi N & Haque I 1994. Legume-*Rhizobium* technology manual. *ILCA Environmental*
484 *Sciences Working Document (ILCA). no. 29.* ILCA, Addis Ababa, Ethiopia.

O'Hara G 2001. Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. *Aust. J. Exp. Agric.,* **41,** 417-433.

Peoples M, Brockwell J, Herridge D, Rochester I, Alves B, Urquiaga S, Boddey R, Dakora F, Bhattarai S & Maskey S 2009. The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis,* **48,** 1-17.

Philpotts H 1976. Filter mud as a carrier for *Rhizobium* inoculants. *J. Appl. Bacteriol.,* **41,** 277-281.

Purcino H, Sé N, Vr'ana M, Scotti MF & Vargast IMM 2000. Response of *Arachis pintoi* to inoculation with selected rhizobia strains in Brazilian Cerrado. *Pasturas Tropicales,* **25,** 26-29.

Ronner E, Franke AC, Vanlauwe B, Dianda M, Edeh E, Ukem B, Bala A, van Heerwaarden J & Giller KE 2016. Understanding variability in soybean yield and response to P-fertilizer and *rhizobium* inoculants on farmers' fields in northern Nigeria. *Field Crop Res.,* **186,** 133-145.

Sertsu S & Bekele T (eds) 2000. *Procedures for soil and plant analysis. National Soil Reseach Center* EARO, Addis Ababa, Ethiopia.

Sinclair TR, Marrou H, Soltani A, Vadez V & Chandolu KC 2014. Soybean production potential in Africa. *Glob. Food Sec.,* **3,** 31-40.

Somasegaran P & Hoben H 1985. *Methods in Legume-rhizobium technology; NIFTAL, MIRCEN,* University of Hawaii Press, Maui, Hawaii.

Taylor GJ 1991. Current views of the aluminum stress response: the physiological basis of tolerance. *Curr. Top. Plant Biochem. Physiol.,* **10,** 57-93.

Thies JE, Singleton PW & Bohlool BB 1991. Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. *Appl. Environ. Microbiol.,* **57,** 19-28.

Thuita M, Pypers P, Herrmann L, Okalebo RJ, Othieno C, Muema E & Lesueur D 2012. Commercial rhizobial inoculants significantly enhance growth and nitrogen fixation of a promiscuous soybean variety in Kenyan Soils. *Biol. Fert. soils,* **48,** 87-96.

Unkovich M, Herridge D, Peoples M, Cadisch G, Boddey B, Giller K, Alves B & Chalk P 2008. *Measuring plant-associated nitrogen fixation in agricultural systems,* Australian Centre for International Agricultural Research (ACIAR), Cannbera, Australia.

Van Reeuwijk L 2002. *Procedures for soil analysis,* International soil reference and information centre (ISRIC), Wageningen, The Netherlands.

VSN International 2014. GenStat for Windows, 17th edn. VSN International, Hemel Hempstead, UK.

Walkley A & Black IA 1934: An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil sci.,* **37,** 29-38.

White JG 1966. Introduction of lucerne into acid soils. *Crop and Pasture Improvement (Selection and Breeding),* pp 105-113. New Zealand Grassland Association, Alexandra, Newzealand

527 Table 1. Location and selected physical, chemical and biological properties of soils at the
528 experimental sites.

	Jimma, Ababiya farm	Jimma, Seifu farm	Jimma, Research Centre	Bako	Assossa, Research Centre	Assossa, farm
Latitude	0.7º42.770'	0.7º42.633'	0.7º06.668'	9º04.503'	10°03.251'	10°01.237'
Longitude	37º00.461'	37º00.305'	36º07.867'	36º59.620'	34°59.412'	34°45.613'
Altitude (m)	1781	1767	1753	1755	1588	1578
%Clay	64	71	44	61	71	64
%Silt	24	19	12	19	15	10.0
%Sand	12	10	44	27	14	26
pH H_20	4.37	4.26	4.53	4.39	4.81	4.62
P mg/kg	4.92	4.72	2.12	4.94	5.28	8.21
CEC meq/100g	17.19	17.92	19.6	14.55	34.7	31.6
%OC	2.05	1.98	2.07	1.56	2.96	2.61
Ex. Acidity[A]	0.46	0.81	1.74	0.78	0.18	0.42
Total N %	0.16	0.15	0.45	0.1	0.18	0.14
Rhizobia MPN cfu/g soil	250	16	$>10^6$	ND*	$1.4x10^3$	$>10^6$

529 *ND= Not detectable with the MPN procedure used. See methods section for further
530 details.
531 [A] Exchangeable acidity (Van Reeuwijk 2002).
532

533 Table 2. Soil samples collected from major soybean growing areas of Ethiopia and the
534 number of strains isolated from the soils using soybean cv. Clark as a host plant.

Soil collection areas	Soils sampled	Rhizobial strains isolated
Southern Ethiopia	40	14
Jimma and Illubabor zone	40	36
Bako area	43	11
Assossa zone	31	19
Total	154	80

535

536 Table 3. Effectiveness of selected rhizobial strains inoculated on to soybean grown in N-free
537 conditions in a controlled environment, compared with controls and reference strains. Means
538 followed by different letters are significantly different at P ≤ 0.05.

Treatment	%N	mg N/ shoot	% Effectiveness	Rank*
Strain 532c	3.3 ± 0.15a	135 ± 7.6a	113	HE
N-fed treatment	2.9 ± 0.03bc	107 ± 1.4b	100	
CB1809	3.0 ± 0.05b	110 ± 1.9b	100	HE
ES4	3.0 ± 0.11ab	110 ± 5.7b	99	HE
ES3	2.8 ± 0.09 cd	82 ± 3.8c	81	HE
ES2	2.6 ± 0.04d	69 ± 2.7d	73	E
ES1	2.5 ± 0.04d	65 ± 30d	69	E
Control	0.8 ±0.01e	11 ± 0.4e	-	
$LSD_{0.05\%}$	0.24	11.96		

539 *Rank indicates: Effective (E) or Highly Effective (HE).

540

Table 4. Aboveground biomass including grain at harvest AGB, kg/ha, %N, total shoot N, percentage of shoot N fixed, %Ndfa, and total shoot N fixed by soybean at harvest following application of different inoculants at Bako. Means in a column followed by different letters are significantly different at P≤0.05.

Treatment	AGB kg/ha	%N	Total Shoot N kg/ha	$\%N_{dfa}$	Fixed N in shoots kg/ha
Control	1325 ± 206c	4.2 ± 0.4a	50± 5d	0.7 ± 0.4c	1 ± 1c
Strain 532c	2693 ± 204a	8.4 ± 2a	226 ± 26a	55 ± 7a	126 ± 26a
ES1	1609 ± 402bc	4.5 ± 0.4a	78 ± 4d	5 ± 0.7c	4 ± 1.5c
ES2	2025 ± 235b	7.6± 1.5a	164 ± 15abc	12 ± 6c	22 ± 15bc
ES3	2091 ± 383ab	8 ± 2a	191 ± 66ab	35 ± 9b	60 ± 18b
ES4	1467 ± 154bc	6 ± 1a	97 ± 4cd	10 ± 1c	10 ± 1c
23 kg N/ha	1602 ± 136bc	6 ± 1.3a	82 ± 22cd	0.08 ± 0.2c	1 ± 2c
46 kg N/ha	1778 ± 142bc	7 ± 0.4a	130 ± 8bcd	0.7 ± 0.9c	1 ± 0.1c
$LSD_{0.05}$	643	NS	82	14.2	38

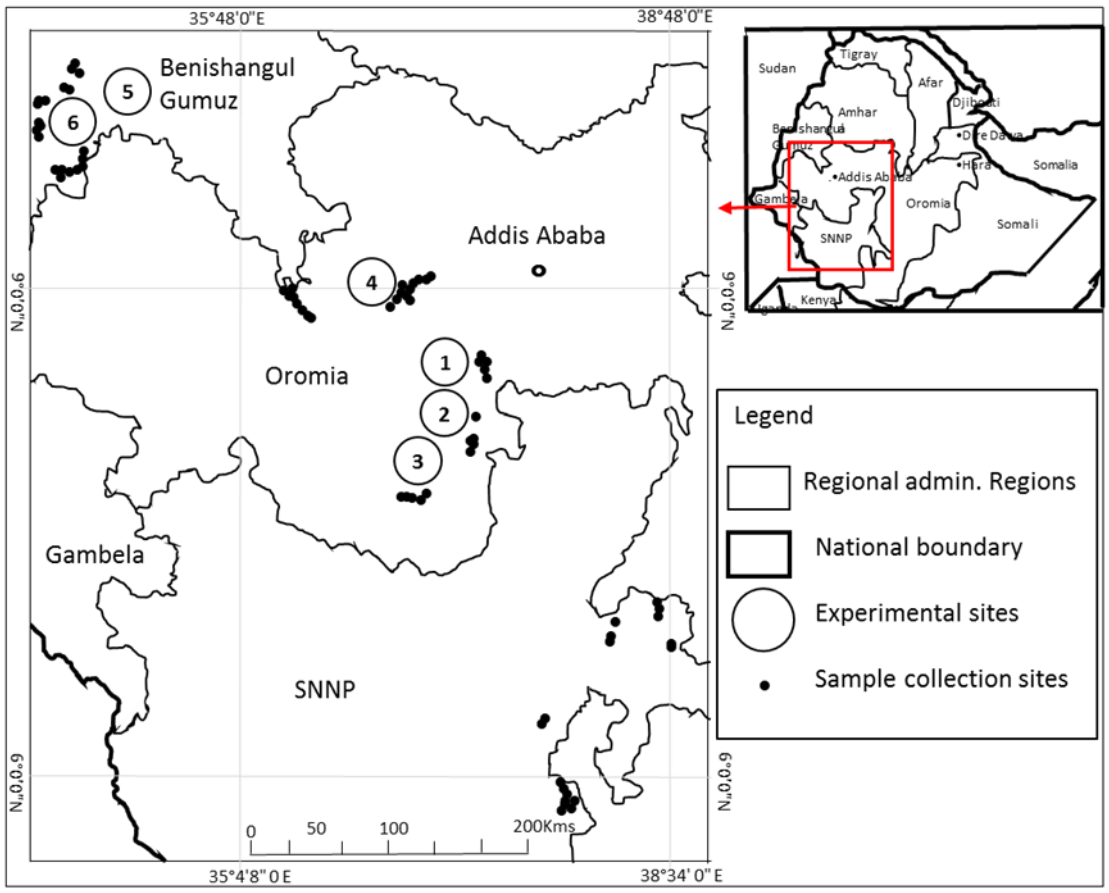
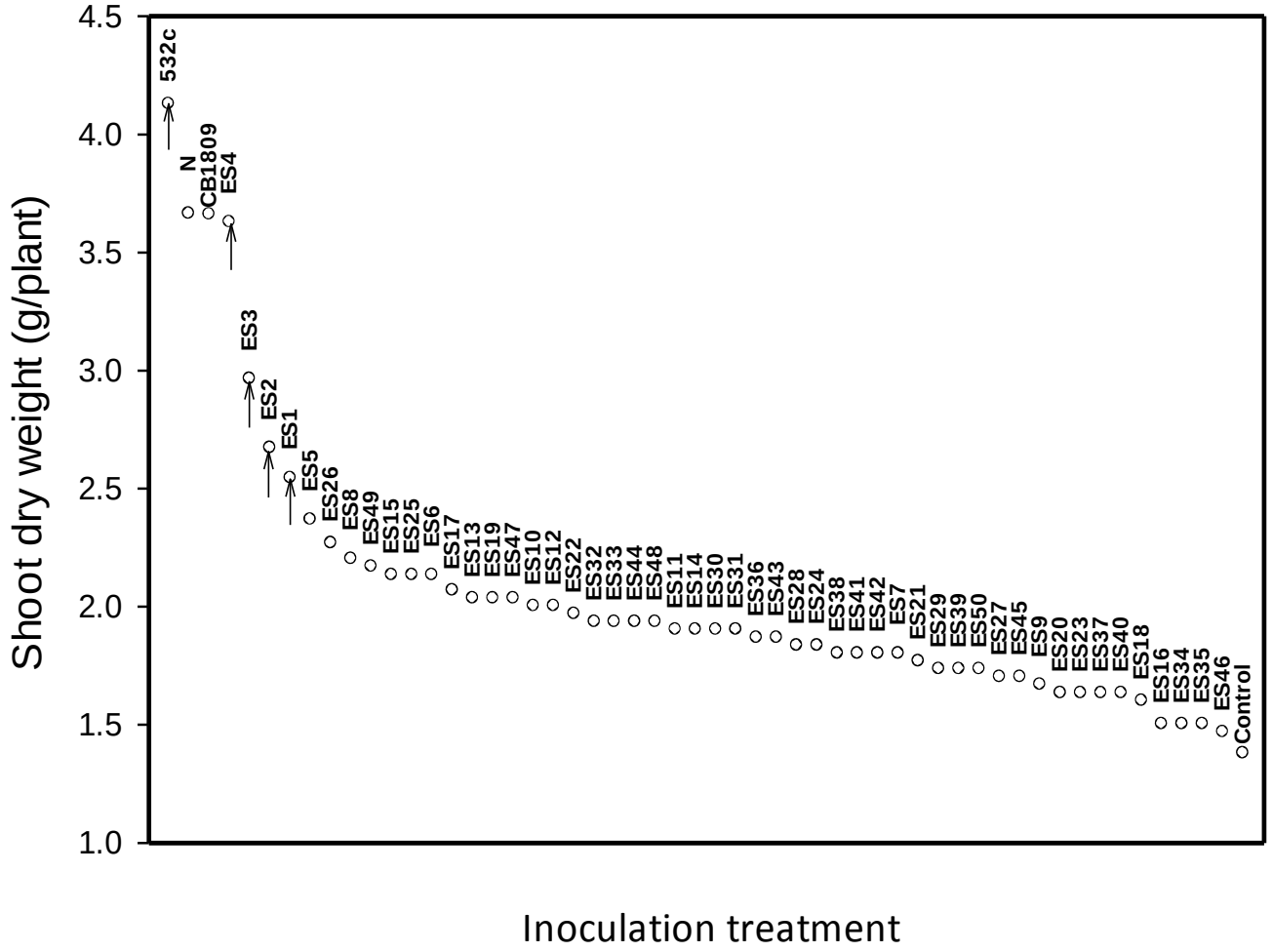


Figure 1. Experimental sites in South Western and West Ethiopia indicated by numbers 1 to 6. The site numbers indicate: 1) Ababiya farm, 2) Seifu farm, 3) research station in the Jimma area, 4) Bako farm, 5) Assossa research Centre and 6) Assossa farm.



strains (1-50) and two commercial reference strains, 532c and CB1809, an N supplied control (N) and a non-inoculated control (Control). LSD = 0.48 at P = 0.05; 3 replicate pots. Arrowed symbols represent strains selected for field experimentation: 532c and four local strains.

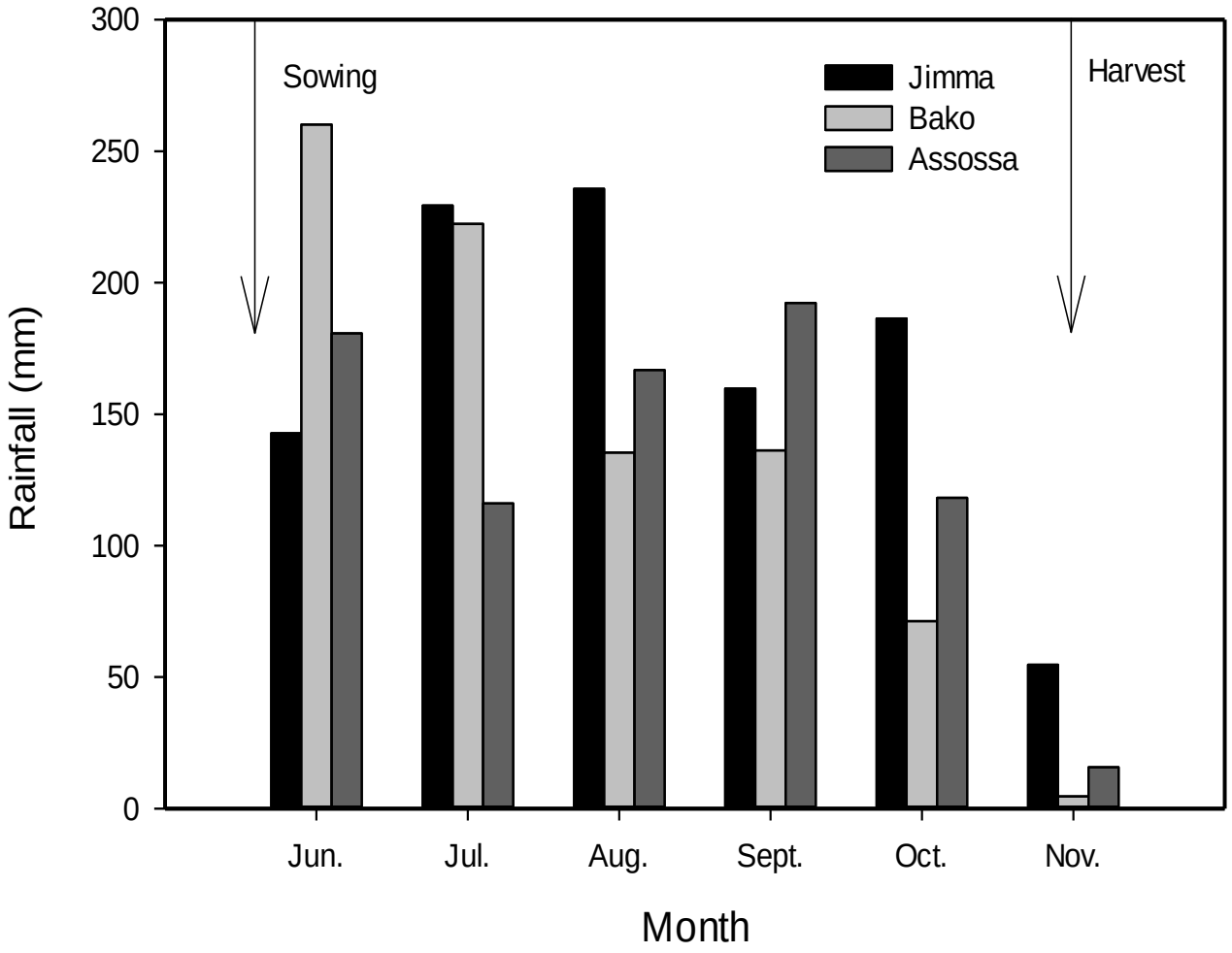


Figure 3. Monthly rainfall distribution across experimental sites during the 2014 growing season. Jimma area represent farms at Ababiya, Seifu and Jimma research stations, Bako area represents an on-farm site, and Assossa area represents sites at the research station and a farm.

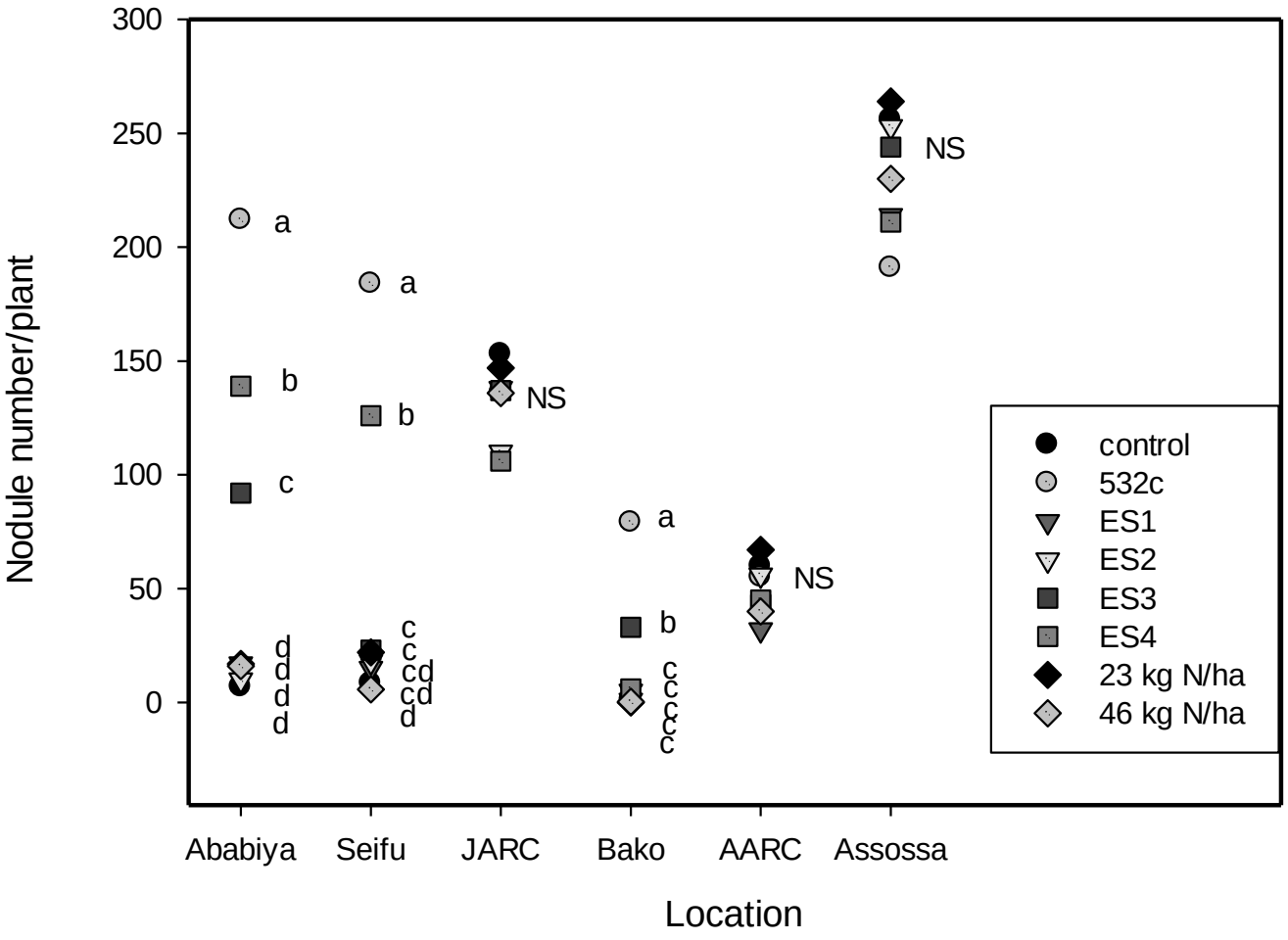


Figure 4. Nodule number per plant of soybean following application of different inoculants and N supply at six experimental sites in major soybean growing areas of Ethiopia, 2014. The means of the treatments for each location is separated by 95% LSD, and means with the same letters are not significantly different.

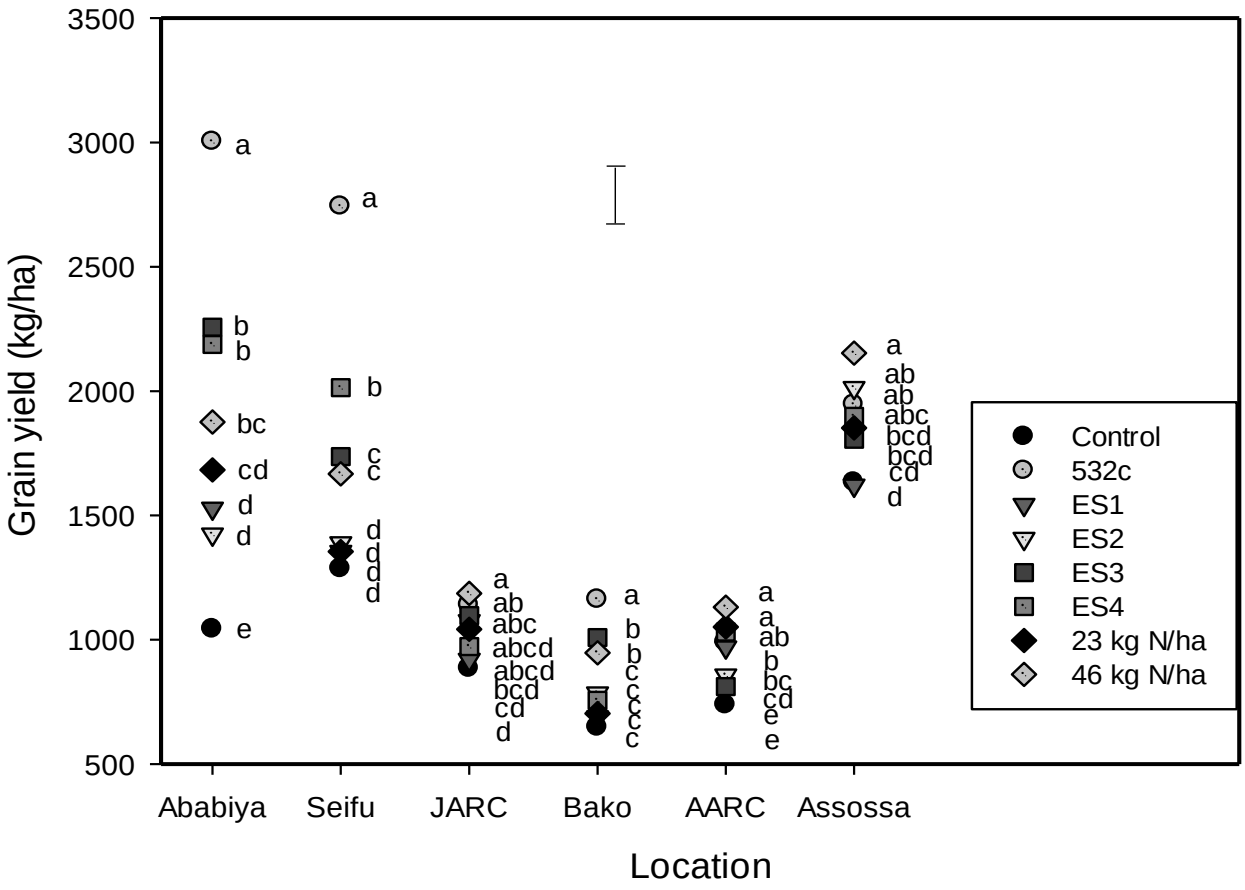


Figure 5. Grain yield of soybean following application of different inoculants and N supply at six experimental sites in major soybean growing areas of Ethiopia in 2014. The means of the treatments for each location is separated by 95% LSD. Means with the same letters are not significantly different.

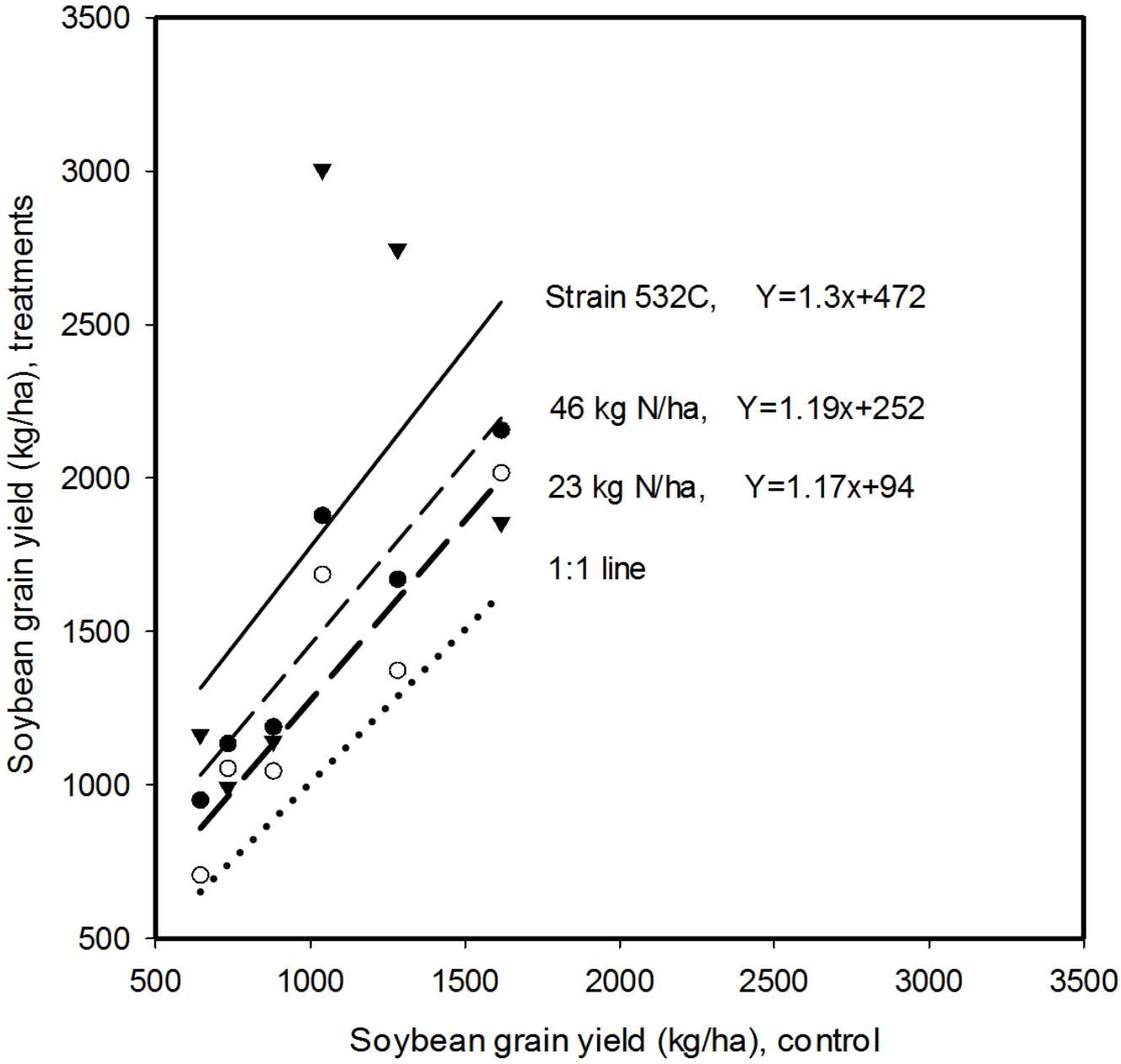
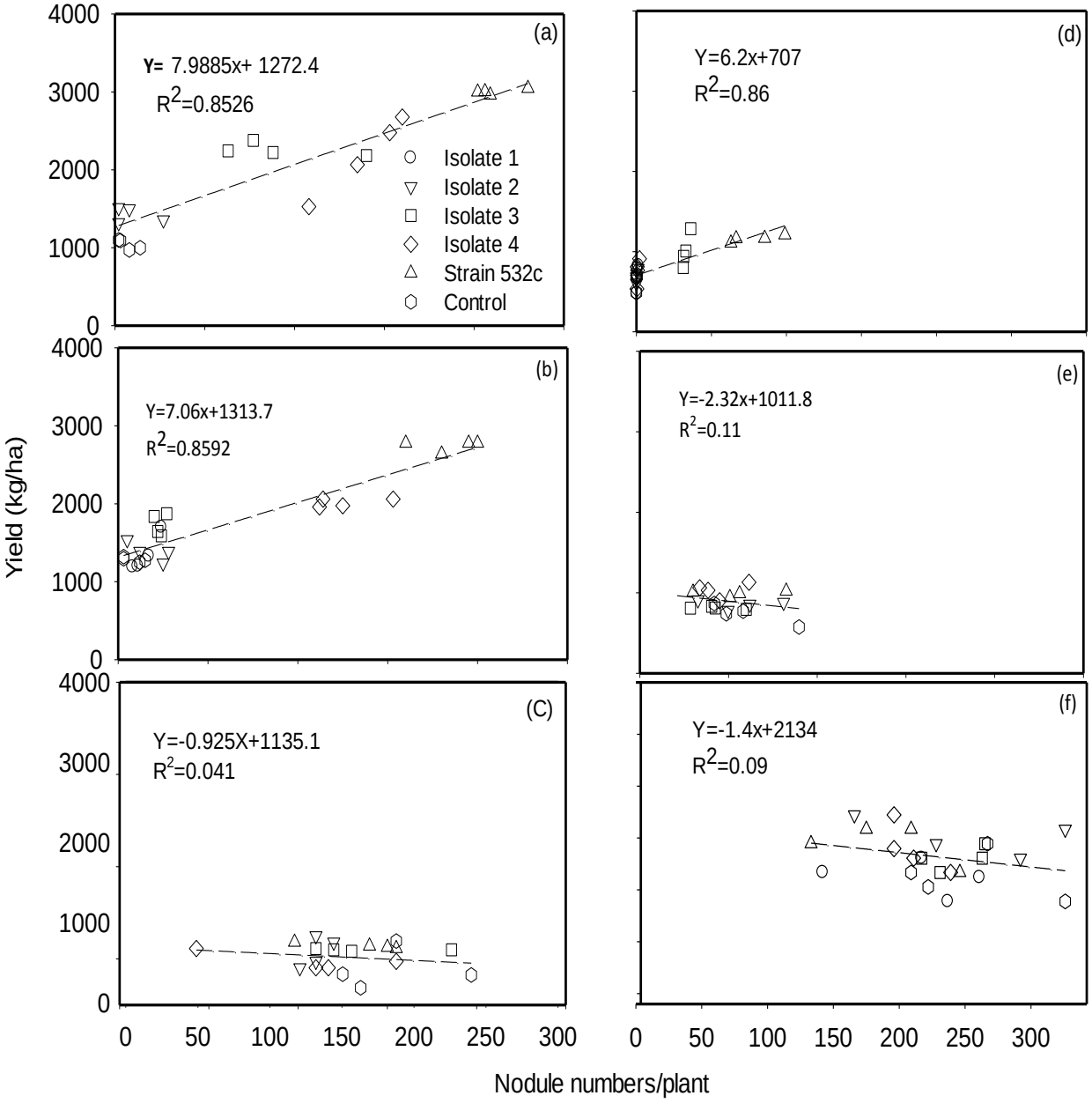


Figure 6. Soybean grain yields following inoculation with commercial strain (closed triangles) or with application of 46 kg N/ha (closed circles), or 23 kg N/ha (open circles) in comparison with the yield of the untreated control across the six experimental sites. A 1:1 dotted line is shown for comparative purposes.



681 Figure 7. Regression analysis of nodule number per plant and grain yield among different
682 treatments at the six experimental sites. Sites are a) Jimma, Ababiya farm, b) Jimma, Seifu
683 farm, c) Jimma, Agricultural Research Centre, d) Bako e) Assossa, Agricultural Research
684 Centre f) Assossa, farm.