

Full Length Research Paper

Water-condition effects on rhizobia competition for cowpea nodule occupancy

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Two indigenous bradyrhizobia strains displaying different natural behaviours towards water regime (strain ORS 3257, nodulating more frequently in favourable-water conditions and strain ORS 3260, in limited-water conditions) were studied for their competitiveness for nodulation of cowpea (Mouride cultivar) under favourable and limited water conditions in non-sterile soil. The nodule occupancy was studied by PCR-RFLP analysis. Both strains showed good competition with other indigenous rhizobia populations under favourable- and limited-water conditions. Competition between the inoculated strains in the mixture varied between water regimes. In non-limited-water conditions, strain ORS 3257 was the best competitor, whereas in limited-water conditions, strain ORS 3260 was the best competitor. Results indicated that screening of strains according to their environmental origin could ensure successful rhizobia inoculation.

Key words: *Vigna unguiculata*, bradyrhizobia, inoculation, competition, limited-water conditions.

INTRODUCTION

Cowpea plays a considerable role in the nutritional balance and economy of the rural population of Senegal. The legume is cultivated in the driest parts of the country: 90% of the cultivated areas are concentrated in the northern and the north-central areas receiving irregular rainfall (100-300 mm per year). However, despite the cultivars' early maturation, cowpea production is often limited by drought, occurring especially in mid-cycle, since it is cultivated under rainfed conditions. Consequently, water deficit constitutes a limiting factor for cowpea yields.

Wild and cultivated cowpea plants are naturally found nodulated in diverse soils and geographical areas.

However, at the farm level, grain yields are often low and inconsistent. This can be due to local strains that are ineffective or in insufficient number. Several studies have reported that cowpea nodulation in tropical soils could not be improved by supplying in larger number selected strains with high symbiotic performance (Awonaike et al., 1990; Gueye, 1983). Among the frequently mentioned causes of failure is the poor competition between introduced strains and indigenous rhizobia (Awonaike et al., 1990; Thies et al., 1991). Nevertheless, Fening and Danso (2002) showed that native isolates are a potentially useful source of highly effective cowpea inoculants and represent an important reservoir in which superior strains could be already adapted to environmental stresses as drought. Recently, Martins et al. (2003) identified a strain, BR 3267, as important resource for the optimisation of biological nitrogen fixation in cowpea in the dry land areas of the Brazilian semi-arid region. Naturally occurring rhizobia are more persistent in

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soils than introduced strains (Fening and Danso, 2002; Sanginga et al., 2000). They can represent a useful source of strains for inoculum production because of their better competitiveness and better adaptation to the environment. The understanding of ecological determinants of natural populations is therefore important because they may assist the selection of rhizobia strains that enhance symbiotic efficiency and introduce the successful establishment of inoculant strains.

Previously Krasova-Wade et al. (2003) studied the diversity of *Bradyrhizobium* strains naturally nodulating three cowpea cultivars (B-21, TN 88-63 and Mouride) in favorable and water-limited conditions. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of 16S-23S rDNA intergenic spacer region (IGS) of 85 nodules DNA distinguished four genetic profiles corresponding to four different strains. The distribution of these IGS types according to water conditions and cowpea cultivars showed that nodulating strain populations appeared more diverse in water-limited condition. More than three quarters of prospected nodules presented the strain ORS 3257. They were formed on all three cultivars and in both water conditions. Only a small part of nodules was distributed between the three other strains, among which ORS 3260 was found mainly in limited conditions on TN 88-63 and Mouride cultivars.

We based on this observation of the natural behaviours of the ORS 3257, nodulating more in favourable-water conditions and the ORS 3260, in limited, to verify the hypothesis that the differences for nodulation of the strains are due to their different adaptations to soil water status. This paper presents a study on competition for cowpea nodulation of the indigenous bradyrhizobia in controlled conditions under different water regimes of soil.

MATERIAL AND METHODS

Soil preparation

Soil was sampled in Bambey locality, where the field experiment described by Krasova-Wade et al. (2003) was carried out. It was sandy, pH 6-6.5 and vernacular dior-modal (Sarr et al., 2001). Soil was sterilized at 120°C for 2 h and placed in 1.4-l sheaths. It was a uniform mixture with topsoil and an estimated moisture concentration of rhizobia at field capacity of 0.294 kg kg⁻¹ three days before sowing.

MPN

Prior to planting, field soil was sampled to determine the most probable number (MPN) of indigenous soil rhizobia able to nodulate cowpea, estimated by serial dilution-plant infection count using siratro (*Macroptilium atropurpureum*) as the trap host. Six successive five-fold dilution series were followed with an initial dilution of 1:50 (Vincent, 1982). Four test plants of five dilutions and five with the last dilution were inoculated with 1-ml aliquots. Seedling tubes inoculated with 1 ml of sterile distilled water served as the control. Siratro seeds were scarified and surface-sterilized with concentrated sulphuric acid for 30 min. After treatment, the seeds were washed several times with sterile water to eliminate any

trace of acid. The seeds were incubated to germinate in sterile Petri dishes containing 0.7% (w v⁻¹) YM agar for 24-48 h in the dark. The seeds were transferred into tubes containing Jensen seedling slant agar for root nodulation trials (Vincent, 1970). Plants were grown under intermittent light (12 h/12 h, day/night) and scored for nodulation after six weeks of incubation (Menyah and Sato, 1996) and the MPN of indigenous rhizobia was determined by statistical tables (Vincent, 1982).

Experiment

The experiment was carried out in Dakar at the experimental station in Laboratoire Commun de Microbiologie (IRD-ISRA-UCAD) during August and September 2002. Plants were grown in 17-l pots constructed from 15-cm diameter polyvinyl chloride tubes cut into 100-cm lengths. One plant was grown in each pot. The trial was done in a randomised complete block. Plants were subjected to two water treatments: favourable and limited conditions. Four inoculation treatments were carried out: with ORS 3257, ORS 3260, the mixture of the two strains (1:1) and the control.

Each experiment unit was composed of three replicates. There were eight treatments with 120 pots distributed among five blocks. *Vigna unguiculata* seeds were surface-sterilised with 96% (v v⁻¹) ethanol for 5 min and rinsed in sterile water. Then they were sterilized with 3.3% (w v⁻¹) Ca(OCl)₂ for 10 min and rinsed in sterile water. To detect rhizobia contamination, the seeds were germinated in the dark in sterile Petri dishes containing 0.7% (w v⁻¹) YM agar at 28°C for 48 h. They were transplanted after germination in the pots.

The water limitation was introduced 10 days after planting by stopping irrigation until the leaf water potential reached -1.5 MPa, measured with a PWSC 3005 pressure bomb (Soilmoisture equipment, USA). Then the plants were irrigated daily with 200 ml until harvesting. Unstressed plants were irrigated daily with 200 ml until harvesting.

Inoculums strains and inoculation

The strains ORS 3257 and ORS 3260 were grown individually in 1000 ml YM (Vincent, 1970) containing (g l⁻¹): mannitol, 10; sodium glutamate, 0.5; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; NaCl, 0.05; CaCl₂, 0.04; FeCl₃, 0.004; yeast extract, 1; pH 6.8. Incubation was at 28°C in aerobic conditions for 3 days. The cell numbers of exponentially growing cultures (approximately 10⁸ cells ml⁻¹) were estimated by optical density (Pharmacia Biotech) at 600 nm and adjusted to the same value by dilution with YM. After transplanting, the seedling of each pot was inoculated with 10 ml of the individual rhizobial culture or with 20 ml of double mixed culture at collar vicinity and on soil surface. Un-inoculated plants were used as the control.

Samplings

During the experiment, three plant samplings were carried out: on day 20, 30 after planting (DAP) and at the harvest (day 74). 5 plants (one plant per experiment unit) per treatment (total of 40 plants) were harvested. Stem length was measured weekly. At each sampling, all aboveground portions divided into the vegetative (leaves, stems) and reproductive parts (pods with enclosed seeds) for 74th day and under-ground portions (roots) were harvested and dried at 60°C for 4 days. Seeds were not separated from the pods. Nodules were harvested, dried at 45°C for 48 h and stored then at ambient temperature.

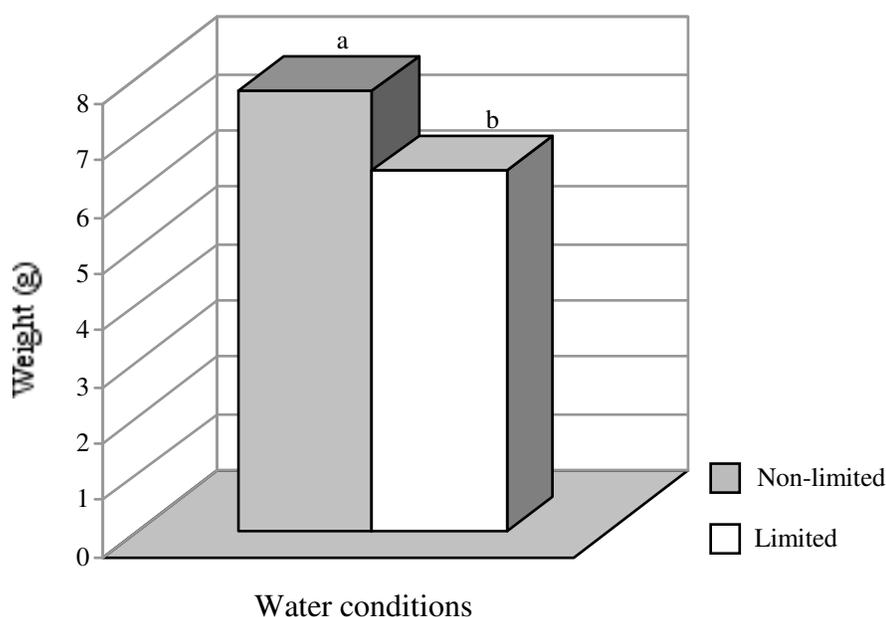
Nodule occupancy

Before analysis, each nodule was treated individually. Nodules were rehydrated in sterile water and surface sterilised by immersion

Table 1. Fisher probability corresponding to significance test of factor effects on cowpea growth under limited- and favourable-water conditions (at 5% level of significance).

Effect	Shdw	Rdw	Noddw	Onenoddw	Nodnm	Stlngth	Repdw
Block	0.0544	0.277	0.2681	0.4081	0.1455	0.0018	0.0325
Date	< 0.0001	< 0.0001	0.0724	0.1896	0.2549	< 0.0001	
Inoc	0.558	0.2324	0.5428	0.2986	0.0045	0.4501	0.6229
Cond	0.0087	0.0426	0.0011	0.069	0.2794	0.3711	< 0.0001
Date*Inoc	0.3074	0.4468	0.5616	0.2428	0.1865	0.7162	
Date*Cond	0.2039	0.0134	0.5995	0.3053	0.3495	0.0255	
Inoc*Cond	0.8035	0.295	0.3818	0.3225	0.6153	0.1715	0.0219
Date*Inoc*Cond	0.0711	0.9591	0.3747	0.1888	0.6109	0.0066	

*Factor interactions: Shdw, shoot dry weight; Rdw, root dry weight; Noddw, nodule weight; Onenoddw, dry weight of one nodule; Nodnm, nodule number; Stlngth, stem length; Repdw, dry weight of reproductive parts; Inoc, inoculum; Cond, water condition. The most important effects appear in bold type.

**Figure 1.** Water regime effect on shoot dry weight during the experiment at $P < 0.05$ (Tukey test).

in 3.3% ($w v^{-1}$) $Ca(OCl)_2$ for 3 min, followed by rinsing in sterile water, then in 96% ethanol for 2 to 3 min followed by rinsing in sterile water. From this stage the nodules were manipulated aseptically. Each nodule was crushed in 150 μ l of sterile water with a plastic pestle sterilised in 96% ethanol in a 1.5-ml Eppendorf tube (Krasova-Wade et al., 2003). Nodule occupancy was determined by PCR-RFLP of 16S-23S rDNA IGS with two enzymes, *Hae*III and *Msp*I, directly on DNA extracts from crushed nodules following the conditions described by Krasova-Wade et al. (2003).

Statistical analysis of growth

The samples from day 20, 30 and 74 after planting were analysed overall, taking into consideration the block, growing day, inoculum and water-condition effects to evaluate the different effects of their interactions. Data underwent an analysis of variance with SAS 8.1 software (SAS Institute Inc., Cary, NC, USA) and Repeated Measures programme for the all samplings. The Tukey method was

used to separate the means at $P < 0.05$ level of significance. Significance difference of interstrain competition for nodule occupancy by inoculant rhizobia was determined by the χ^2 test of Pearson Likelihood Ratio of SAS 8.1 software. Double occupancy by inoculant strains was scored as a separate observation.

RESULTS

Growth and dry-matter yield

Data presented in Table 1 and Figure 1 show that shoot dry biomass was significantly reduced by drought by 17%. Leaf water potential reached -1.5 MPa after 20 days of water stress imposition. The root dry biomass was influenced by water conditions in relation to growth period (Figure 2). On day 74 after planting this parameter

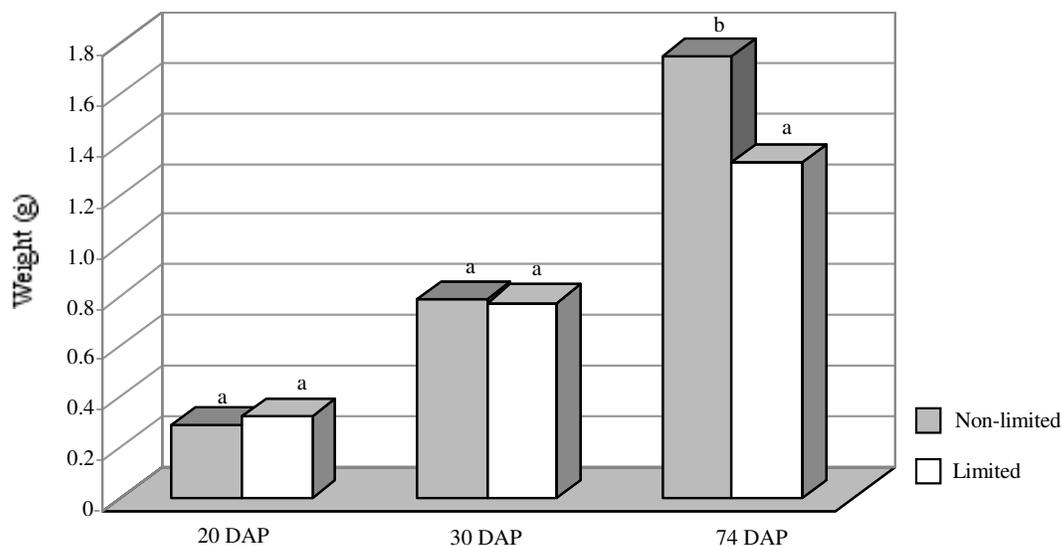


Figure 2. Water regime effect on root dry matter at different dates. Values for each date followed by the same letter are not significantly different at $P < 0.05$ (Tukey test); DAP, days after planting.

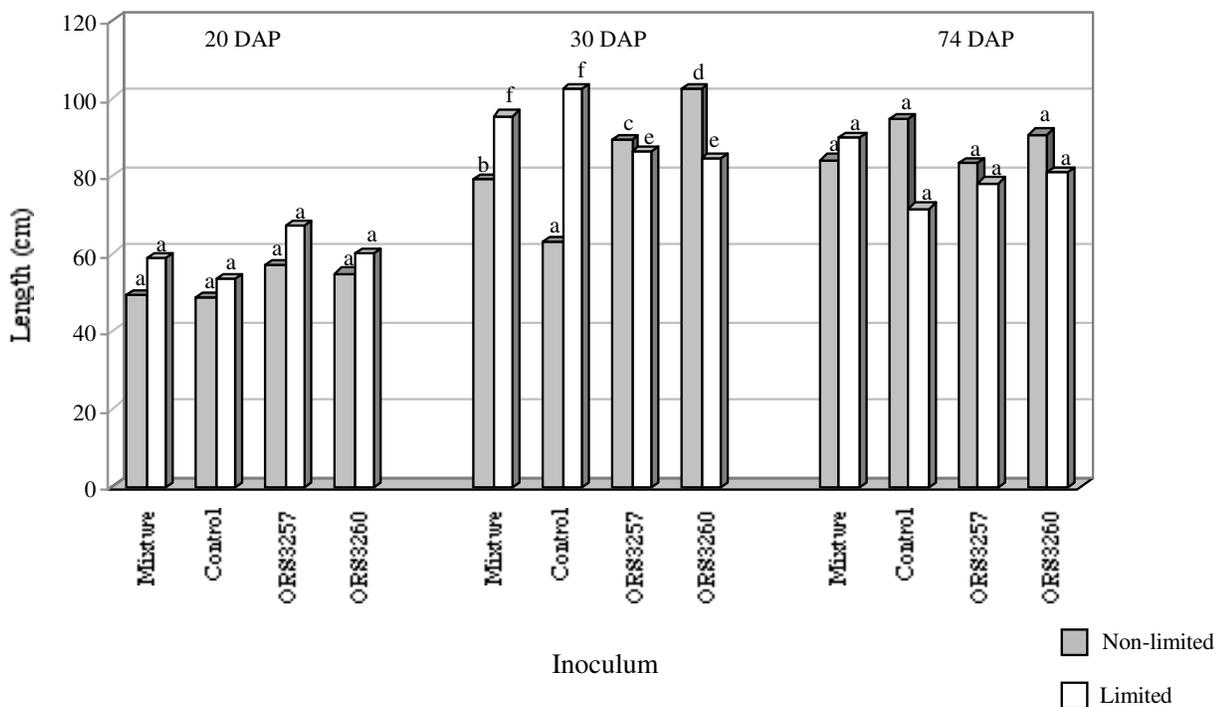


Figure 3. Water regime effect on root length of plants inoculated with different inoculums at different dates. Values for each date and for each water regime followed by the same letter are not significantly different at $P < 0.05$ (Tukey test); DAP, days after planting.

was significantly decreased by water stress. At day 30 of growth, the root length was significantly affected by the inoculated strain and water conditions (Figure 3). Under non-limited-water conditions, the roots of un-inoculated plants were shorter than inoculated ones, independent of

the strain used. Inversely, the roots of stressed un-inoculated plants were the longest, except those of plants inoculated with two strains. The cowpea's response to water deficit appears to be directly related to a reduction of nodule weight (Figure 4). The total nodule weight of

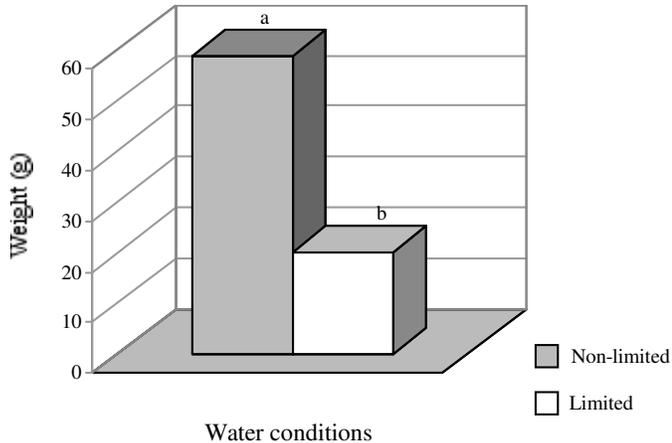


Figure 4. Water regime effect on nodule dry weight during the experiment at $P < 0.05$ (Tukey test).

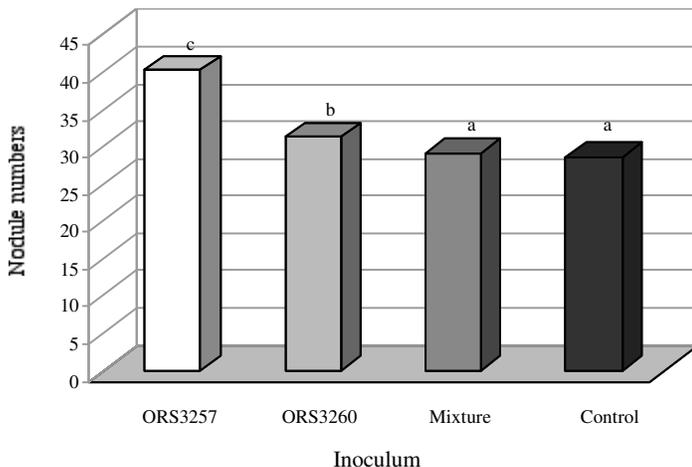


Figure 5. Inoculation effect on nodule number. Values followed by the same letter are not significantly different at $P < 0.05$ (Tukey test).

stressed plants was 66% lower than those of non-stressed plants. Nodule weight of one nodule was not affected. The nodule number was not affected by water stress, but by inoculated strain. Figure 5 shows that the plants inoculated with ORS 3257 formed a significantly higher number of nodules than the ones inoculated with ORS 3260.

Significant interaction was detected between water conditions and inoculum type on pod yield (Figure 6). Whatever the inoculum type, pod yields of non-stressed plants were significantly higher than those of stressed plants. Inoculation with the mixture of ORS 3257 and ORS 3260 tended to increased pod dry biomass in limited-water conditions, whereas in non-limited-water conditions, inoculation with this mixture tended to decreased the yield.

Competition for nodule occupancy

The low number of bradyrhizobia, from 0 to <10 cells per gram of soil, occurred on the sample soil. Table 2 shows the results of bradyrhizobia distribution in the different nodules that were analysed. Under water stress as well as under non-limited conditions, occupancy rate was significantly dependent of inoculum type. The strains ORS 3257 and ORS 3259 were shown to be good competitors in regards to indigenous strains in all water conditions if they are inoculated separately. Conversely, if the strains were inoculated in the mixture, their double nodule occupancy was rarely found in non-limited- ($\chi^2=46.7$; $ddl=2$) as well as in limited-water conditions ($\chi^2=28.7$; $ddl=2$). Competition for nodule occupancy between the inoculated strains in the mixture was significantly related (by descriptive statistical analysis) to water conditions. In non-limited-water conditions, ORS 3257 was the most successful competitor, occupying 50% of the formed nodules. In comparison, ORS 3260 was a weak competitor, occupying 35% of the formed nodules. The presence of this strain in nodules was significantly greater than those of ORS 3257 (50% versus 33%) in limited-water conditions.

The un-inoculated plants were always found to be nodulated. In non-limited-water conditions, 70% of the formed nodules were occupied by other indigenous strains (PCR-RFLP profiles did not correspond to those of the inoculated strains). In limited-water conditions, 63% of the formed nodules were occupied by ORS 3257.

DISCUSSION

The indigenous cowpea isolates ORS 3257 and ORS 3260 and the Mouride cultivar were selected following the results obtained by Krasova-Wade et al. (2003). It was noticed by the authors that rhizobia population appeared to be more diverse in limited-water conditions. ORS 3257 was the most frequently found under favourable-water conditions, whereas ORS 3260 was more frequent under limited-water conditions. The Mouride cultivar was studied because of its good drought tolerance (Sarr et al., 2001) and its ability to be nodulated by a larger population of bradyrhizobia than cultivars B-21 and TN 88-63 (Krasova-Wade et al., 2003).

The inoculation, in our study, did not improve the crop growth, as it was shown in many cases by different authors. Although an important objective of the study was to ascertain responses to drought stress, the water potentials that plants were allowed to reach was not sufficiently low (-1.5 MPa). Results shown that the water stress, nevertheless, actually occurred. They were high enough to cause a decrease of 17% in shoot biomass, 24% in root biomass and from 74% to 89% in pod yield. Figueiredo et al. (1998) studied water-stress effect at different stages of N_2 fixation in cowpea plants and verified the adaptive physiological response to stress.

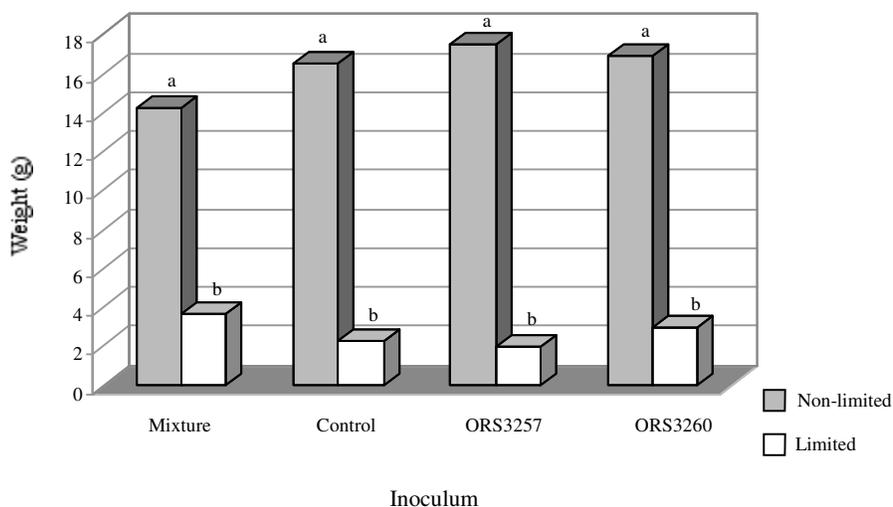


Figure 6. Water regime effect on reproductive part dry weight of plants inoculated with different inoculums. Values for each water regime followed by the same letter are not significantly different at $P < 0.05$ (Tukey test).

Table 2. Nodule occupancy rate of inoculated and indigenous strains obtained by PCR-RFLP analysis of 16S-23S rDNA IGS directly on crushed nodules.

Inoculum	Water conditions	Nodule occupancy (%) by ^a :				χ^2 ^b	Prob ^c
		ORS 3257	ORS 3260	Mixture	Other indigenous strains		
ORS 3257	non -limited	96	0	4	0	46.7	< 0.0001
ORS 3260	non-limited	6	91	3	0		
Mixture	non-limited	50	35	15	0		
ORS 3257	limited	81	14	0	5	28.7	< 0.0001
ORS 3260	limited	10	75	5	10		
Mixture	limited	33	50	13	4		
Control	non-limited	25	5	0	70		
Control	limited	63	11	0	26		

^aPCR-RFLP profiles of crushed nodules were compared with the genetic profiles of inoculated strains for two enzymes, *HaeIII* and *MspI*.

^b χ^2 of Likelihood Ratio Chi-Square of Pearson with 2 degree of freedom.

^cSignificance at $P < 0.05$.

They found a positive interaction between water stress and development stages of N_2 fixation. Water stress applied at the stage of 15-30 days gave the most negative interference, indicating it is possibly a critical period of water stress for cowpea. In our study, the water stress was applied from day 10 to day 30 of growth.

In contrast, the nodule number was not affected by water stress, but by strain inoculation. It is well known now that the indigenous rhizobial populations are one of the environmental factors that influence total nodule occupancy by inoculant strains (Thies et al., 1992). It was found that 50 to 100 rhizobial cells specific to the host plant per gram of soil are enough to produce a good nodulation (Thies et al., 1991). Greater than 50% nodule occupancy by inoculant strains was achieved in 76% of the observations where inoculating rhizobia were applied

at a rate less than 1000 times the size of the indigenous rhizobial population. We found that regarding the infection area, the ratio between the indigenous population (<10 cells per gram of soil) and the inoculation rate was greater than 1:1000. With this ratio, we often obtained at least 75% recovery rates for the inoculated strains if they were inoculated separately.

The inoculated strains were found naturally nodulating cowpea in the zone where the soil was sampled (Krasova-Wade et al., 2003). The fact that the strains used always nodulated the un-inoculated plants confirms these observations. The diversity of nodulating strains appeared to be larger in limited-water conditions and strain ORS 3257 formed more nodules on un-inoculated plants than strain ORS 3260 in both water conditions. This is in accordance with previous results (Krasova-

Wade et al., 2003). However, we showed that the natural balance could be modified by inoculation of the strains having more cells than the existing cell number in the soil and by environmental factors. When strain ORS 3260 was inoculated in favourable- and limited-water conditions, it forms 91 and 75% of nodules, respectively; a few were formed by the strain ORS 3257. When the strains were inoculated in the mixture, at least 13% of nodules were formed by both strains, whatever the water conditions. Under favourable-water conditions ORS 3257 appears to be a more successful competitor. Under limited water conditions, ORS 3260 appears to be the best. It seems that a better adaptation to environment contributes to better competition. Studying various growth and physiological parameters, it suggested that an interaction exists between the competitive strains in relation to various environmental factors, which could reveal harmful or beneficial effects (Sreekumar and Sen, 1989).

Many authors showed an existence of variability in cultivar-strain interactions (Fall et al., 2003; Krasova-Wade et al., 2003; Ndiaye et al., 2000; Robinson et al., 2000; Sanginga et al., 2000) and in symbiotic effectiveness of *Bradyrhizobium* strains (Awonaike et al., 1990; Fening and Danso, 2002; Gueye, 1983). The data presented here indicate that one of the most important aspects of cowpea-bradyrhizobia interactions is the origin (environmental conditions) of inoculated bradyrhizobia. Consequently, the optimal conditions to compete for ORS 3257 will be the non-limited-water conditions, whereas ORS 3260 will have a better effect under limited-water conditions.

In conclusion, in this work, we tested and confirmed our hypothesis that if indigenous strains are re-introduced in the soil they behave in accordance to their environmental origin. To optimise competitiveness of inoculated strains in field conditions, it appears very important to carry out the strain selection in consideration to their geographical location, soil characteristics and climate conditions. We showed, moreover, that it is possible to reconstitute environment conditions and their effects on rhizobium behaviour in controlled laboratory conditions.

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