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Effect of *Rhizobium* sp. inoculation on N_2-fixing and photosynthetic activities of two cowpea [*Vigna unguiculata* (L.) Walp.] genotypes

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Abstract

Time course of symbiotic N_2-fixing and photosynthetic activities during vegetative growth from 30 d after plantation until pod set was measured in the CB5 and 7964 cowpea [*Vigna unguiculata* (L.) Walp.] genotypes of contrasting senescence traits. At emergence, seedlings were inoculated with a "non-cowpea miscellany" *Rhizobium* strain generally used to inoculate *Cicer arietinum*. Maximum N_2-fixing activity occurred in inoculated CB5 and 7964 plants about 54 and 68 d after plantation, respectively. A similar temporal shift of maximum was found for net photosynthetic rate (P_N), confirming a good coordination between the two processes. A higher P_N was found from the first measurements in inoculated plants of both genotypes as compared with uninoculated plants. Apparently, the maximum activity of both N_2-fixation and P_N was timed to occur at a particular stage of plant ontogeny correlating the high N supply with the high N demand by the plant. *Rhizobium* inoculation did not significantly affect partitioning coefficients of biomass to various plant organs but extended leaf longevity by about 10 d in the CB5 genotype, retarding thus the monocarpic senescence.

Additional key words: acetylene reduction; leaf area; monocarpic senescence; plant ontogeny; ureides.

Introduction

Biological nitrogen fixation performed by *Rhizobium*-legume symbiosis can provide about half of the total N required for global agriculture (Elkan 1992, Dakora and

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Keya 1997). This might explain why many investigations have been devoted to the enhancement of this process at the microbiological, ecophysiological, agronomical, and, more recently, molecular levels (Shantharam and Mattoo 1997). This symbiosis is specific, that is every rhizobial strain has a defined host range (Young and Johnston 1989). The specificity is due to the exchange of low-molecular-mass signal molecules between the plant and the rhizobia that controls, to some extent, the expression of nodulation genes (Dénarié *et al.* 1992, Hungria and Stacey 1997). However, a developing interest in biodiversity and the emergence of powerful molecular biology techniques have evidenced that the degree of specificity may vary greatly among different types of rhizobia. Some leguminous plants, mostly tropical species, may nodulate with many different strains of both slow- and fast-growing rhizobia (Neves and Rumjanek 1997). Cowpea, like most of the tropical legumes, is non-selective in the *Rhizobium* species it requires for effective symbiosis; it is a broad host-range legume that can be nodulated by several fast-growing rhizobial strains (Martins *et al.* 1997), although it is characteristically nodulated by strains described as "cowpea-miscellany" bradyrhizobia (Jordan 1984, Eaglesham *et al.* 1987, Thies *et al.* 1991). Therefore, better knowledge and utilisation of different types of *Rhizobium* species might contribute to both the identification of strains with potential for use as cowpea inoculants and the evaluation of their symbiotic effectiveness to increase the input of biologically-fixed nitrogen.

The suitability of a *Rhizobium* strain as a successful inoculant can be estimated by the percentage of nodulation and the N_2-fixing activity of plant roots. Time course of nitrogen fixation has been studied for soybean, fababean, and common bean (Zapata *et al.* 1987a,b, Peña-Cabriales *et al.* 1993). However, the results obtained with one legume species may not be applicable to another species because of the specificity of symbiosis. Moreover, biological fertilisation requires the optimisation of inoculation techniques to obtain good timing of the interaction between N_2-fixation and other plant processes and thus to enhance plant productivity. More information is also required on the interaction of rhizobial activity with the growth dynamic of plant components such as the root and shoot, and also with the dynamic of important physiological processes such as photosynthesis and transpiration.

The present study was conducted to ascertain the capacity of a "non-cowpea-miscellany" *Rhizobium* to nodulate two cowpea genotypes different in monocarpic senescence (Gwathmey *et al.* 1992). To evaluate the efficiency of symbiosis, the N_2-fixing activity of plant roots has been measured by means of both acetylene reduction assay and ureides' concentration in xylem sap, because the acetylene reduction activity of decapitated root systems may be significantly lower than that measured in intact plants (Mague and Burris 1972). On the other hand, ureides transported in the xylem of cowpeas represent about 80 % of the total N fixed by the root nodules (Herridge *et al.* 1978). Moreover, P_N measurements and allometric analyses of biomass allocation patterns were used to study the effect of *Rhizobium* inoculation on carbon fixation activity and plant ontogeny and to investigate the co-ordination between these plant processes and the diazotrophic activity.

Materials and methods

Two cowpea [*Vigna unguiculata* (L.) Walp.] genotypes with contrasting senescence traits, supplied by the Department of Botany and Plant Sciences of the University of California, Riverside, were used: CB5 showing normal monocarpic senescence and 7964 showing delayed leaf senescence (Gwathmey *et al.* 1992). The *Rhizobium* NA936 strain, supplied by the Department of Agrobiotechnology, ENEA-Casaccia, Rome, was grown in the yeast-mannitol medium (Vincent 1970), pH = 6.8, for 24 h at 28 °C.

Seeds were germinated at 28 °C in a growth cabinet and then sown in 3 000 cm^3 pots filled with sand and clay-loam soil (1 : 1, m : m). At emergence, 30 plants of each genotype were inoculated with the *Rhizobium* culture at the final concentration of about 10^7 colony forming units per plant, while uninoculated plants were provided with 2 mM nitrogen ($NaNO_3$). All plants were weekly supplemented with 200 cm^3 of N-free Jensen mineral solution (Vincent 1970) and a daily irrigation with tap water replenished the pot mass loss due to evapotranspiration.

Plants were grown until pod set in a glasshouse where the night/day temperature was controlled to maintain a minimum and a maximum of 18 and 30 °C, respectively. Growth photon flux densities in the glasshouse were 30 % lower than the typical daily full sunlight fluxes during the early summer months (May–July) in the mediterranean area.

Four replicates were sampled for plant growth analysis and measurements of physiological activities at 30, 40, 48, 54, 60, 68, and 75 d after planting. P_N was measured using a *LiCor 6400* (*LiCor,* USA) portable IRGA analyser. A central part of the last fully expanded leaf was clamped into a cuvette and irradiated with red radiation of 1200 $\mu mol\ m^{-2}\ s^{-1}$ until a steady CO_2 uptake was reached and maintained for 5 min. At least three measurements were done for each sample and a constant relative humidity of 60 % and a temperature of 28 °C were imposed during the measurements.

After the P_N measurements, plants were cut just below the first node and separated into leaves and stem. The leaf area was measured using a leaf area meter *LI-3000* (*LiCor,* USA), and then each plant part was dried at 65 °C until mass constancy. Exudates from the cut ends of the stumps were collected to determine the ureide concentration in xylem sap (Elowad *et al.* 1987). Stumps were then harvested and separated into roots, nodules, and stem. Roots were carefully rinsed under water to remove as much soil as possible and were oven-dried for plant biomass determination, as well as residual stems.

Fresh nodules cut from the roots were incubated for 1 h at 28 °C in sealed bottles in an atmosphere of 10 % acetylene to detect N_2-fixation by means of the acetylene reduction assay (ARA). The ethylene produced was measured by a *Perkin Elmer Sigma3B* gas chromatograph [*FID*, 80–100 mesh *Porapak Q* column (2 m×3 mm i.d.) at 50 °C with N_2 carrier gas at a flow rate of 417 $mm^3\ s^{-1}$].

Values are reported as the means ± standard error of at least four samples, unless otherwise indicated, and the statistical analysis of results was performed by the oneway analysis of variance.

Results

Inoculation of cowpeas with the *Rhizobium* NA936 strain resulted in the nodulation of plant roots that was observed in each sample after 30 d of growth. Uninoculated plants only developed few and small nodules due to some indigenous *Rhizobium* present in the soil used to fill the pots. The N_2-fixing activity of inoculated plants was measured by means of both ARA and ureides concentration in xylem sap at different stages of plant development (Fig. 1*A,B*), while uninoculated plants were only tested with the second method (values not shown). Diazotrophic activity in all uninoculated plants was very low and significantly (p<0.005) differed from that of inoculated plants of both genotypes that similarly reached maximum activity at flowering.

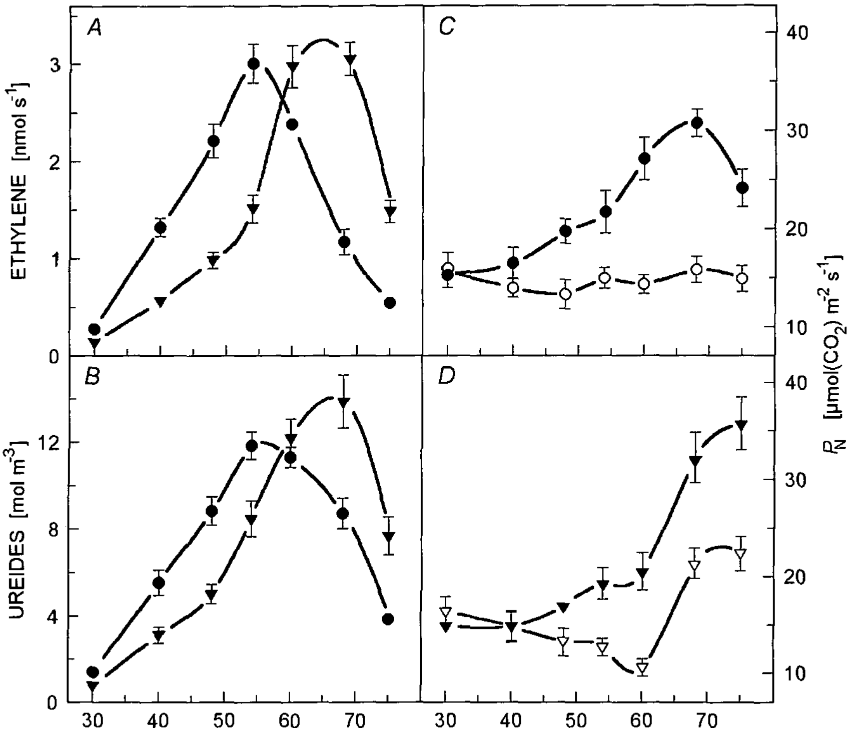

Fig. 1. Symbiotic N_2-fixation (*A*, *B*) and net photosynthetic rate, P_N (*C*, *D*) during vegetative growth of CB5 (●) and 7964 (▼) cowpea [*Vigna unguiculata* (L.) Walp.] genotypes inoculated (●, ▼) or not (O, ▽) with *Rhizobium* NA936 strain. Diazotrophic activity was measured by means of the acetylene reduction assay (*A*) and the ureide concentration in xylem sap (*B*). Means of four plants ± SE (in some cases smaller than symbol size).

However, the N_2-fixation reached the maximum more quickly in CB5 than in 7964 plants and with an anticipation of about 10 d. The acetylene reduction activity and the ureide concentration in xylem sap exhibited similar trends (Fig. 1*A*,*B*) and were well correlated ($r = 0.98$ with $p<0.05$).

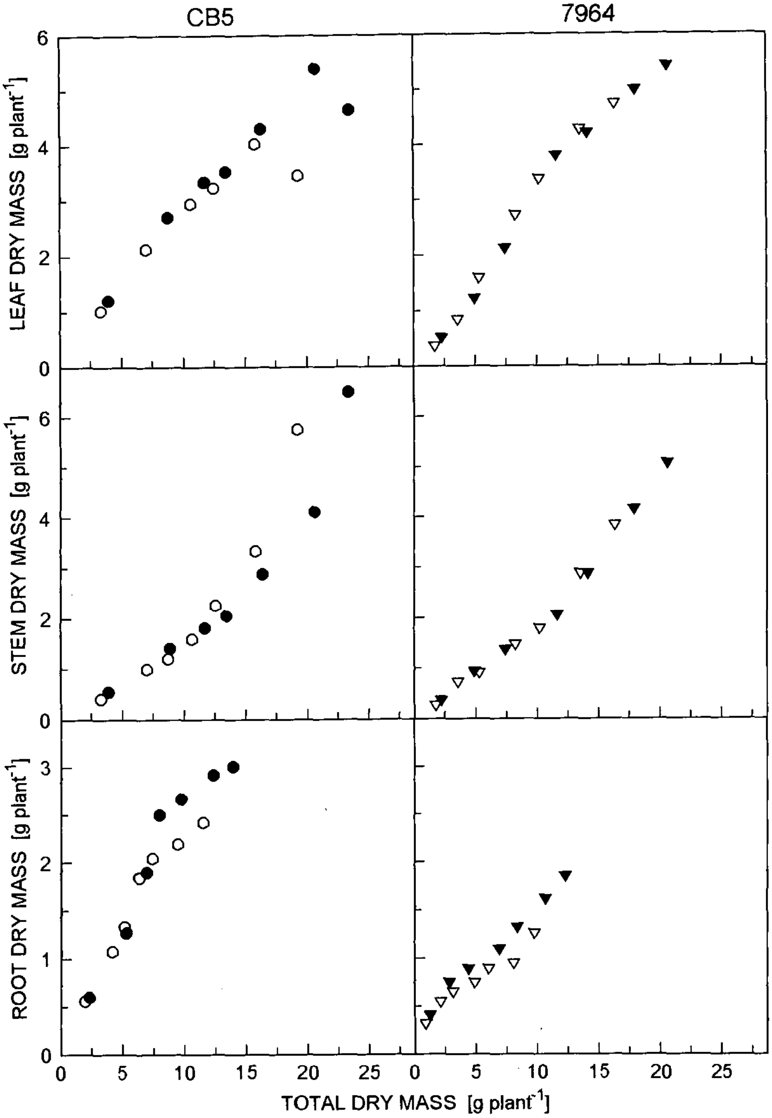

Fig. 2. Allometric analysis of dry biomass during vegetative growth of CB5 (*left*) and 7964 (*right*) cowpea [*Vigna unguiculata* (L.) Walp.] genotypes inoculated (●, ▼) or not (○, ▽) with *Rhizobium* NA936 strain. Means of four samples with standard error varying between ±1 and ±11 %.

Plant inoculation with the *Rhizobium* strain induced a significant increase ($p<0.01$) of P_N in both genotypes at 40 d after planting (Fig. 1*C,D*) and the maximum activity was higher in 7964 than in CB5. In the latter genotype the maximum activity was achieved earlier and then a decrease was observed, similarly as for N_2-fixation. In the uninoculated plants of both genotypes, photosynthesis declined slightly in the early stages of growth. Thereafter, P_N oscillated around 15 μmol(CO_2) m^{-2} s^{-1} in CB5 plants, while in the 7964 genotype photosynthetic activity showed a rapid increase at 60 d after planting, with a trend similar to that of the inoculated plants. Transpiration rate had a general time course comparable to that of P_N except that its maximum was much less pronounced (values not shown).

Total biomass of inoculated plants of both genotypes was always higher than that of uninoculated ones (Fig. 2) and the differences were significant at the 0.05 level. Allometric measurements did not show some specific effects of rhizobial inoculation on root, stem, and leaf biomass accumulation for any plants during the early stages of growth. Conversely, after about 50 d of growth a significantly ($p<0.05$) higher biomass accumulation was observed in the inoculated roots of both genotypes and in the leaves of inoculated CB5 plants.

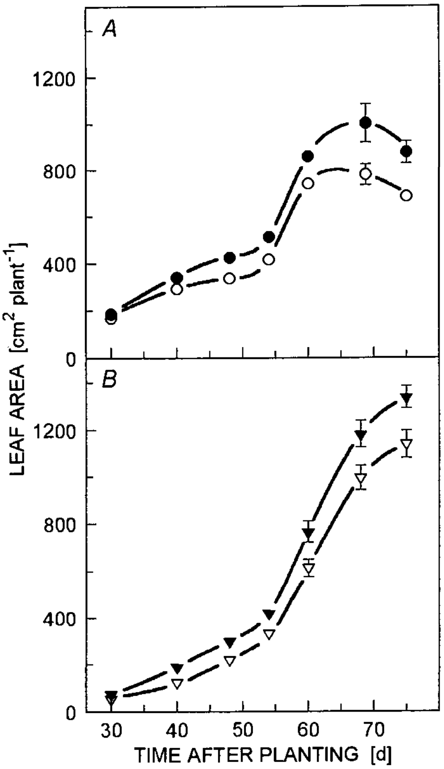

Fig. 3. Total leaf area measured during vegetative growth of CB5 (*A*) and 7964 (*B*) cowpea [*Vigna unguiculata* (L.) Walp.] genotypes inoculated (●, ▼) or not (○, ▽) with *Rhizobium* NA936 strain. Means of four plants ± SE (in some cases smaller than symbol size).

A similar significant effect was in the total leaf area of both genotypes (Fig. 3). In addition, inoculated plants of the CB5 genotype showed a delay in the typical senescent decline of total leaf area. The ratio of total leaf area to total leaf biomass did not significantly differ over time between inoculated and uninoculated plants (values not shown), confirming that an increased nitrogen fixation and availability corresponded to an increased carbon utilisation for growth.

Discussion

The *Rhizobium* NA936 strain was successful in inducing an efficient nodulation in the CB5 and 7964 cowpea genotypes and a relevant N_2-fixation activity was detected mainly at flowering. A good correlation was observed between the concentration of ureides in xylem sap and acetylene reduction, and a substantial correspondence of the two methods for measuring N_2-fixation activity was also confirmed, as already reported for various plant species (McNeil and LaRue 1983, Elowad *et al.* 1987). The maximum diazotrophic activity was reached in CB5 plants about 14 d earlier than in the 7964 genotype that exhibited delayed senescence traits. This seems to confirm that a short cycle of plant growth requires an early and fast efficiency of nodules and that interaction between the bacterium and the host plant is genotype dependent.

The nodulation process is started by the mutual exchange of molecular signals between the bacterium and the host plant that exudes specific nodulation inducers to control the specificity of the plant toward inoculant strains (Hungria and Stacey 1997). In our experiment, the temporal shift of the maximum diazotrophic activity between the different cowpea genotypes might suggest that the exudation of inducers is timed to optimise the nodule activity in response to the plant requirement for N that reaches maximum soon after flowering, *i.e.*, at fruit development (Summerfield *et al.* 1977, Peoples *et al.* 1983).

Fruit development requires a great availability of N and an eligible important source of this highly required element may be ribulose-1-5-bisphosphate carboxylase (RuBPC), since it is the most abundant soluble protein in plants (Osaki *et al.* 1995). In the blossom leaves of senescent plants a probably hormone regulated signal triggers the degradation mechanism of this protein leading the leaf to eventually loose photosynthetic capacity and viability. In the CB5 cowpea genotype this degradation process might be prevented by an exogenous N supply available from increased fixation of atmospheric N_2 at flowering, as reported by Summerfield *et al.* (1977) and Neves *et al.* (1982).

A temporal shift of maximum activity was also observed in P_N of inoculated plants of the two genotypes and a declining P_N closely related to decline in N_2-fixation was observed in the CB5 senescent genotype. The establishment of an efficient symbiosis clearly affected P_N which was significantly higher than that of NO_3-fed plants in both legumes, thus indicating that a high P_N requires a symbiotic N_2-fixation. On the other hand, our results were also in agreement with the studies showing that plants can increase their P_N to compensate for the needs of their microbial partners (Paul and Clark 1989). The mutual relationship between biological N_2-fixation and photosynthesis was investigated in soybean where the photosynthate supply to the nodules limited N_2-fixation (Pandey 1996, Shantharam and Mattoo 1997) and the control of both photosynthesis and diazotrophic activity was integrated by a common signal transduction system (Joshi and Tabita 1996, Hungria and Stacey 1997).

The temporal shift of maximum P_N of inoculated CB5 plants might also be associated with the increased duration of a functional leaf area and the increased accumulation of biomass in leaves. Some investigations have already addressed this link. Peoples *et al.* (1983) report a strong correlation between the peptide hydrolase activity and the degradation of RuBPC occurring in cowpea during senescence in blossom leaves and suggest a regulation of this enzyme by a senescence signal from fruits. Conversely, Hayati *et al.* (1995), in a study on the effect of carbon and nitrogen supply on soybean seed filling and senescence, conclude that it is not the N demand that triggers leaf senescence but some processes in the leaf itself. This argument merits further examination because the existence of a senescence signal is certain but little is known about its nature and action mechanism. However, an increase of leaf area duration in response to *Rhizobium* inoculation on the CB5 genotype suggests that an adequate supply of N by inoculated bacteria might interact with that signal. Moreover, the efficiency of root nodules after flowering, although reduced, might indicate that nodules senescence occurred at a phase of plant growth later than that we examined. When in cowpea the older leaves senesce, the young leaves, the branches, and the nodulated roots continue to grow and are physiologically active (Neves *et al.* 1982).

Another important point of our investigation is plant ontogeny as affected by biological N_2-fixation. Allometric measurements evidenced that *Rhizobium* inoculation did not affect the biomass allocation to the main sinks with the exception, at flowering and thereafter, of roots in both genotypes and leaves in CB5, since the biomass content of these plant components was significantly higher than that of NO_3^- fed plants.

In conclusion, inoculation of the *Rhizobium* NA936 strain: (*a*) induced an effective symbiotic N_2-fixation in the two cowpea genotypes that exhibited similar maximum activity; (*b*) resulted in a diazotrophic activity that increased more slowly in the 7964 delayed leaf senescence genotype during vegetative growth, reaching its maximum later than the CB5 senescent genotype, thus suggesting that plant ontogeny might directly affect nodular activity; (*c*) *vice-versa*, N_2-fixation induced a longer duration of the functional leaf area in the senescent CB5, most likely by means of a considerable supply of N to leaves that limited RuBPC degradation; (*d*) maxima of N_2-fixation and P_N were timed with the same temporal shift in both cowpea genotypes, thus indicating a good correlation between these two physiological processes.

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