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The use of GUS-reporter gene to study the effect of *Azospirillum-Rhizobium* coinoculation on nodulation of white clover

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Abstract The *gusA*-marked *Azospirillum lipoferum* T1371, constructed by inserting transposon mTn5SSgusA20 from *Escherichia coli* S17-1 λ-pir into the genome of *Azospirillum lipoferum* 137, was used to evaluate its effect on nodulation of white clover with and without *Rhizobium* inoculation. When inoculated alone, *Azospirillum* colonized the tap roots, secondary roots and root hairs. The combined inoculation of white clover with *Rhizobium leguminosarum* bv *trifolii* and *A. lipoferum* enhanced the number of nodules by 2–3 times from 5 to 20 days after inoculation (DAI). The combined inoculation also enhanced acetylene reduction activity by 2.3–2.7 times at 20 DAI. Moreover, *Azospirillum* was observed colonizing the tap root, root hairs and sites near or on the nodules. These results suggest that the formation of additional infection sites by *A. lipoferum,* with a combined inoculation, may be the mechanism that will enhance nodulation and nitrogen fixation of white clover.

Key words Acetylene reduction activity · *Azospirillum-Rhizobium* coinoculation · GUS-reporter gene · Nodulation · White clover

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Introduction

There have been many reports of the beneficial effects of *Azospirillum* spp. on the growth and yield of nonleguminous plants. Bacteria can colonize the plant root surface and penetrate into the root interior (Schank et al. 1979; Michiels et al. 1989). Moreover, coinoculation of *Rhizobium* spp. and *Azospirillum* spp. enhances the nodule number and grain yield of various leguminous crops (Sarig et al. 1986; Yahalom et al. 1987). Coinoculation also increases the shoot length, dry weight, number of root hairs and root diameter of alfalfa (Itzigsohn et al. 1993).

In white clover (*Trifolium repens*), the effects of combined *Rhizobium* and *Azospirillum* on nodulation depend on a precise *Rhizobium/Azospirillum* cell ratio (Plazinski and Rolfe 1985b). The ratio between *Rhizobium leguminosarum* bv *trifolii* and *Azospirillum* at 1:500, 1:1000 and 1:1500, mixed before inoculation, inhibits the nodule formation. A cell ratio of 1:2000 stops the formation of white clover nodules (Plazinski and Rolfe 1985c). However, the non-nodulated plants show no nitrogen deficient symptoms but form more root hairs and lateral roots (Plazinski and Rolfe 1985a). Thus, it is suggested that when coinoculated with *Rhizobium, Azospirillum* stimulates the formation of epidermal cells that become infected root hair cells, or creates additional infection sites that are later occupied by rhizobia (Plazinski and Rolfe 1985c; Yahalom et al., 1987).

In this study, we used a GUS-reporter gene to clarify the effect of *Azospirillum-Rhizobium* coinoculation on white clover nodulation.

Materials and methods

Bacteria strains

The bacteria strains used in this study were *Azospirillum lipoferum* 137, (kindly supplied by Dr. Lyudmila Vassyuk, Research Institute for Agricultural Microbiology, St. Petersburg, Russia), *gusA*-marked *A. lipoferum* T1371 (Tchebotar et al., manuscript

submitted), *Rhizobium leguminosarum* bv. *trifolii* ANU 843 (provided by Dr. Ivan R. Kennedy, University of Sydney, Australia), and *gusA*-marked *R. l.* bv. *trifolii* ANU 843 (Tp3) (provided by Dr. Robert Ridge, International Christian University, Tokyo, Japan).

Plant culture and inoculation

White clover (*Trifolium repens*) cv. California ladino seeds were surface sterilized with 70% alcohol for 5 min, followed by 5% H_2O_2 for 5 min. Seeds were then washed for 2 min with sterile deionized water. This was done five times. Sterilized seeds were germinated on petri plates with 2% agar under dark conditions at 28 °C. Seedlings were transplanted, at 3 seedlings pouch^{-1} to sterile Seed Pack Growth Pouches (MEGA International, Minneapolis, Minn.). Sterile water and half-strength N-free nutrient solution (Akao and Kouchi 1989) were supplied alternately to each pouch. Seedlings were kept in a greenhouse with a day/night temperature of 28/23 °C and a 14 h photoperiod.

For inoculation tests, *gusA*-marked *A. lipoferum* T1371 and *A. lipoferum* 137 were grown overnight at 28 °C on Döbereiner Agar sucrose medium with and without 20 μg spectinomycin ml^{-1}, respectively (Vassyuk et al. 1990). *R. l.* bv. *trifolii* ANU 843 was grown overnight at 28 °C on yeast mannitol agar (YMA) plates, and *R. l.* bv. *trifolii* ANU 843 (Tp3) was grown on YMA plates with 50 μg spectinomycin ml^{-1}. Each strain was centrifuged at 10000 rpm, 4 °C for 10 min. Pellets were washed twice with sterile saline solution, and cell density was adjusted to 4.0×10^7 cells ml^{-1} with sterile deionized water. Single or dual (1:1 cell ratio) inoculation of *Rhizobium* spp. and *Azospirillum* spp., at 1 ml pouch^{-1}, was done 3 days after transplanting the white clover.

Nodulation and acetylene reduction assay

The nodulation and colonization patterns of white clover roots by *Azospirillum* spp. and *Rhizobium* spp. were observed 5, 10 and 20 days after inoculation (DAI). Five replications were used for each observation. Exhumed roots, with or without nodules, were placed in test tubes with a GUS assay solution composed of 10 ml of a phosphate buffer (3.9 ml of 0.2 M NaH_2PO_4, 6.1 ml of 0.2 M Na_2HPO_4), 20 μl of 10% sodium dodecyl sulfate, and 100 μg of X-Gluc ml^{-1}. Air in the test tubes was removed under vacuum for 30 min. The test tubes were then incubated overnight at 37 °C. Roots and nodules were observed under a light microscope (Nikon Optiphot) equipped with a camera. Acetylene reduction activity (ARA) at 20 DAI was determined following the procedure of Francisco et al. (1992). Analyses of variance were computed using IRRISTAT 93-1 (IRRI, Philippines) with log transformation, and means were compared using least significant difference.

Results and discussion

Previous reports have shown that coinoculation of *Rhizobium* and *Azospirillum* enhanced the nodulation and yield of leguminous plants (Plazinski and Rolfe 1985b; Yahalom et al. 1987). However, it is not clear what mechanism regulates this phenomenon. The formation of additional infection sites on the clover roots is one of the possible modes of action (Plazinski and Rolfe 1985c). Hence, it is very important to detect the sites of *Azospirillum* spp. attachment to the root surface when it is inoculated alone or in combination with *Rhizobium* spp. In this study, we used *gusA*-marked *A. lipoferum* T1371, which has similar characteristics with that of the wild strain, to visually observe its colonization pattern in white clover roots and evaluate its effect on nodulation.

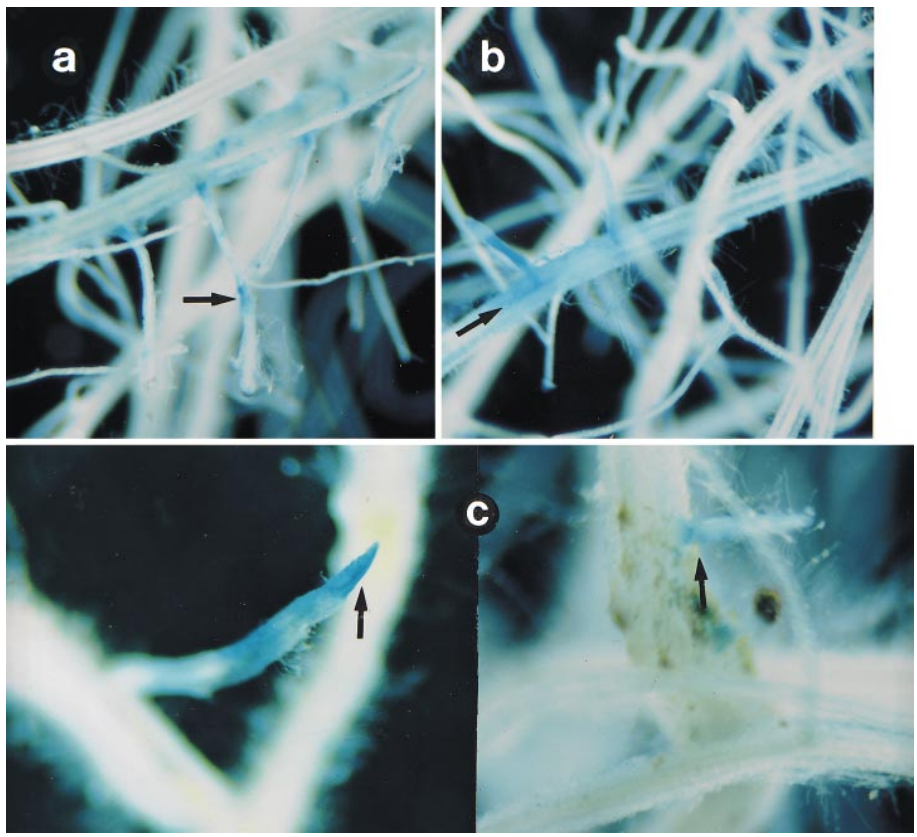

Fig. 1a–c Colonization of white clover roots by *gusA*-marked *Azospirillum lipoferum* T1371 at 5, 10, and 20 days after inoculation (DAI). Blue staining (*arrows*) indicates GUS (β-D-glucuronidase) activity of the bacteria. The colonization of the main roots, secondary roots, and portions of the roots hairs by *Azospirillum* is sparse at 5 DAI (**a**) and 10 DAI (**b**) but a strong colonization of the root tips and root hairs is observed at 20 DAI (**c**)

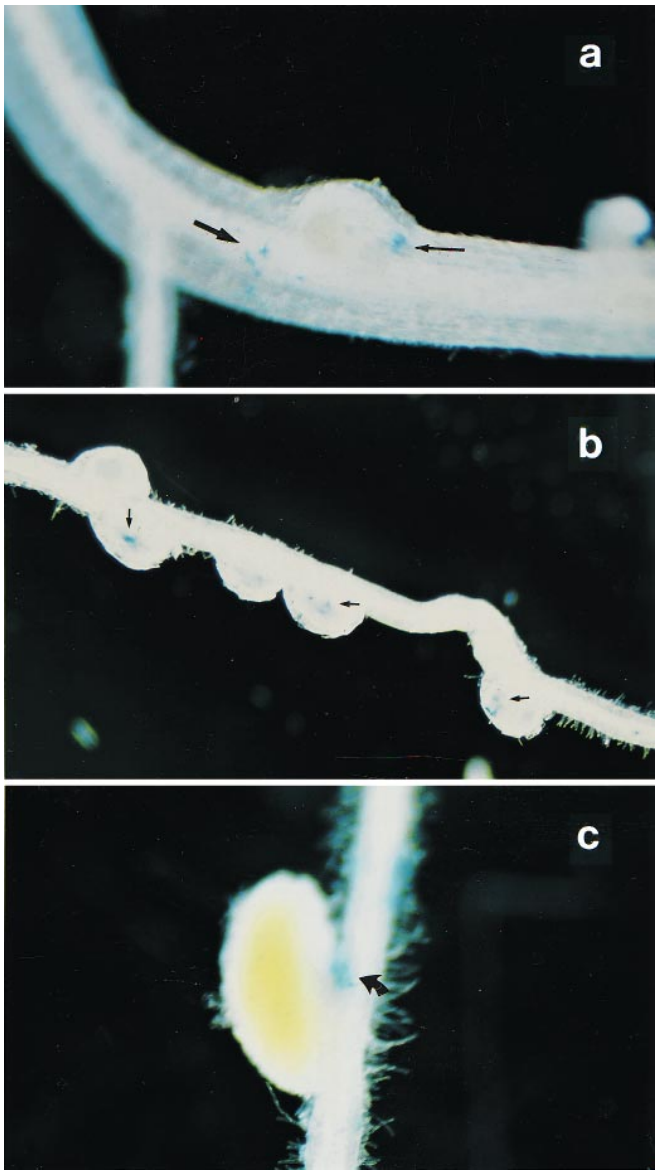

Fig. 2a–c Young root nodules of white clover coinoculated with *Rhizobium leguminosarum* bv *trifolii* ANU 843 and *gusA*-marked *Azospirillum lipoferum* T1371 at 10 DAI (**a, b**) and 20 DAI (**c**). Blue-stained *A. lipoferum* T1371 (*arrows*) mostly colonized the surface near the nodule

Figure 1 shows the ability of *Azospirillum* spp. to colonize white clover roots. In all replications, the *gusA*-marked *A. lipoferum* T1371 colonized the tap roots, secondary roots, and some parts of the root hairs at 5 and 10 DAI. At 20 DAI, strong staining was observed on the root tips and root hairs suggesting an abundant colonization of the bacteria. On the other hand, when coinoculated with *R. l.* bv *trifolii* ANU 843, *A. lipoferum* T1371 colonized the tap root, root hairs, and sites near or on the nodules (Fig. 2).

Similar colonization patterns were observed in cereal-*Azospirillum* associations (Patriquin and Dobereiner 1978; Umali-Garcia et al. 1980). Patriquin et al. (1983) have suggested that *Azospirillum* can invade the intercellular spaces by penetrating root hairs or regions of branches. *Azospirillum* showed pectinolytic activity in culture (Tien et al. 1981; Vassyuk et al 1990). This may have facilitated the bacterial attachment to the root surface and its penetration into the root interior. Thus, we conclude that the enhanced nodule numbers in treatments with combined inoculations were a result of the formation of more infection sites for *Rhizobium.*

Plazinski and Rolfe (1985c) and Yahalom et al. (1987) reported that inoculation enhanced the number of nodules when *Azospirillum* was applied before or after *Rhizobium.* However, nodule formation was inhibited when *Rhizobium* spp. and *Azospirillum* spp. were applied in a mixture (Plazinski and Rolfe 1985c). In this study, we applied *Rhizobium* and *Azospirillum* in a 1:1 cell ratio, and we observed enhanced nodule numbers from 5 to 20 DAI instead of inhibition (Fig. 3). The effect was more evident at 5 DAI when clover plants treated with a combined inoculation produced nodules

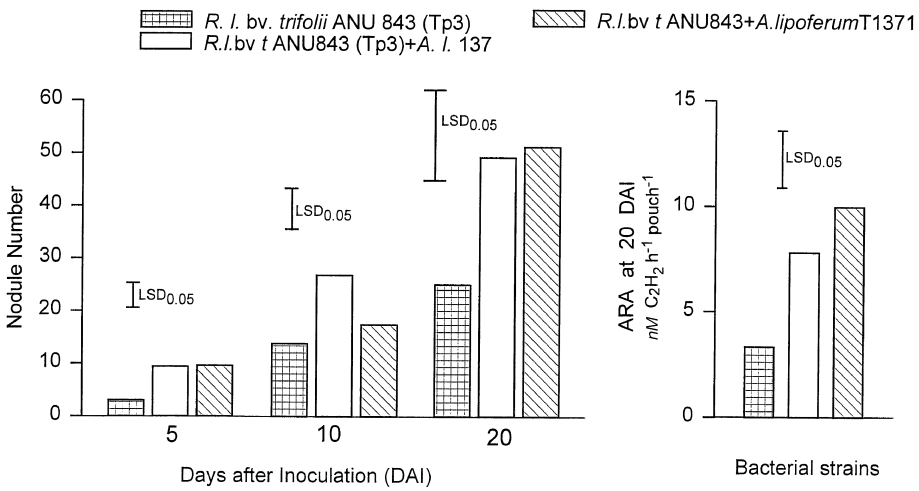

Fig. 3 Nodulation of white clover from 5 to 20 DAI and acetylene reduction activity (*ARA*) at 20 DAI as influenced by *Rhizobium* spp. and *Azospirillum* spp. inoculation

more than 3-fold when compared with plants inoculated with *R. l.* bv *trifolii* ANU 843 (Tp3) alone (control). Coinoculation doubled the number of nodules at 10 and 20 DAI. Moreover, ARA determined at 20 DAI was significantly higher (2.3–2.7) with combined inoculation than with the control. These results could be partly due to strain-cultivar specificity. Similar effects have been observed for combined *Rhizobium-Azospirillum* inoculation in lupine and alfalfa (Dr. L. F. Vassyuk, personal communication). The question of whether the *Rhizobium/Azospirillum* cell ratio has any effect on the number of nodules is yet to be studied.

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