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Influence of *Azospirillum* Strains on the Nodulation of Clovers by *Rhizobium* Strains

JACEK PLAZINSKI* AND BARRY G. ROLFE

Genetics Department, Research School of Biological Sciences, Australian National University, Canberra City, 2601, Australia

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Mixed cultures of several *Azospirillum* and *Rhizobium trifolii* strains caused either an inhibition or stimulation of nodule formation on plant hosts as compared with nodulation of plants inoculated with *R. trifolii* alone. *Azospirillum* strains affected the nodulation process at a precise cell ratio (*R. trifolii*/*Azospirillum* cells) and time of inoculation. All *Azospirillum* strains used showed a variation in their ability to inhibit or enhance nodulation by *R. trifolii* strains. When nonviable cell preparations of *Azospirillum* strains were used for mixing experiments, no effect on nodulation was observed. A decrease in the effectiveness of normally Nod$^+$ Fix$^+$ *R. trifolii* strains was observed when an *Azospirillum* strain caused an increase in nodule number.

There is little information available regarding ecological interactions of bacterial diazotrophs which are able to colonize the root zones of leguminous or nonleguminous plants and subsequently fix nitrogen either in a symbiotic or an associative manner. Nevertheless, there are many reports on interactions between different bacterial strains, although these are mainly concerned with various *Rhizobium* strains (5, 10, 12, 13, 15, 16, 18, 20–22, 26).

Until recently little was known about the nitrogen-fixing *Azospirillum* bacteria and their association with plants (1, 25). Currently, the genus *Azospirillum* has attracted the attention of microbiologists, agronomists, and ecologists due to its ability to fix nitrogen in association with certain nonleguminous plants (1, 7, 25).

There have been reports that soybeans (*Glycine max*) gave increased yields after inoculation with a mixed culture of *Rhizobium japonicum* containing either *Azotobacter vinelandii* or *Azospirillum brasilense* (6, 12, 22). Rai (15) has reported an increase in grain yield, nodule dry weight, and nitrogenase activity of chick pea nodules when plants were inoculated with *A. brasilense* together with a *Rhizobium* strain. Iruthayathas et al. (10) have observed a substantial increase in the nodulation, N_2 fixation, and shoot dry-matter production by winged beans and soybeans when these plants were inoculated with *Rhizobium-Azospirillum* combined cultures. Recently, an enhancement of the nodulation of several legumes by *Azospirillum* addition has been reported (S. Sarig, Y. Kapulnik, and Y. Okon, Abstr. 3rd Int. Symp. N_2-Fixation in Non-Legumes, 1984, p. 45).

No studies have been made to detect any beneficial effects when *Rhizobium trifolii* and *Azospirillum* strains are brought into interaction in clover pastures.

In this paper, we report the negative effect of *Azospirillum* strains on the *R. trifolii*-clover symbiosis, when studied under the artificial conditions of an agar plate assay (19).

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used are listed in Table 1.

Media and culture conditions. The following growth media used have been previously described: nutrient agar (NA [2]), Bergersen medium (BMM [18]), and tryptone-yeast extract medium (TY [3]). All media were supplemented either with kanamycin (200 μg ml^{-1}), streptomycin (200 μg ml^{-1}), spectinomycin (20 to 100 μg ml^{-1}), or carbenicillin (100 μg ml^{-1}), when required.

Bacteria were grown on agar plates at 29°C.

Cell mixture experiments. *Azospirillum* strains were maintained on NA, and *R. trifolii* strains were maintained on BMM. Before inoculation, *Azospirillum* and *R. trifolii* strains were grown on TY plates for 24 and 16 h, respectively. Bacteria were scraped off the plates and suspended in 1 ml of liquid F medium (8). For mixed inocula the strains were first diluted in F medium and then mixed at appropriate cell ratios on the F plates that the plants were grown on (0.1 ml of each strain suspension) before planting. The undiluted cell suspensions were 1×10^8 to 5×10^8 cells ml^{-1}. The viable-cell number was measured as CFU ml^{-1} on BMM plates.

Seed inoculation and plant assays. Commercial seeds of white clover (*Trifolium repens* [New Zealand white clover 5826]) and subterranean clover (*Trifolium subterraneum*) were used. Surface sterilization was as follows: seeds were washed for 5 min with 96% ethyl alcohol and 5 min with 0.2% $HgCl_2$, washed three times with sterile distilled water, soaked for 10 min in water and 5 min with 12% sodium hypochlorite, washed five times with sterile water, soaked for 10 min in water, and finally washed again with water.

Bacterial strains were inoculated onto clover by using the rapid plate-screening method (19). Inoculated plates were incubated vertically in a growth cabinet with a 22°C 18-day and 19°C night and with an average light intensity of 340 microeinsteins m^{-2} s^{-1}.

Preparation of dialyzed cell suspensions. A suspension of *R. trifolii* ANU794 was washed with liquid F medium. At the same time a culture of *A. brasilense* SP107 was washed once with liquid F medium, and the cell suspension was pipetted into a 9-mm-width dialysis bag and sealed. Two seedlings were inserted into Fahraeus slides (8), inoculated with the *R. trifolii* strain, and immersed in a 150-ml beaker containing liquid F medium. The dialysis bag containing *A. brasilense* SP107 was added to the beaker.

Plant responses were continuously assayed from 1 to 6 weeks for their general appearance, nodule formation, and nitrogen fixation ability.

Preparation of a nonviable-cell suspension. Liquid F medium suspensions (ca. 1×10^7 to 5×10^7 cells ml^{-1}) of *A.*

* Corresponding author.

TABLE 1. Bacterial strains used

Strain	Relevant characteristics[a]	Source or reference
R. trifolii		
ANU794	Prototrophic N_2-fixing parental strain Sm^r; gives a Nod^+ Fix^+ response on both white and subterranean clovers	6
ANU329	Nod^+ Fix^+ Sp^r mutant of ANU794	19
ANU844	Sp^r mutant of ANU843	6
ANU845(pRt032)	Sym plasmid-cured Nod^- Sp^r mutant of ANU843 carrying the subcloned nodulation genes of ANU843 into the plasmid vector pKT240	P. R. Schofield
ANU850	Sym plasmid-cured Nod^- Sp^r mutant of ANU843, carrying pJB5JI plasmid[b]	M. A. Djordjevic
ANU870	Sym plasmid-cured Nod^- Sp^r mutant of ANU843, carrying pBR1AN plasmid[b]	M. A. Djordjevic
ANU1030	Prototrophic N_2-fixing, highly ineffective strain; Fix^- on subterranean clovers	J. C. Burton (Nitragin Co.)
T1 Km^r	Km^r mutant of N_2-fixing T1 strain	Laboratory isolate
T1 Sp^r	Sp^r mutant of N_2-fixing T1 strain	23
A. brasilense		
SP7[c]	Prototrophic, N_2-fixing, Cb^r, Nir^- strain	4
SP107	Prototrophic, Sm^r, Cb^r, N_2-fixing, Nir^- strain	4
SP245	Prototrophic, Sp^r, Cb^r, N_2-fixing, Nir^- strain	J. Dobereiner
A. lipoferum SP59[c]	Prototrophic, Cb^r, N_2-fixing, Nir^+ strain	4
Azospirillum spp. SP242	Prototrophic, Sm^r, Cb^r, N_2-fixing, Nir^+ strain previously classified as *A. lipoferum* but does not use glucose as a carbon source	J. Dobereiner

[a] Sp^r, Spectinomycin resistant; Sm^r, streptomycin resistant; Km^r, kanamycin resistant; Cb^r, carbenicillin resistant; Nir^+, able to denitrify; Nir^-, no ability to denitrify.
[b] The characteristics of both plasmids have been previously described (for pJB5JI, see reference 11; for pBR1AN, see reference 17).
[c] Strains SP7 and SP59 are strains ATCC 29145 and ATCC 19707, respectively.

brasilense SP107 and SP245 were boiled for 15 min in a microwave oven. Afterwards, 0.1 ml of each suspension was plated out onto BMM plates to detect any cell viability. Of the heat-killed cell suspension, 0.1-ml samples were mixed on F plates with an equal volume of a *R. trifolii* suspension before plant inoculation.

Test for bacteriocin production. The method used for testing bacteriocin production by *Rhizobium* and *Azospirillum* strains has been described previously (9).

Experimental design and statistical analysis. For each experiment at least eight replications were done. Uninoculated controls were included in each replication. Analyses of variance and comparisons of means were done separately on all data to detect significant differences between the treatment means of each latin square at the 0.05 level of probability. Nodulation was expressed as a percentage of the control.

RESULTS

Inhibition of nodulation. When five different *Azospirillum* strains were mixed with nine different *R. trifolii* strains and these mixtures were inoculated onto clover, inhibition of nodulation was observed in five of the *R. trifolii* strains (Table 2). In the case of three *R. trifolii* strains (ANU794, ANU870, and T1 Km^r), the inhibition of nodulation by all *Azospirillum* strains ranged from 25 to 100%. Two other *R. trifolii* strains (ANU844 and ANU845 [pRt032]) showed varying inhibition of nodulation by several *Azospirillum* strains on white clover plants only (Table 2). Before inoculation of the plants, *R. trifolii* and *Azospirillum* strains were mixed in different cell ratios from 1:1 to 1:2,500. When *R. trifolii* ANU794 and *A. brasilense* SP107 were mixed together before inoculation of the plants at a cell ratio of 1:2,000, 100% inhibition of nodulation on white clover plants

TABLE 2. Inhibitory effect of *Azospirillum* strains on clover nodulation when *R. trifolii* and *Azospirillum* strains were inoculated together

Azospirillum strain	Effect of inoculation[a] with *R. trifolii* strain on type of clover[b]:																	
	ANU794		ANU329		ANU844		ANU845 (pRt032)		ANU850		ANU1030		ANU870		T1 Km^r		T1 Sp^r	
	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC
SP7	I_{100}	I_{100}	NI	NI	NI	NI	NI	NT	NA	NI	NI	NI	I_{100}	I_{100}	I_{100}	I_{100}	NI	NI
SP59	I_{100}	I_{100}	NI	NI	I_{75}	NI	NI	NT	NA	NI	NI	NI	I_{100}	I_{100}	I_{75}	NI	NI	NI
SP107	I_{100}	I_{100}	NI	NI	I_{50}	NI	I_{25}	NT	NA	NI	NI	NI	I_{100}	I_{100}	I_{75}	I_{25}	NI	NI
SP242	I_{100}	I_{100}	NI	NI	I_{75}	NI	NI	NT	NA	NI	NI	NI	I_{100}	I_{100}	I_{50}	I_{100}	NI	NI
SP245	I_{100}	I_{100}	NI	NI	I_{75}	NI	NI	NT	NA	NI	NI	NI	I_{100}	I_{100}	I_{75}	I_{100}	NI	NI

[a] Values used were significantly different ($P = 0.05$) from those for the control and were means for a minimum of 12 plants taken from at least three experimental repeats. I, Inhibition of nodulation expressed as a percentage of *R. trifolii* nodulation activity alone (I_{100} is 100% inhibition); NI, no inhibition; NT, not tested; NA, not applicable.
[b] WC White clover plants; SC, subterranean clover plants.

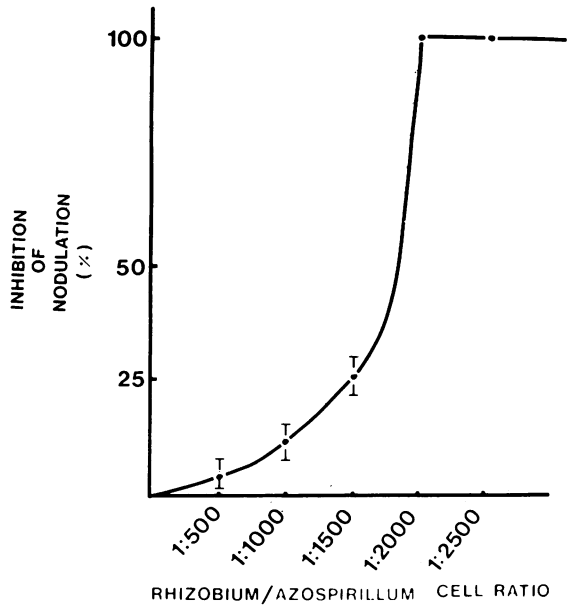

FIG. 1. Inhibition of nodulation by mixed cultures of *R. trifolii* ANU794 and *A. brasilense* SP107. Both strains were inoculated onto white clover at the same time in different cell ratios. Plant response to the bacterial mixed cultures was assayed after 4 weeks. The values were significantly different ($P = 0.05$) from those of control plants and represented data from 16 plants and six experimental repeats.

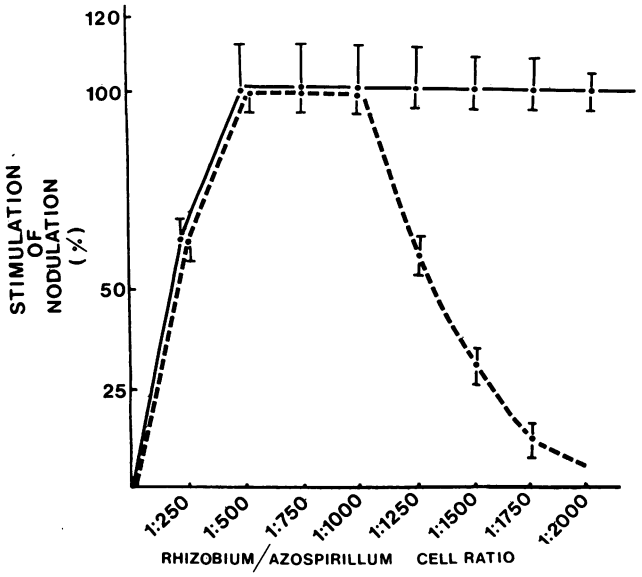

FIG. 2. Stimulation of nodulation by mixed cultures of *R. trifolii* ANU794 and *A. brasilense* SP107 and SP245. *A. brasilense* strains were inoculated onto white clover three days after *R. trifolii* strains. Plant response to inoculum was assayed (counting of the nodule number and measuring of the nitrogen fixation ability by the acetylene reduction method) 4 weeks after *Azospirillum* addition. The values were significantly different ($P = 0.05$) from control plants and represented data from 16 plants and six experimental repeats. The continuous line represents the stimulation of nodule formation after strain SP245 was added to plants previously inoculated with strain ANU794. The dashed line shows stimulation of nodulation when SP107 was added to strain ANU794.

occurred (Fig. 1). Cell ratios (*R. trifolii*/*A. brasilense*) of 1:500, 1:1,000, or 1:1,500 produced less inhibition of nodulation, namely, 10, 15, and 25%, respectively (Fig. 1).

Stimulation of nodulation. Of nine investigated *R. trifolii* strains, seven showed stimulation of nodule formation when *Azospirillum* isolates were added to the plants 1 to 6 days before or after inoculation with *R. trifolii*. This increase of nodule number varied from 25 to 100% for the same *R. trifolii* strain and depended on the *Azospirillum* strain used (Table 3). When white clover plants were inoculated first with *R. trifolii* strain ANU794 and then *A. brasilense* strain SP107 was added 3 days later, stimulation of nodule formation was observed (Fig. 2). A maximum stimulation (100%) was detected only when the ratios of *R. trifolii*/*A. brasilense* cells were between 1:500 and 1:1,000. However, this increased nodule number usually produced a non-nitrogen-fixing (Fix^-) phenotype (no nitrogenase activity in C_2H_2 reduction tests). Generally these plants formed more lateral roots, and nodules were equally distributed over both the main tap root and lateral roots. Clovers inoculated with *R. trifolii*/*A. brasilense* ratios of 1:1 to 1:100 developed normal nitrogen-fixing (Fix^+) plants. When *A. brasilense* SP245 was added to *R. trifolii* ANU794 at a ratio (*R. trifolii*/*A. brasilense*) of 1:500, 100% stimulation of nodule formation occurred, and this was maintained even with cell inoculum ratios of 1:2,000 (Fig. 2).

Effect of inoculation timing on nodulation of white clover. The timing of the addition of strains SP107 and SP245 had profound effects on the nodulation capacity of strain ANU794. When strains SP245 and ANU794 were simultaneously inoculated onto clover, no nodules were formed (Fig. 3). However, if either strain was first inoculated into clover and the second strain was added 24 h later, a marked

TABLE 3. Stimulatory effect of *Azospirillum* strains on clover nodulation when *Azospirillum* cells were added 1 to 6 days before or after *R. trifolii* cells

Azospirillum strain	Effect of inoculation[a] with *R. trifolii* strain on type of clover[b]:																	
	ANU794		ANU329		ANU844		ANU845 (pRt032)		ANU850		ANU1030		ANU870		T1 Km^r		T1 Sp^r	
	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC
SP7	S_{100}	S_{100}	S_{50}	NT	S_{100}	S_{100}	NS	NT	NT	S_{50}	NS	NS	S_{50}	S_{25}	NS	NS	NS	NS
SP59	S_{100}	S_{100}	S_{50}	NT	NS	NS	S_{25}	NT	NT	S_{25}	NS	NS	S_{25}	S_{25}	NS	NS	NS	S_{25}
SP107	S_{100}	S_{100}	S_{50}	NT	NS	S_{25}	NS	NT	NT	S_{50}	NS	NS	NS	S_{25}	NS	NS	NS	S_{25}
SP242	S_{100}	S_{100}	S_{50}	NT	NS	S_{50}	NT	NT	NT	NS	NS	NS	S_{25}	NS	NS	NS	NS	NS
SP245	S_{100}	S_{100}	S_{50}	NT	NS	S_{75}	S_{25}	NT	NT	S_{25}	NS	NS	S_{25}	S_{25}	NS	NS	NS	S_{25}

[a] Values used were significantly different ($P = 0.05$) from those for the control and represented data from a minimum of 12 plants taken from three experimental repeats. *Azospirillum* strains were inoculated onto plants 72 h after *R. trifolii* strains. S, Stimulation of nodule formation expressed as a percentage of the nodule number formed by *R. trifolii* alone; NT, not tested; NS, no stimulation.

[b] WC, and SC are defined in footnote *b* of Table 2.

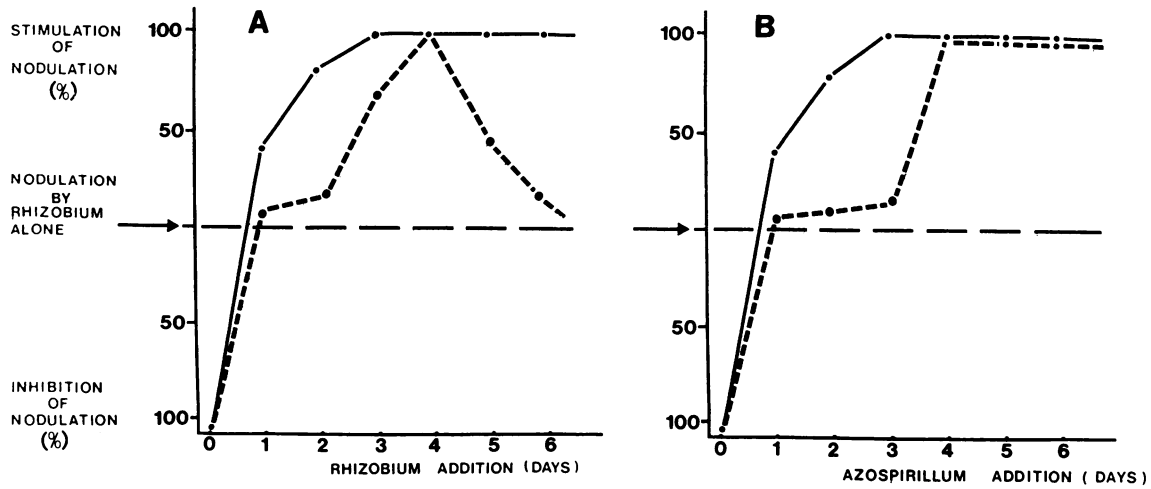

FIG. 3. Effect of inoculation timing on white clover nodulation. (A) Effect when plants were inoculated first with *Azospirillum* cells and then *R. trifolii* ANU794 was added at 1-day intervals; (B) effect when plants were inoculated first with *R. trifolii* ANU794 and then with *Azospirillum* cells at 1-day intervals. The *Rhizobium/Azospirillum* initial cell ratio was 1:2,000, and the final results were scored on 6-week-old plants. The values are means of 12 plants from three experimental repeats. The continuous line represents strain SP245, and the dashed line represents strain SP107.

stimulation of nodule formation was observed (Fig. 3A, B). A 100% stimulation of nodule formation occurred if either strain (*A. brasilense* SP245 or *R. trifolii* AU794) was added to plants 3 days after inoculation by the other strain. This elevated level of nodule number was found even when 6 days elapsed between the initial inoculation and the addition of the second strain.

This marked stimulation of nodulation was also found when *A. brasilense* SP107 was added to clover plants 3 days or more after the original inoculation with strain ANU794. When strain SP107 was added to plants before strain ANU794, maximum stimulation of nodulation occurred when *R. trifolii* cells were inoculated 4 days later. This elevated level of nodulation was lost if *R. trifolii* cells were added at later times (Fig. 3A). Thus, the interaction of strain SP107 added before strain ANU794 led to a transient peak response.

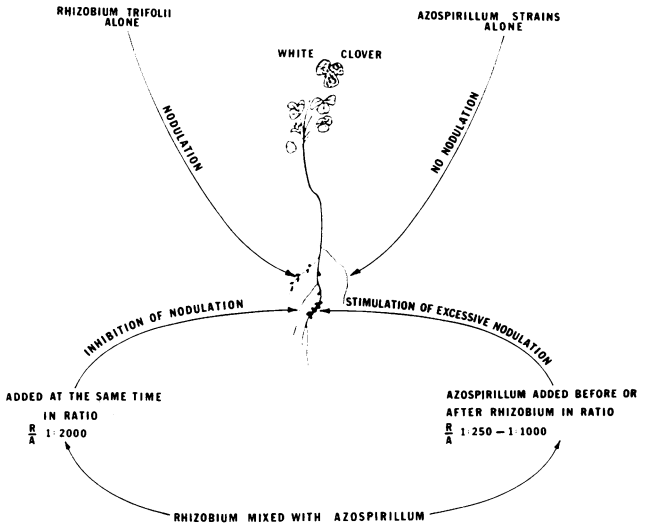

FIG. 4. The scheme presents changes in white clover nodulation when plants were inoculated with *R. trifolii* ANU794 and *A. brasilense* SP107 in different time and cell ratios. Stimulation of excessive nodulation expresses the surplus of nodules that leads to a Fix$^-$ plant phenotype. $\frac{R}{A}$, *Rhizobium/Azospirillum* cell ratio.

Effect of nonviable cell suspensions on the nodulation process. *A. brasilense* SP107 and SP245 were heat killed and checked for viability before they were mixed with *R. trifolii* ANU794. No inhibition or stimulation of nodulation was detected when either of the killed-*A. brasilense* strain suspensions was mixed with the *R. trifolii* strain and inoculated onto white clover plants.

Effect of a dialyzed cell suspension on the nodulation process. When the *A. brasilense* SP107 suspension was placed into a dialysis bag and immersed into liquid F medium containing *R. trifolii* ANU794 and a Fahraeus slide of white clover seedlings, no inhibition or stimulation of nodulation was observed.

Bacteriocin production test. *R. trifolii* ANU794, ANU329, ANU870, and T1 Km^r and *A. brasilense* SP107 and SP245 were tested for bacteriocin production, but no inhibition zones were observed. In addition, when strain ANU794 was mixed with either strain SP107 or SP245 and the strains were grown together in liquid media, no inhibition of growth was detected for either inoculum strain.

DISCUSSION

Our studies have shown that the presence of various *Azospirillum* strains could significantly influence the *Rhizobium*-legume symbiotic interaction. *Azospirillum* strains could totally inhibit nodulation under the following conditions: (i) when plants were inoculated with cell mixtures containing both *Azospirillum* and *Rhizobium* strains; and (ii) when the strains were mixed in cell ratios of about 1:2,000 (*Rhizobium* strains/*Azospirillum* strains).

R. trifolii ANU794 nodulates rapidly, with nodules detected on white clover plants in under 3 days (unpublished data). Moreover, root hair curling on inoculated seedlings can be observed 18 to 24 h after inoculation with ANU794. When nodulation was inhibited, no marked root hair curling was observed. Thus, inhibition of nodulation, due to the presence of *Azospirillum* strains, is exerted rapidly after inoculation.

It is also known that *Azospirillum* strains can colonize roots of some tropical grasses within a few hours of inoculation (25). Because *Azospirillum* cells may colonize the root hairs before *Rhizobium* bacteria, it is possible that the presence of *Azospirillum* cells makes infection sites on clover root hairs unavailable for *Rhizobium* cells. Both bacterial species can be readily found associated with the clover root hairs. *Rhizobium* strains could be isolated from clover roots that had been previously inoculated with *Rhizobium-Azospirillum* mixed cultures. When these strains were purified and inoculated again on clover, effective nodules were induced by the *Rhizobium* (J. Plazinski, and B. G. Rolfe, submitted for publication). *Azospirillum* strains not only inhibit nodulation but also stimulate nodule formation on clovers. These phenomena occurred only when *Azospirillum* strains were added 24 h or more before or after *Rhizobium* inoculation of clover plants. This finding indicates the presence of a timing window, which determines either the total inhibition of nodulation or a marked increase in nodule number. This is a very unusual observation. A possible explanation may be that the addition of *Azospirillum* cells may prime the plant, creating additional infection sites which are later occupied by *Rhizobium* cells. Perhaps *Azospirillum* cells produce an excretable compound(s) which creates new infection sites.

These diverse interactions between *A. brasilense* SP107 and *R. trifolii* ANU794 are summarized in Fig. 4. These interactions require viable *Azospirillum* cells since heat-killed cells were unable to alter the nodulation pattern of strain ANU794 in any detectable manner. Similarly, it was found that neither *Azospirillum* nor *Rhizobium* cells produced any detectable bacteriocins or growth-inhibiting agents when these cells were mixed and grown together on various liquid or solid media.

It is interesting that the spectinomycin-resistant mutants (ANU329 and T1Sp) of strains TA1 and T1 do not show *Azospirillum*-induced inhibition of nodulation but still retain the capacity to be stimulated to form more nodules on white clovers (ANU329 was not tested on subclovers) and subterranean clovers (T1Sp).

Where there is an increase in nodulation, a Fix^- response is produced on clover. It has been previously reported (14, 24) that delayed inoculation of clovers generally gives a Fix^- plant response. In these cases, more lateral roots appear, and an increase in nodule number (mainly on lateral roots) occurs. When *Azospirillum* strains caused an increase in the number of nodules formed, these were mostly found on the lateral roots of the inoculated clovers. Thus, the effect of the stimulation of nodulation paralleled that observed with delayed inoculation. This also reflects the involvement of the plant in response to the two interacting bacterial species. All negative effects resulted when either a stimulation or an inhibition of clover nodule formation occurred in the presence of *Azospirillum* strains. This result contrasts with other reports of *Azospirillum-Rhizobium* interactions (10, 15, 22). Therefore, the relevance of our work to the field situation awaits further studies.

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