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Interaction Between a Non-Nodulating and an Ineffective Mutant of *Rhizobium trifolli* Resulting in Effective (Nitrogen-Fixing) Nodulation

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A noninvasive (non-nodulating) mutant of *Rhizobium trifolii* when mixed with an ineffective (non-nitrogen-fixing) mutant gives rise to effective (nitrogen-fixing) nodules.

Rhizobium trifolii strain NA34 and derived substrains have had an interesting history in rhizobial research and application. Early use as a clover inoculant had to be discontinued when field experience (Jenkins et al. [5], with the clonal derivative SU295) and laboratory checks revealed that the culture contained a majority of noninvasive sublines (J. M. Vincent, unpublished data) as well as invasive but ineffective small colony forms (D. Marshall, personal communication). At that time, three forms were available: (i) large colony, invasive and effective; (ii) large colony, not invasive; and (iii) small colony, invasive but ineffective. Substrains of strain NA34 used in studying the possible relationship between root hair adhesion and invasiveness (1, 2) parallel the last two (ii and iii above).

More recently, all of the large-colony substrains held in the collection at Sydney University have proved to be noninvasive, whereas the small colony cultures have maintained a fully invasive but ineffective N_2-fixing capacity. The genetic basis for these different mutant types is unknown. However, experiments designed to determine whether the invasiveness of the small-colony form could be combined with a possibly cryptic effectiveness in the large-colony noninvasive substrain were made by mixing both strains in equal proportions and then inoculating white clover seedlings.

The strains used here which were representative of the non-nodulating and the ineffective isolates were: SU846 (ex NA34 via SU304/11), small colony, invasive, ineffective; SU847 (ex NA34 via SU304/1), large colony, noninvasive. Strain SU846 forms small, ineffective (non-nitrogen-fixing) nodules on white clover. These nodules form fairly readily after inoculation but fail to continue to develop in size. A number of these nodules were isolated from different plants and fixed and prepared for examination under the electron microscope (Fig. 1). Sections through these nodules showed that they consisted mainly of large, vacuolated plant cells with no bacteroids or bacteria present. Nor was there any evidence of bacteroid degradation (Fig. 1A). However, there were several small clusters of plant cells which did contain bacteroids (Fig. 1B and C). These plant cells also contained large amounts of presumably starch deposits, and often the bacteroids appeared to have an incomplete morphological development (Fig. 1D). These features are often observed in some *Rhizobium* strains which have defective bacteroid development (Bad) phenotypes (J. M. Vincent, Proc. Steenbock-Kettering Int. Symp. Nitrogen Fixation, in press), and therefore strain SU846 is thought to be a probable Bad mutant. The lack of attachment to root hair surfaces by the large-colony noninvasive form (1, 2) equivalent to strain SU847 (1) indicates that it has a defective root adhesion phenotype (Roa) (Vincent, in press). Furthermore, no reversion of their symbiotic phenotype was ever observed with these two strains under any condition used.

In the first set of mixing experiments both strains were mixed and concentrated together onto membrane filters (Millipore Corp.) for 1 to 3 days to enable possible conjugation between the mutants to take place. The cells from the filters were suspended in water, diluted, and used to inoculate white clover plants to select for possible "effective (nitrogen-fixing) recombinants". Healthy plants did in fact develop with typically effective nodules at the same time as parent controls were either ineffectively nodulated (SU846) or not nodulated (SU847) (Table 1) (Fig. 2). In fact, most of the plants showed this phenomenon and were effective.

Different nodules which were large, pigmented, and had the appearance of being Eff^+

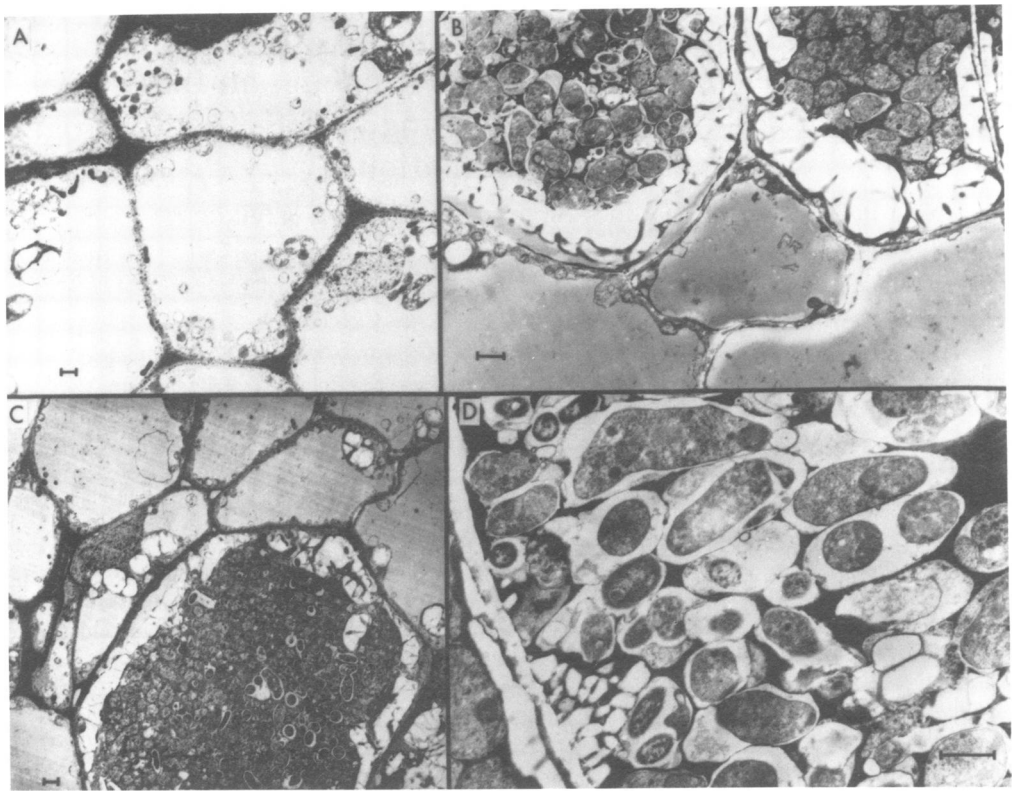

FIG. 1. *Electron micrographs of nodule tissue from ineffective nodules formed by strain SU846 on white clover plants. (A) Large vacuolated cells, not infected with bacteroids or bacteria; (B and C) clusters of bacteroid-containing nodule cells with large amounts of presumably starch deposits in peripheral cytoplasm; (D) bacteroids of strain SU846, showing incomplete morphological development. Scale: bar represents 2 μm.*

TABLE 1. *Effect of mixed rhizobial strains on the inoculation of white clover*

Inoculating strains	Result on white clover
SU846 (Bad)	Eff⁻ nodulation
SU847 (Roa)	Non-nodulated
SU846 and SU847 (ratio 1:1 mixture)	Eff⁺ nodulation
SU846 (diluted) and SU847 (ratio, 1:100 mixture)	Eff⁺ nodulated plants (reduced frequency)
SU846 UV irradiated	No nodules
SU846 UV irradiated and SU847 (ratio, 1:1 mixture)	No nodules
SU846 heat killed	No nodules
SU846 heat killed and SU847 (ratio, 1:1 mixture)	No nodules
SU846 filtrate and SU847	No nodules
T1 Nm5 (Noi)	No nodules
T1 Nm5 and SU847	No nodules
T1 Rif16 (Bad)	Eff⁻ nodulation
T1 Rif16 and SU847	Eff⁻ nodulation
T1 Rif33 (Nop)	Eff⁻ nodulation
T1 Rif33 and SU847	Eff⁻ nodulation
L4Sp (Noi)	No nodules
L4Sp and SU847	No nodules

[a] White clover (*Trifolium repens*, N.Z. white clover 5826) plants were treated and cultured on petri dishes by the method of Rolfe and Gresshoff, Aust. J. Biol. Sci., in press) using Fåhraeus medium (6). SU846, and SU847 and T1 are strains of *R. trifolii*; L4Sp is a spectinomycin-resistant mutant of *R. leguminosarum*. Eff⁺ phenotype, effective (nitrogen-fixing); Eff⁻, ineffective (non-nitrogen-fixing). Drug resistance markers; Sp, spectinomycin; Nm, neomycin; Rif, rifampin. Phenotype of mutant strains; defective bacteroid development (Bad); loss of root adhesion (Roa); lack of nodule induction (Noi); reduction of nodule persistence (Nop). Strain SU846 was killed by heating a culture to 100°C or irradiating with ultraviolet light until no surviving colony formers were present.

were isolated from various plants and examined by the bacteroid isolation techniques (3, 4). This technique checks the success of surface sterilization of a nodule. Moreover, protoplasts of nodule cells containing bacteroids can be isolated completely free of extra-protoplast bacteria by serial dilutions. The bacterial contents of individual plant protoplasts were plated onto protective media, and the nodule contents were also diluted and plated. It was found that most of the plant protoplasts had only large-colony-forming cells present and that they were sensitive to a phage which plates on both SU846 and SU847.

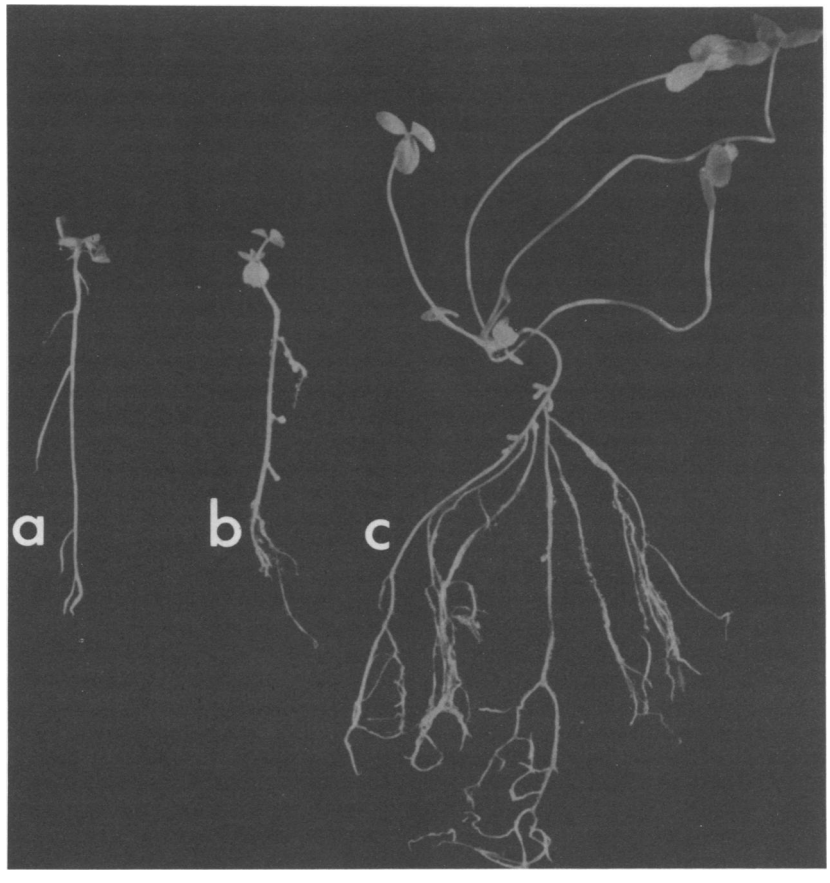

FIG. 2. *Response on white clover; (a) non-nodulation by strain SU847, (b) ineffective nodulation by strain SU846, and (c) Eff⁺ nodulation after inoculation by a mixture of both strains SU846 and SU847 in equal proportions. Plants photographed 5 weeks after inoculation.*

In addition, these large-colony-forming cells were unable to renodulate white clover. A low frequency of plant protoplasts had small-colony-forming cells present. These cells were phage sensitive and reformed small colonies on restreaking as well as ineffective nodules on white clover plants. A sufficient number of colonies was tested from different plant protoplasts and different nodules and plants to enable the conclusion to be made that genetic exchange between strains SU846 and SU847 did not occur. Thus, in some way the presence of the invasive, ineffective strain SU846 permits the entry into the plant of the noninvasive strain SU847 and the subsequent expression of effectiveness. The method of growth (on liquid or solid medium) or prior interstrain contact did not affect the phenomenon. Cell ratios in favor of the noninvasive substrain (100:1) still permitted joint occupancy, although it reduced the frequency of effectively nodulated plants (Table 1). However, if the SU846 cells were either heat killed or heavily irradiated with ultraviolet irradiation and then mixed with SU847 cells, no nodulation resulted. Similarly, if filtrates were made of strain SU846 cultures grown under different growth conditions or in various media and mixed with strain SU847, no nodulation was observed (Table 1).

The success achieved by strain SU847 in the presence of strain SU846 on white clover has, so far, proved specific to these related substrains of strain NA34, in that it has not occurred with invasive but different ineffective mutants of *R. trifolii* strain T1 or with the invasive (nonnodulating) *R. leguminosarum* strain L4 (Table 1). This rhizobial strain interaction between SU846 and SU847 also occurs on subterranean clover. At present, all attempts to show that the invasive strain SU846 can "actively help" the related noninvasive strain SU847 have failed, and it appears likely that strain SU847 can ef-

fectively nodulate plants by "sneaking" into the root hair along with its invading partner.

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LITERATURE CITED

1. **Dazzo, F. B., and D. H. Hubbell.** 1975. Antigenic differences between infective and noninfective strains of *Rhizobium trifolii*. Appl. Microbiol. **30**:172–177.
2. **Dazzo, F. B., C. A. Napoli, and D. H. Hubbell.** 1976. Adsorption of bacteria to roots as related to host specificity in the *Rhizobium*—clover symbiosis. Appl. Environ. Microbiol. **32**:166–171.
3. **Gresshoff, P. M., M. L. Skotnicki, J. F. Eadie, and B. G. Rolfe.** 1977. Viability of *Rhizobium trifolii* bacteroids from clover root nodules. Plant Sci. Lett. **10**:299–304.
4. **Gresshoff, P. M., and B. G. Rolfe.** 1978. Viability of *Rhizobium* bacteroids isolated from soybean nodule protoplasts. Planta **142**:329–333.
5. **Jenkins, H. V., J. M. Vincent, and L. M. Waters.** 1954. The root-nodule bacteria as factors in clover establishment in the red basaltic soils of the Lismore District, New South Wales. III. Field Inoculation Trails. Aust. J. Agric. Res. **5**:77–89.
6. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria, I.B.P. Handbook No. 15, Blackwell Publishers, Oxford, and Edinburgh.