

## Inoculation of Woody Legumes with Selected Arbuscular Mycorrhizal Fungi and Rhizobia To Recover Desertified Mediterranean Ecosystems

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Received 3 August 1992/Accepted 3 November 1992

**Revegetation strategies, either for reclamation or for rehabilitation, are being used to recover desertified ecosystems. Woody legumes are recognized as species that are useful for revegetation of water-deficient, low-nutrient environments because of their ability to form symbiotic associations with rhizobial bacteria and mycorrhizal fungi, which improve nutrient acquisition and help plants to become established and cope with stress situations. A range of woody legumes used in revegetation programs, particularly in Mediterranean regions, were assayed. These legumes included both exotic and native species and were used in a test of a desertified semiarid ecosystem in southeast Spain. Screening for the appropriate plant species-microsymbiont combinations was performed previously, and a simple procedure to produce plantlets with optimized mycorrhizal and nodulated status was developed. The results of a 4-year trial showed that (i) only the native shrub legumes were able to become established under the local environmental conditions (hence, a reclamation strategy is recommended) and (ii) biotechnological manipulation of the seedlings to be used for revegetation (by inoculation with selected rhizobia and mycorrhizal fungi) improved outplanting performance, plant survival, and biomass development.**

The natural equilibria in a given natural ecosystem can be disturbed by changes in the activity of natural agents (climatic, geomorphic, or paleotectonic processes, etc.). Consequently, the structure, morphology, and species diversity of the climax vegetation can be degraded, a process which occurs concomitantly with generalized damage to the biological, chemical, and physical status of the soil (11, 15). The decline in soil and plant productivity, particularly in areas subjected to moisture deficiency, ultimately leads to a desert situation (desertization). Such a desert situation appears to be irreversible. In addition, anthropic activities, such as badly controlled overgrazing, nonregulated cultivation techniques, deforestation, etc., can cause or accelerate the development of desertlike conditions (desertification) (14, 21). Desertification is a complex and dynamic process (1, 2, 14, 21) which obviously has a negative environmental impact, particularly in arid, semi-arid and subhumid areas of the world, where the process is claiming several million hectares annually (11, 15). The characteristics of desertification include (11, 15, 21) loss or disturbance of the vegetation cover, increase in soil erosion, loss of available nutrients and organic matter, loss of microbial propagules, and/or diminution in microbiota activity, which affects suitable nutrient cycling. These determinants act either as causes or effects, thereby creating a downward spiral, the result of which is the progressive degradation of both soil fertility level and vegetation. This causes a decline in plant productivity and in the degree of soil protection against further activity from erosive agents (11, 15). The establishment of a suitable plant cover is known to improve the chemical, physical, and biological properties of the soil (11, 15, 21); however, the scarcity of microbial propagules in the eroded soil (9, 12, 13, 23, 24) can become a handicap to plant

establishment because the formation of a dynamic rhizosphere is critical, particularly in low-nutrient ecosystems.

Woody legumes are useful for revegetation of water-deficient ecosystems that have low availability of N, P, and other nutrients (8, 10, 17) because of their ability to develop symbiotic associations with both rhizobial bacteria and mycorrhizal fungi. *Rhizobium* or *Bradyrhizobium* spp. have been isolated from root nodules of different woody legumes, but there is little information concerning selection of microsymbionts to realize the full potential of a given woody legume-rhizobium combination to maximize biological N<sub>2</sub> fixation (10, 17). The scarcity of available P and the imbalance of trace elements in desertified ecosystems actually limit legume establishment and N<sub>2</sub> fixation (5). Thus, mycorrhizae have been found to increase legume performance (4). Moreover, woody legumes usually exhibit a considerable degree of dependence on mycorrhizae to thrive in stressed situations (9, 12, 13, 18, 20, 24). Arbuscular mycorrhizae, which are by far the most widespread mycorrhizae in nature, are the most common mycorrhizae in nodulated, N<sub>2</sub>-fixing legumes (4). After biotrophic mutualistic colonization of plant roots, the microsymbiont develops an extraradical mycelium that links the roots and the soil environment and helps the plant to use more efficiently soil nutrients, particularly those that diffuse slowly toward the root surface, such as phosphate and trace elements (4, 22). In addition, the symbiosis enhances the ability of the plant to become established and cope with stress situations (nutrient deficiency, drought, trace element imbalance, soil disturbance), which are typical in desertification situations (5, 6, 23).

Therefore, interacting mycorrhizal and rhizobial symbioses appear to be important for revegetation. However, as soil erosion tends to reduce mycorrhizal propagules (21), it could be critical to reintroduce such propagules to improve the recovery rate of disturbed ecosystems. First, mycor-

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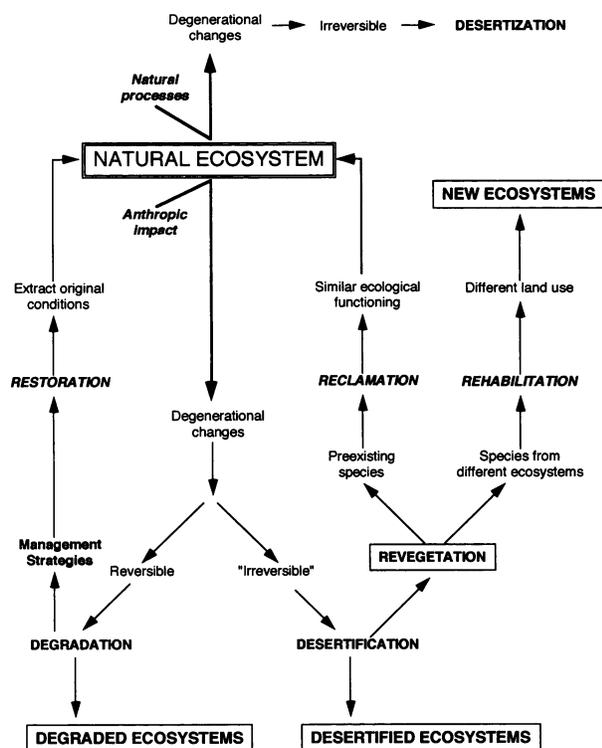


FIG. 1. It is known that anthropic activities can cause or accelerate the natural degeneration (desertization) of an ecosystem. As a result of human-mediated effects, a natural ecosystem can be either merely degraded or desertified, depending on the severity of the disturbance (2). A degraded ecosystem can be returned to the original conditions through appropriate management technologies of the soil-plant system (restoration) (2). Desertified ecosystems seem to be irreversible in theory. However, suitable strategies for revegetation, based on ecological principles that act as feedback mechanisms for regeneration, can be used to recover vegetation. The use of preexisting species could return the desertified ecosystem to an ecological functioning similar to that in the original situation (reclamation) (2). Conversely, in another management strategy exotic species or local species from different ecosystems are used. New stable and sustainable ecosystems are then engineered, but they have a different land use (rehabilitation) (2).

rhizal fungi that exhibit symbiotic efficiency and physiological compatibility with the test legume (functional compatibility [22]) must be selected.

Degraded ecosystems are common in the Mediterranean climate regions. Particularly in southeast Spain, low precipitation and human impact have resulted in fragile ecosystems that exhibit increased erosion and desertification. These desertified ecosystems are good representatives for testing

reclamation and rehabilitation programs (2, 11, 15). The main objective of this investigation was to assess the significance and effectiveness of plant-microbe symbioses as a component of an appropriate revegetation strategy (Fig. 1).

## MATERIALS AND METHODS

**Host legumes and test soil.** The revegetation experiment was performed in a representative desertified ecosystem in Granada province in southeast Spain. The soil at this site is a neutral calcareous soil that is very low in assimilable P and N. The topographic and physiochemical characteristics of the soil have been published previously (6). A number of woody species that are commonly used in revegetation programs in Mediterranean regions were assayed, including two native shrubs (*Anthyllis cytisoides* and *Spartium junceum*) and four exotic tree legumes (*Robinia pseudoacacia*, *Medicago arborea*, *Acacia caven*, and *Prosopis chilensis*) (5, 6).

**Screening for suitable plant species-microsymbiont combinations.** The isolation and characterization of the microsymbionts were studied previously (6, 17); the data from the previous studies allowed selection of the most effective rhizobia for each test plant (Table 1). The mycorrhizal fungi used in tests for functional compatibility (22) with the test legumes were then selected as described below. The following four mycorrhizal fungi were tested: *Glomus mosseae*; *Glomus fasciculatum* from the collection of our laboratory; and a *Glomus* sp. and a *Scutellospora* sp. isolated from the rhizosphere of almond and the rhizosphere of *Spartium* sp., respectively, which were obtained from the ecosystem in southeast Spain. To determine the levels of functional compatibility of these fungi with each host legume, a pot experiment was carried out by using native topsoil (2 kg of air-dried soil per pot) collected from the site in southeast Spain. This soil was steam sterilized. Five-day-old uniform seedlings of each test legume that were obtained from surface-sterilized seeds (3, 18) and were germinated on moist filter paper were inoculated with the selected rhizobial strains (Table 1) to form N<sub>2</sub>-fixing host-symbiont systems for the different mycorrhizal fungi screened. The corresponding strain of rhizobial inoculum was provided by 1 ml (about 10<sup>8</sup> cells) of a culture in Allen medium (3) that was applied to the seedling roots.

Mycorrhizal inoculation was accomplished by placing into each pot 10 g of an air-dried mycorrhizal fungus inoculum obtained from our stock culture collection. The inocula consisted of thoroughly mixed rhizosphere samples containing spores, hyphae, and mycorrhizal onion root fragments. This inoculation method produced similar levels (70 to 80%) of mycorrhizal colonization of onions for the four mycorrhizal fungi. The test legumes were grown for 2 months under greenhouse conditions (3). The parameters used to establish the level of functional compatibility (22) were those

TABLE 1. *Rhizobium* and *Bradyrhizobium* strains selected for effectiveness (N<sub>2</sub> fixation, biomass production, and total N content) for each of the woody legumes tested

Plant species	Rhizobial strain	Isolated from:
<i>R. pseudoacacia</i>	<i>Rhizobium</i> sp. strain GRH11	<i>R. pseudoacacia</i>
<i>Acacia caven</i>	<i>Bradyrhizobium</i> sp. strain GRH2	<i>Acacia cyanophylla</i>
<i>P. chilensis</i>	<i>Bradyrhizobium</i> sp. strain GRH2	<i>Acacia cyanophylla</i>
<i>M. arborea</i>	<i>Rhizobium meliloti</i> GRH4B	<i>Medicago sativa</i>
<i>S. junceum</i>	<i>Bradyrhizobium</i> sp. strain B 3824	<i>Vigna</i> sp.
<i>Anthyllis cytisoides</i>	<i>Rhizobium</i> sp. strain G4	<i>Lotus corniculatus</i>

described by Azcón et al. (3). The parameters measured were plant biomass production, nodulation, and nutrient (N and P) concentrations and contents in plant shoots. In this study the total N content was particularly emphasized. This parameter took into account well-balanced effects on N concentration and biomass production as an expression of mycorrhizal efficiency for each of the legume-*Rhizobium* combinations tested (3).

**Production of plantlets with optimized mycorrhizal and nodulation status.** Two-week-old seedlings that were obtained from seeds of the six test legumes germinated in moistened sand were the plants that were inoculated with the selected microsymbionts. The seedlings were grown in 2-liter plastic bags containing nonsterilized native test soil. At transplanting one-half of the seedlings received the selected rhizobial and mycorrhizal inocula, which were prepared as described above. The other seedlings were as noninoculated controls, although they may have become symbiotic by being naturally colonized by the native rhizobia and mycorrhizal fungi present in the nonsterilized soil. The plants were grown without added nutrients for 1 month in a controlled greenhouse (temperatures range, 19 to 25°C; photoperiod consisting of 16 h of light and 8 h of dark; relative humidity, 70 to 90%) and for an additional 2 months in an open shadehouse (temperatures range, 9 to 30°C; photoperiod consisting of 11 to 12 h of light and 12 to 13 h of dark; relative humidity, 50 to 65%). At transplanting nodulation and mycorrhizal colonization were measured by using the most common standard procedures (3).

**Establishment of the woody legumes in the desertified ecosystem.** When the plantlets were 3 months old, they were planted at our field site. A total of 20 terraces (30 m long by 1.5 m wide) were prepared by following the level curves of the soil. In this experiment we used a completely randomized two-factor factorial design that included (i) plantlets inoculated with selected microsymbionts, and (ii) uninoculated, but naturally symbiotic plantlets. The plantlets were placed 2 m apart to prevent any possibility of cross-contamination of microsymbionts between adjacent plants that received different treatments. A qualitative estimate at transplanting revealed that all of the plantlets, whether they were inoculated or not, were mycorrhizal (mean percentage of the root length colonized, 40 to 60%), as estimated by standard methods (3), and nodulated. The objective of the long-term field experiment was to determine the effectiveness of the symbioses. There were no apparent plant growth responses to inoculation at transplanting.

Plantlets were transplanted into the plots at the research site in April 1989 and did not receive any treatment during the experiment. The mean annual precipitation ranged from 260 to 318 mm. The minimum temperature was 25°C for some weeks in July and August. Additional climatological characteristics are described elsewhere (14). Plant survival was monitored at least twice annually, and the data reported below were data from the June recordings.

## RESULTS

**Screening for the appropriate plant species-microsymbiont combinations.** Figure 2 summarizes the data for the selection trial used to ascertain the most suitable mycorrhizal fungi for each legume-rhizobium combination. Data are given for four of the six host legumes because of previous descriptions that established the compatibility of *G. mosseae* with either *Acacia caven* or *P. chilensis* (6). The level of mycorrhizal effectiveness was estimated on the basis of the plant re-

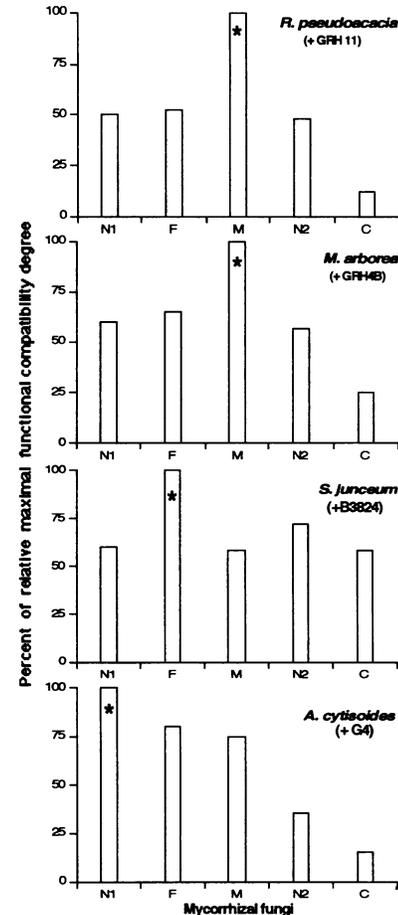


FIG. 2. Screening of mycorrhizal fungi for functional compatibility with different test legumes. The *Rhizobium* strains selected for the plant species (Table 1) are indicated in parentheses. N1, unidentified *Glomus* sp. isolated from rhizosphere (almond) soil in the region; M, *G. mosseae*; F, *G. fasciculatum*; N2, unidentified *Scutellospora* sp. isolated from rhizosphere (*Spartium junceum*) soil in the region; C, uninoculated controls. The asterisks indicate significant effects at the 5% level compared with the second-most-effective endophyte in the ranking of functional compatibility for each test plant species.

sponse as biomass production and nutrient (P and N) content. As indicated above, the main parameter used to estimate functional compatibility was the N content (which was calculated on the basis of biomass dry weight and N concentration in plant tissues). The data for level of mycorrhizal colonization, which ranged from 40 to 60%, roughly correlated with the plant response data.

**Outplanting and plant establishment evaluation.** The results of the 4-year trial showed that (i) only the native species survived the local environmental conditions after 2 years and (ii) the biotechnological manipulation of the seedlings by inoculating them with selected microsymbionts improved outplanting performance and survival (Fig. 3). Microsymbiont inoculation also had an effect on plant growth. Indeed, the final data (June 1992) revealed that the still growing native shrub legumes inoculated with selected microsymbionts had at least twice as much above-ground biomass as the corresponding controls.

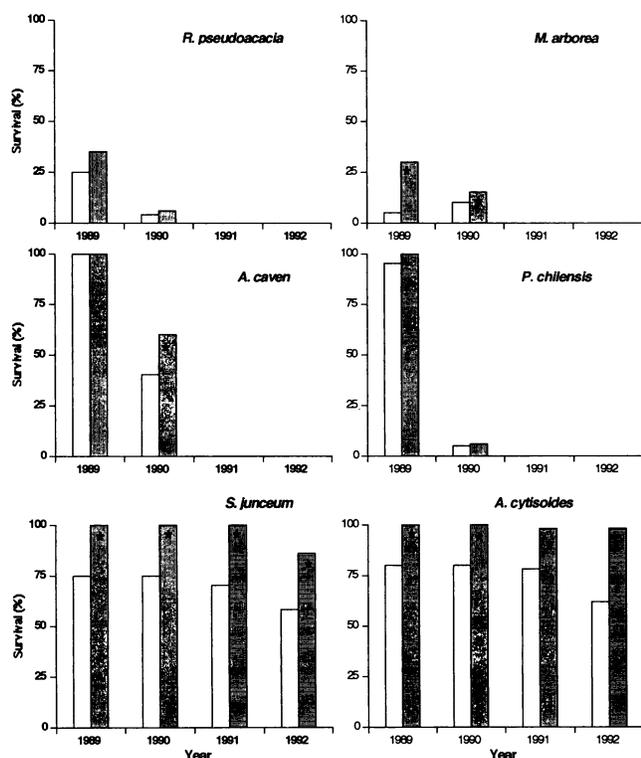


FIG. 3. Establishment of woody legumes in the desertified ecosystem. The shaded columns indicate the responses of plants inoculated with selected microsymbionts. The open columns indicate the responses of control plants that were symbiotic because they were naturally colonized by native rhizobia and mycorrhizal endophytes. The asterisks indicate significant effects (at the 5% level) of microbial inoculation for each monitoring date.

## DISCUSSION

From a microbiological point of view, the following two main points deserve discussion: (i) whether the inability of the exotic woody legumes to become established can be associated, at least in part, with inappropriate management of microbial symbiosis; and (ii) whether the effectiveness of selected microsymbionts for improving outplanting performance and survival of native legumes increases the possibility that this biotechnology (microsymbiont inoculation) can be used in revegetation strategies for desertified lands.

It is known that local edaphic and climatic conditions, which affect ecophysiological relationships, preclude the long-term establishment of species that are exotic for an ecosystem. In this investigation appropriate microsymbionts that are important for legume establishment were provided. However, none of the endophytes which we used were isolated from the desertified ecosystem tested. This could explain the failure of the mycorrhizae to get the exotic legumes established despite the fact that adequate colonization levels (50 to 60% of the root length) were measured. However, considering that the same exotic mycorrhizal isolates helped the native legumes become established, we suggest that climatic and edaphic conditions other than those involving symbiosis can preclude the establishment of legumes that are not native to an area.

The effectiveness of microsymbionts in improving outplanting performance of native shrub legumes was evident.

In the case of *S. junceum*, the mycorrhizal endophytes selected were not endophytes that were isolated locally from the disturbed site, and as also found in another study (16), they were effective in promoting plant growth and they adapted to the ecosystem, as shown in the test for functional compatibility (5, 22). For *Anthyllis cytisoides* the fungus which was used was isolated from the test soil.

The presence and role of natural endophytes should be considered. Despite the scarcity of mycorrhizal propagules in the test soil (5), the ubiquitousness and lack of specificity of these microorganisms ensure some colonization levels. In the case of rhizobial bacteria, it is also well known that the relationships between woody legumes and rhizobia are rather promiscuous and often nonspecific (17, 20). Moreover, rhizobia isolated from legume trees can nodulate herbaceous legumes and vice versa (17). This could explain the natural nodulation level found in the control plants.

There is in fact a great deal of genetic diversity among the rhizobia that are able to nodulate a given tree legume (7), just as there is a great deal of genetic diversity among mycorrhizal fungi (22), a situation which is expressed in the wide spectrum of symbiotic host responses. Thus, appropriate selection for either mycorrhizal fungi or rhizobia must be carried out under local environmental conditions. This was done in our experiment, and the effectiveness of the selected microbial inoculants for promoting long-term establishment and development of native legumes is clear.

Since the two native shrubs which we used are participants in the natural plant succession in the area, (6, 11, 14, 15), they can be used as the basis for trying to accelerate such succession (11, 15, 21). Therefore, reclamation strategy (2) (Fig. 1) can be proposed, particularly with *Anthyllis cytisoides*, which is a more drought-tolerant species. The technique involving artificial acceleration of natural succession for revegetation can be followed by replanting randomly spaced groups of shrubs according to the natural pattern and structure of the climax ecosystem (11, 15, 21). While rhizobial inocula can be easily formulated, mass production of mycorrhizal inocula has well-known difficulties (4). Nevertheless, mycorrhizal biotechnology can be integrated into nursery and revegetation management, which is based mainly on the use of transplants of vegetatively produced plant material. It appears that appropriate microsymbiont management can help legumes (4, 19) to promote the stabilization of a self-sustaining ecosystem (9, 24). The mycorrhizal shrubs could serve as sources of inocula for the surrounding area and improve N nutrition in semiarid ecosystems for non-N-fixing vegetation.

## ACKNOWLEDGMENTS

This study was supported initially by the LUCDEME Project (CSIC-ICONA) and later was included in and sponsored by CICYT project NAT-91-1127.

We thank A. Francia and S. Cuadros for technical help.

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