ADVANCES IN RHIZOBIAL RESEARCH – PROGRESS PRIORITIES IN TEMPERATE AREAS

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Introduction

Rhizobia are well known for their capacity to establish a symbiosis with legumes. During this symbiosis the bacteria inhabit root nodules where they reduce atmospheric nitrogen and make it available to the plant. Biological nitrogen fixation (BNF) is an important source of nitrogen and the various legume crops and pasture species often fix as much as 200-300 kg nitrogen per hectare (Peoples et al., 1995). Globally, symbiotic nitrogen fixation has been estimated to amount to at least 70 million metric tons of nitrogen per year (Brockwell et al., 1995). Furthermore, in many cases nitrogen fertilizers are not efficiently used by crops and the environmental costs are high due to nitrogen losses from fertilizers (Peoples et al., 1994). The contribution of BNF has been suggested to be more open to management than fertilizer nitrogen (Peoples et al., 1995). Moreover, legumes stimulate the soil microflora and may favour the proliferation of plant pathogen antagonists while rhizobia may promote plant growth (Chabot et al., 1996; Schloter et al., 1997). Natural plant communities, legume crops, pastures, tree plantations and various integrated cropping systems such as alley cropping, intercropping and crop rotations can gain from nitrogen inputs by BNF (Wani et al., 1995; Thomas, 1995; Sanginga et al., 1995; Ikerra et al., 1999; Lehmann et al., 1999).

Nitrogen Fixation Potential

Rhizobial strains can greatly differ in their ability to fix atmospheric nitrogen. In the case of a symbiosis between one soybean cultivar and 20 strains of *Bradyrhizobium japonicum*, N fixation ranged from 38 to 76% N derived from the atmosphere (Hardarson et al., 1984). Several methods are available to measure nitrogen fixation, each having advantages and disadvantages. Such techniques include the determination of dry matter yield, the total N difference method, the acetylene reduction assay and ^{15}N methodologies being most suitable for evaluating nitrogen fixation efficiency (Hardarson and Danso, 1993).

The Rhizobium competition problem

Numerous rhizobial strains have been identified that show high nitrogen-fixing ability. Nevertheless, attempts to increase legume yields in agricultural fields by inoculation with superior strains often failed due to the inability of many inoculant strains to compete with indigenous rhizobial strains for nodule formation on the host plant. Important parameters affecting inoculation success are the inoculum size, i.e. the amount of *Rhizobium* cells added to the seed or the soil, and the size of the indigenous soil populations being able to nodulate the host (Thies et al., 1991). Besides the indigenous population size, the population structure plays an important role and environmental factors as well as agricultural practises may contribute to field dominance. In addition, the plant genotype plays an essential role in selecting the microsymbiont (Cregan and Keyser, 1989) and different genotypes may prefer more or less effective rhizobial partners. The method of rhizobial inoculation has been reported to affect the nodulation pattern (Danso and Bowen, 1989) demonstrating that soil inoculation gives increased nitrogen fixation over seed inoculation.

Several suggestions have been made how to overcome the rhizobial competition problem. In order to avoid competition for nodulation plant breeding programmes have been carried out using two approaches. The first approach has been directed towards the selection of highly effective combinations of host plant and bacterial cultivar (Alwi et al., 1989) or the development of lines with a restricted nodulation range that are able to bypass the native soil rhizobia (Montealegre and Kipe-Nolt, 1994). The second approach is to screen for plants that are nodulated by the most effective strains present in a natural soil population (Herridge and Rose, 1994). Regarding the bacterial symbiont, genetic engineering has been used to produce inoculant strains with enhanced competitive abilities (Triplett, 1990; Martinez-Romero and Rosenblueth, 1990; Novikova and Pavlova, 1993). In addition, dominant field strains may be used for inoculation and the recently developed molecular techniques may facilitate this approach.

Reliable and fast methods are required to assess the competitive abilities of bacterial strains and to assess their performance in various environments. The use of marker genes for identification of rhizobial strains has several advantages such as a high degree of specificity and the fact that the assay can be carried out on intact nodulated root systems. The reporter genes, *gusA* and *celB*, encoding ß-glucuronidase and a thermostable ß-glucosidase, respectively, have been demonstrated to be particularly convenient for *Rhizobium* competition studies (Sessitsch et al., 1996; Sessitsch et al., 1997; Sessitsch et al., 1998).

Rhizobium taxonomy

Early *Rhizobium* taxonomy has been mainly based on the nodulation host range (Fred et al., 1932), although overlapping host ranges have already been reported more than fifty years ago (Wilson, 1944). The development of molecular techniques accelerated the taxonomic evaluation and led to the identification of many new rhizobial genera. Based on the sequence of the 16S rRNA gene rhizobia could be grouped in the alpha subdivision of the *Proteobacteria* (Young and Haukka, 1996) and several genera have been defined including *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium* and *Mesorhizobium*.

The first described *Rhizobium* species, *R. leguminosarum*, can be grouped in three biovars: *R. leguminosarum* bv. trifolii that nodulated clover, *R. leguminosarum* bv. viciae that nodulates pea and faba bean, and *R. leguminosarum* bv. phaseoli nodulating common bean (Jordan, 1984). Various common-bean nodulating species have been described such as *R. etli* (Segovia et al., 1993), *R. tropici* (Martinez-Romero et al., 1991), *R. gallicum* (Amarger et al., 1997; Sessitsch et al., 1997a) and *R. gardinii* (Amarger et al., 1997). *R. hedysari* obtained from *Hedysarium coronarium* nodules has been characterized based on various fingerprinting techniques as well as phenotypic properties (Squartini et al., 1993; Selenska-Pobell et al., 1996). Other described species include *R. galegae* (Lindström, 1989), *R. huakuii* (Chen et al., 1991), *R. hainanense* (Chen et al, 1997), *R. huautlense* (Wang et al., 1998), and *R. mongolense* (van Berkum et al., 1998).

Two species of *Bradyrhizobium* are well known to nodulate soybean, *B. japonicum* (Jordan, 1982) and *B. elkanii* (Kuykendall, 1992). Xu et al. (1995) described another soybean nodulating species, *B. liaoningiensis*, consisting of extremely slow-growing strains. In addition, yet unnamed species have been found that nodulate other legumes than soybean (Young and Haukka, 1996).

The genus *Azorhizobium* includes strains that are very distinct from other rhizobia in many characteristics and *A. caulinodans* is the only species characterized up to now nodulating the roots and stems of *Sesbania rostrata* (Dreyfus et al., 1988). Recently, *Allorhizobium* *undicola* has been described as a new genus and species nodulating *Neptunia natans* (de Lajudie et al., 1998).

Sinorhizobium includes *S. fredii*, *S. meliloti*, *S. teranga* and *S. saheli*. *S. fredii* comprises fast-growing strains nodulating soybean, although strains of this species are also able to nodulate and fix nitrogen on various legumes (Krishnan and Pueppke, 1994). *S. meliloti* was isolated from alfalfa, while *S. teranga* and *S. saheli* have been isolated from various tree legumes such as *Sesbania* and *Acacia* species (de Lajudie et al., 1994). *S. medicae* members are able to nodulate various alfalfa species but show a different host range than *S. meliloti* strains (Rome et al., 1996).

Rhizobium diversity

Recently, studies have aimed to uncover the nature of rhizobial symbionts in their native environments as it has been recognized that one of the major problems in the application of BNF technology is the establishment of introduced inoculant strains. In addition, molecular tools have become available to analyse diversity and population structure of bacteria. The 16S rRNA gene sequences are an indispensable parameter in *Rhizobium* taxonomy and methods based on differences in ribosomal RNA genes have been frequently applied to species identification (Laguerre et al., 1994). Nevertheless, the conservative nature of 16S rRNA genes limits its use for discrimination at the strain level. The intergenic spacer between 16S and 23S rRNA genes was described to be more variable (Massol-Deya et al., 1995) and RFLP of the PCR-amplified IGS was used for the characterization of *Rhizobium* strains (Nour et al., 1994; Selenska-Pobell et al., 1996; Sessitsch et al., 1997b). The development of the polymerase chain reaction (PCR) led to new fingerprinting methods. Arbitrary oligonucleotide PCR primers of random sequence (RAPD) have been used to generate strain-specific fingerprints of *Rhizobium* (Selenska-Pobell et al., 1995; Paffetti et al., 1996). In addition, PCR primers based on short intergenic repeated sequences have been designed to fingerprint bacteria (de Bruijn et al., 1992; Versalovic et al., 1991) and this approach became a frequently employed technique for analysing bacterial communities (Laguerre et al., 1996; Sessitsch et al., 1997b)

Although the microsymbionts of plants other than crop species have been neglected for a long time, efforts have been undertaken to analyse rhizobia associated with economically less important leguminous plants such as nitrogen fixing trees (McInroy et al., 1999). Furthermore, the diversity of rhizobia occurring on native shrubby legumes in Southeastern Australia was recently investigated revealing a respectable diversity among the isolated strains (Lafay and Burdon, 1998). A high diversity has been found among bacteria establishing a symbiosis with common bean in European soils (Herrera-Cervera et al., 1999; Amarger et al.,1997; Sessitsch et al., 1997b), however, a molecular analysis revealed that the European strains are of American origin (Sessitsch et al. 1997a). Rhizobia nodulating alfalfa have demonstrated a tremendous diversity in various soils (del Papa et al., 1999; Hartmann et al., 1998; Paffetti et al., 1996). In an Italian field a population of 96 *Rhizobium meliloti* isolates, which were phenotypically indistinguishable, proved to consist of 55 different strains when analysed by RAPD-PCR. (Paffetti et al., 1999). Mendes and Bottomley (1998) demonstrated recently that a population of *R. leguminosarum* bv. trifolii was heterogenously distributed among different size classes of soil aggregates. This distribution was influenced by cover crop treatment and sampling time indicating that microsites exist in soils, which vary in their suitabilities to support growth and protection of bacteria.

Genetic manipulation of Rhizobium

Recently research efforts have been directed towards the use of genetic engineering in order to overcome problems related to the application of BNF. Essentially two objectives have been addressed – to increase the nitrogen fixation efficiency or to enhance the competitive ability of inoculant strains. Bosworth et al. (1994) engineered a *S. meliloti* strain by adding additional copies of *nifA* and *dctABD*, which proved to increase alfalfa yield under agricultural conditions. The rationale of this strategy was based on the positive regulatory role that *nifA* plays in the expression of the *nif* regulon and the fact that a supply of dicarboxylic acids from the plant is required as a carbon and energy source for nitrogen fixation by *Rhizobium* bacteroids. Furthermore, nodulation of alfalfa, nitrogen fixation and plant growth was increased by specific DNA amplification of symbiotic DNA regions in *Sinorhizobium meliloti* (Castillo et al., 1999). A *Rhizobium etli* mutant that overproduces the *Bradyrhizobium japonicum* symbiotic terminal oxidase has shown significantly enhanced symbiotic performance as judged by the determination of nitrogenase activities of plants inoculated with this mutant (Soberon et al. 1999).

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