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Tesi di Dottorato di Ricerca in

Scienze e Tecnologie delle Produzioni Agro-Alimentari

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Resistance to abiotic stress in *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti* 1021

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- INTRODUCTION -

1. NITROGEN FIXATION

Virtually all plants live in intimate association with microorganisms, which can colonize the surfaces of plants (epiphytic colonization) or occupy spaces within plants tissues (endophytic colonization). Many types of microbes live in close association with host plants and benefit from these associations by obtaining carbon and other nutrients from their hosts.

The rhizosphere (the zone that surrounds the roots of plants) and roots are easily colonized by microbes (Bolton et al., 1992; Bowen and Rovira, 1999), since sources of carbon and minerals are very abundant in this zone (Curl and Truelove, 1986; Walker et al., 2003). Plants exude high levels of nutrients from their roots: low-molecular-weight root exudates (such as amino acids, organic acids, sugars, aromatiscs and other secondary metabolite) and high-molecular-weight root exudates (such as polysaccharides and proteins) (Marschner, 1995). This complex mixture of organic compounds results in the "rhizosphere effect" (Bolton et al., 1992).

A large variety of bacteria, fungi, protozoa, and nematodes colonize the rhizosphere (Bolton et al., 1992; Bowen and Rovira, 1999). These organisms may exist as free-living organisms in the soil or attached to surface of roots.

Soil bacteria belonging to various genera of the order Rhizobiales (collectively called rhizobia) are able to invade legume roots in nitrogen-limiting environments, leading to the formation of a highly specialized organ, the root nodule. The α-proteobacteria include various nitrogen-fixing plant symbiont, such as *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (*Ensifer*). In the root structures the bacteria, through the induction of the nitrogenase complex, are able to convert

atmospheric dinitrogen into ammonia, which is used by the plant as a nitrogen source, while the plant supplies the carbon source for the reduction of nitrogen.

2. RHIZOBIA-PLANT SYMBIOSIS

2.1. Rhizobia- plant interaction

Nodule formation is a complex process that requires a continuous and adequate signal exchange between the plant and the bacteria, of which we only have a fragmentary knowledge.

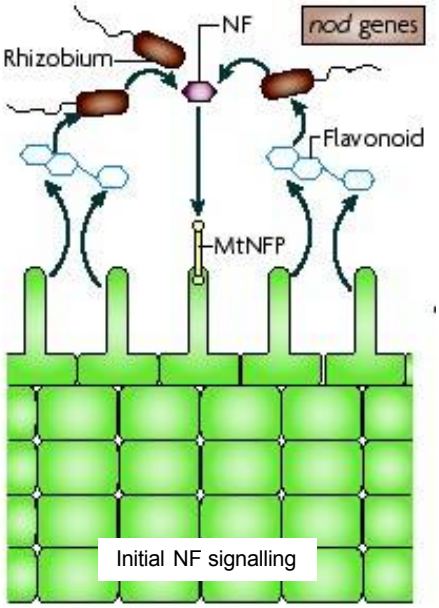
The early and essential step in the plant-microbe interactions involves bacterial chemiotaxis toward plant roots. Rhizobia are attracted by root exudates and colonize the plant root surface. Specific flavonoid compounds present in the exudates activate the expression of the bacterial nodulation (*nod*) genes involved in the synthesis and secretion of Nod-factors (NF), lipochito-oligosaccharides that are recognized by the plant. The genes specifically required for interaction with a host plant are carried on the symbiotic plasmids pSymA and pSymB in *Sinorhizobium meliloti* (Galibert et al., 2001), and on a chromosomally located symbiotic island in *Bradyrhizobium japonicum* and *Mesorhizobium loti* (Kaneko et al., 2000; Kaneko et al., 2002).

Nod factors, together with additional microbial signals such as polysaccharides and secreted protein, allow bacteria attached to root hairs to penetrate the root. For many rhizobia, primary target sites for infection are young growing root hairs, but there are no exclusive loci for rhizobial attachment (van Rhijin and Vanderleyden, 1995). Binding of rhizobia to plant surface is thought to take in two steps (Matthysse and Kijne, 1998).

The first one is a weak and reversible binding step that involves same bacterial polysaccharides (Brencic and Winans, 2005). The second binding step requires the synthesis of bacterial cellulose, which causes a tight, irreversible binding and formation of bacterial aggregates on the host surface (Matthysse, 1983; Robertson et al., 1988).

Specific adherence of compatible rhizobia was proposed to be mediated by specific binding of particular polysaccharide structures present on the bacterial cell surface to host plant lectins (van Rhijin and Vanderleyden, 1995). The synthesis and release by a legume host of Nod factors induces developmental changes in the plant (Downie and Walker, 1999; Stougaard ,2000; Geurt and Bisseling, 2002). The tip of a root hair, to which rhizobia are bound, curls back on itself, trapping the bacteria within a pocket of host cell wall (Fig. 1). After entrapment, a local lesion of the root hair cell wall is formed by hydrolysis of the cell wall. The plasma membrane invaginates and the host plant deposits new cell wall material around the lesion forming a tube. The bacteria proliferate in this zone forming an intracellular-infection thread (Brencic and Winans, 2005). The infection thread grows toward the inner tangential wall of the root hair cell tip by a process of tip growth (van Rhijin and Vanderleyden, 1995).

a



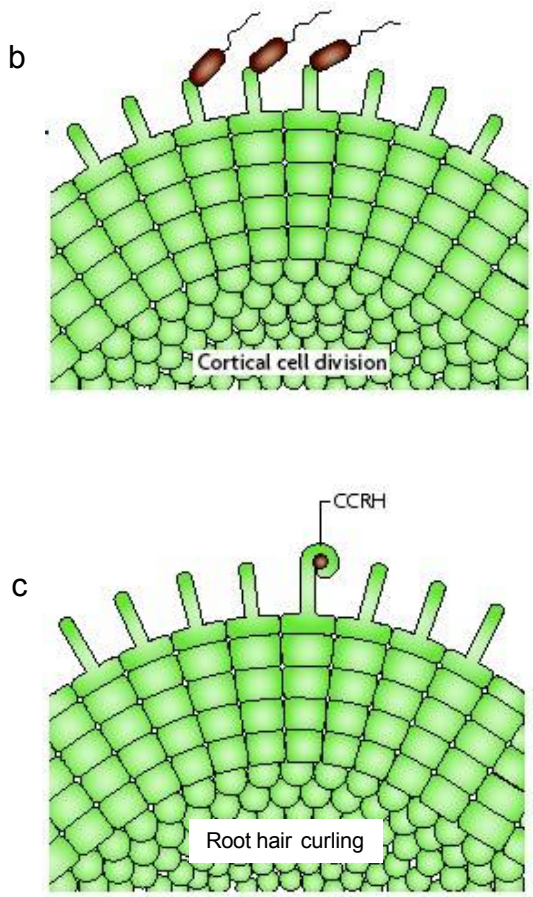


Fig. 1. The initial signalling dialogue between *Sinorhizobium meliloti* and *Medicago truncatula*. **a,** The induction of rhizobial nod genes requires plant flavonoids. The nod gene products produce Nod factor (NF), which is initially perceived by the M. truncatula MtNFP receptor; **b,** Root hair curling and cortical cell division; **c,** formation and colonization of curled root hair (CCRH) (Jones et al., 2007).

At the same time with formation of the infection thread, particular cortical cells divide to form a nodule primordium, and the infection thread grows toward these primordia (Vasse and Truchet, 1984; Wood and Newcomb, 1989).

2.2. Nodule formation

The sequence of rhizobia-induced cell division and cell invasion varies for different legumes, and this has important consequences for nodule morphogenesis; in fact nodules induced by rhizobia can be of two types, determinate and indeterminate.

Legumes that form determinate nodules are typically tropical in origin and include *Glycine max* (soybean) and *Lotus japonicus* (Gage, 2004). In these host plants, cortical cell division occurs just beneath the epidermis. These cells are invaded by rhizobia before they become meristematic and therefore rhizobia can spread by dividing within the cytoplasm of host cells, which are, in their turn, dividing. Thus the nodule meristem involves a mixture of infected and uninfected cells derived from the root cortex, together with uninfected cells derived from the root pericycle. This form of nodule development results in the formation of spherical nodules in which meristematic activity is only a transient. In fact, they are characterized by a persistent meristem in which all cells in the interior of the nodule proliferate, differentiate and senesce synchronously (Brewin, 1991) (Fig. 2).

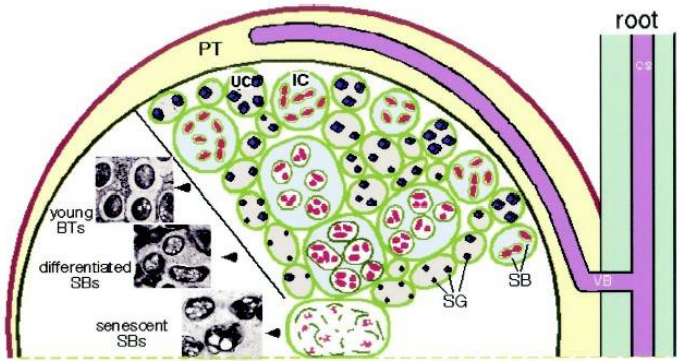


Fig. 2. Scheme of the determinate globose nodule. CS, central stele; VB, vascular bundle; PT, peripheral tissue; BTs, bacteroids; SBs, symbiosomes; SG, starch grain; IC, invaded cell; UC, uninvaded cell (Patriarca et al., 2002).

Medicago sativa (alfalfa), *Medicago truncatula*, *Pisum sativum* (pea), *Vicia* species (vetches), and *Trifolium* species (clovers) have historically been used as models for

studying the formation of indeterminate nodules (Gage, 2004). Indeterminate nodules appear elongated because they are characterized by a continuously growing nodule meristem at the distal end and they have zones of tissue at different stages of development (Jones et al., 2007). While the meristem is active, rhizobia are released from the infection threads into the plant cell cytoplasm, where they differentiate into their endosymbiotic form, the bacteroids. These bacteroids are able to reduce dinitrogen into ammonia, which is used by the plant. In return, the bacteria are supplied with carbohydrates in a protected environment. It is possible to distinguish five steps in bacteroid differentiation (types 1 to 5) in the four different histological zones of the indeterminate nodule (Vasse et al., 1990; Luyten and Vanderleyden, 2000) (Fig. 3).

Zone I is situated at the apical part of the nodule and contains meristematic tissue devoid of bacteria.

Zone II is the infection zone. In this zone the bacteria penetrate the root cells via infection threads. Bacteria released from the infection threads are called type 1 bacteroids. They are cells characterized by a large periplasmic space. Type 1 bacteroids divide and resemble free-living bacteria by size and cytoplasm content. In the proximal part of zone II there are type 2 bacteroids. Type 2 bacteroids are eloganted cells; in fact their periplasmic and peribacteroid spaces are reduced. The bacteroid differentiation occurrs by stop of the cell division and of the DNA replication, but this one occurs only after a few rounds of replication, when bacteria are released into the plant cytoplasm. Therefore, bacteroids have an increased DNA content comparing to the free-living cell.

Interzone II-III is a very restricted zone, containing three to four layers of cells in the mature nodule. It contains type 3 bacteroids, which stopped elongating and which are about seven times longer than free-living bacteria. The cells do not fix nitrogen in this

zone, but transcription of the nitrogen fixation genes (*nif* and *fix*) starts here, since the nodule parenchyma forms the oxygen diffusion barrier.

Zone III spreads over eight to twelve cell layers. In the distal zone the type 4 bacteroids (bacteroids fully differentiated) are present. Nitrogen fixation takes place since leghaemoglobin, capable to bind oxygen molecules, is produced, protecting the nitrogenase.

In the proximal zone III the last step of bacteroid differentiation occurs. The bacteroids (type 5) become variable in morphology and stop fixing nitrogen.

Zone IV is the senescence zone and it is located proximal to the point of attachment to the plant root. Here, both symbiotic partners degrade and the number of bacteroids gradually decreases. Ghost membranes of plant and bacteroid origin are the ultimate result of the senescing process.

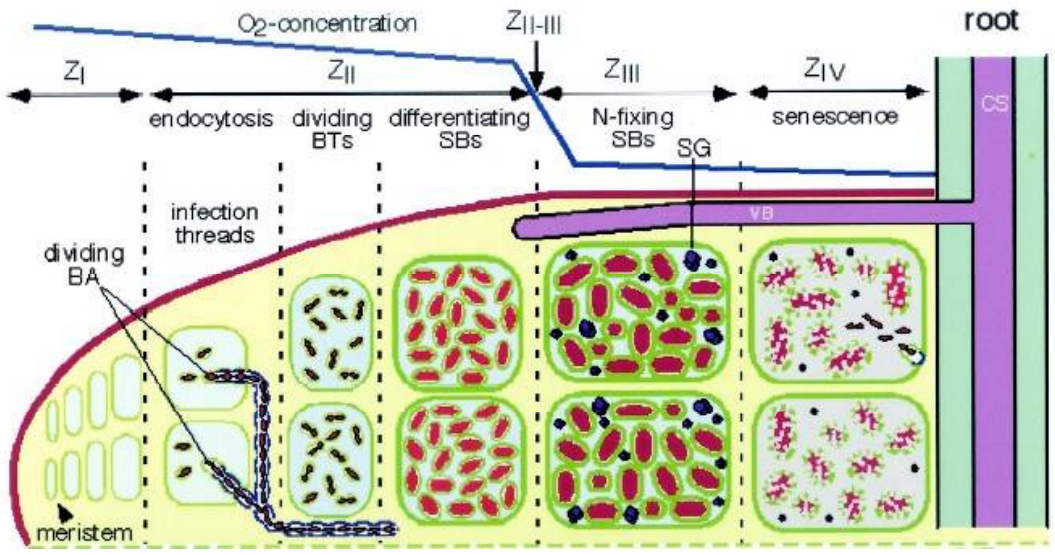


Fig. 3. Scheme of the indeterminate elongated nodule. BA, bacteria; BTs, bacteroids; SBs, symbiosomes; SG, starch grain; CS, central stele; VB, vascular bundle. The nodule zones (Z) are indicated (Patriarca et al., 2002).

3. RHIZOBIAL GENES FOR INFECTION AND NODULATION

3.1. Production of exopolysaccharides during nodule invasion

The structure and function of bacterial cell surface components may play an important role during infection, as a protection against host responses and new physiological conditions.

After bacteria enter a root hair, they begin to travel along an infection thread toward a developing nodule. The initiation and extension of the infection thread depends on the production of specific exopolisaccharides (EPS) by the bacteria (Becker and Pühler, 1998; Fraysse et al., 2003). EPS are extracellular polysaccharides that accumulate on the surface with little or no cell association and are one of the major compounds of the bacterial outer surface. The location of EPS contributes to cell protection against environmental influences, surface attachment, nutrient gathering and antigenicity. An array of different structure can be formed on the basis of the variation in structure. Strain-specific EPS are polymers of repeating units of seven, eight or nine sugar residues, linked with non-carbohydrate substitutions such as acetate, pyruvate or succinate (Luyten and Vanderleyden, 2000). The synthesis of EPS originates from cytoplasmic sugars, and its biosynthesis therefore involves mechanisms of assembly, polymerisation and export. However, the structural diversity suggests a function as signal molecules, important during cell-cell communication.

The three known EPS that are important for symbiosis are a cyclic neutral glucan, succinoglycan (also known as EPSI), and EPS II. The first of these is encoded by the *ndvAB* operon, while succinoglycan is encoded by a 24-kb cluster of *exo* genes and EPS II is encoded by a 32-kb cluster of *exp* genes (Becker and Pühler, 1998). In *S. meliloti*,

the last two clusters are located on the large symbiotic plasmid pSymB (Galibert et al., 2001). EPS and their role in symbiosis have been studied intensively in *S. meliloti*.

EPS production depends on the concentration of available phosphate, which might be sensed by the bacteria during the process of nodulation (Mendrygal and Gonzales, 2000; Zhan et al., 1991). Phosphate concentration is very low in the soil (typically 1 to 10 μM) and considerably higher within plant tissues (10 to 20 mM). EPS II is produced preferentially under low-phosphate conditions, whereas succinoglycan synthesis is stimulated at high concentrations of phosphate (Mendrygal and Gonzales, 2000). This suggests that inside the plant, bacteria produce succinoglycan, which is consistent with the observation that although both EPS can mediate nodule invasion, succinoglycan is much more efficient in this process (Pellock et al., 2000).

Mutations in *exo* genes, which abolish the EPSI production, cause severe defects in symbiosis in *S. meliloti* (Jones et al., 2007). While, in a different host-symbiont pair, involving vetch (*Vicia sativa*) and *R. leguminosarum*, the structure of the bacterial exopolysaccharide appears to be less critical for infection thread extention (Laus et al., 2005).

3.2. Nodulation genes

Nod factors synthesized by bacteria consist of a β-1,4-linked *N*-acetyl-D- glucosamine backborne with four or five residues. Nod factors are synthesized and exported from the bacteria by the products of the *nod* genes (Downie, 1998). The *nod* genes are expressed in response to specific polycyclic aromatic compounds called flavonoids, which are released by plants into the rhizosphere (Brencic and Winans, 2005). Each rhizobial species possesses specie-specific *nod* gene; therefore it produces a different set of Nod

factors, which play a critical role in host specificity (Downie, 1998; Cullimore et al., 2001).

The *nodABC* genes are present in all rhizobia and are essential for the nodulation. In fact these genes are required for production of the basic Nod factor, since they encode for the enzymes involved in the synthesis of the chitin oligomer backbone (John et al., 1993; Geremia et al., 1994; Rohrig et al., 1994). In most species, the *nod ABC* genes are part of a single operon. Inactivation of these genes abolishes the ability to elicit any symbiotic reaction in the plant, including root hair curling, infection thread formation, cortical cell division, and nodule formation, regardless of the host, the mode of infection, the type of nodule development and the nodule location (Long, 1989; Martinez et al., 1990).

NodD gene is expressed constitutively (Mylona et al., 1995). NodD proteins are members of the LysR family of transcriptional regulators (Schell, 1993). They are thought to be direct receptors of the plant-released signals and to be flavonoid-dependent transcriptional activators of *nod* promoters (Schlaman et al., 1998). Many species of rhizobia possess more than one copy of the *nodD* gene, and the properties of different *nodD* genes vary within the same strain as well as from one rhizobia species to another. Some strains possess two to five copies of *nodD* genes (Fellay et al., 1995; Schlaman et al., 1998) and may in addition possess one or two copies of another LysR-type regulator gene called *syrM* (symbiotic regulator) (Michiels et al., 1993; Swanson et al., 1993; Hanin et al., 1998). Different NodD proteins may differ in their affinity for various *nod* boxes and may also have different flavonoids specificities (Brencic and Winans, 2005).

Other nodulation genes have been identified, which are not functionally or structurally conserved among rhizobia. These genes are necessary for the nodulation of a particular

host plant. Mostly mutations in these genes result in alteration or extension of the host range (van Rhijin and Vanderleyden, 1995). *nodEF* mutants have a delayed nodulation phenotype and show low number of infection threads in *Sinorhizobium meliloti* (Demont *et al.*, 1993) and nodulate white and red clover poorly but they have acquired the ability to infect and nodulate peas in *R. leguminosarum* bv. *trifolii* (Spaink et al., 1989). In *Rhizobium* sp. strain NGR234 and in *R. tropici* CIAT899, mutation of *nodS* causes a loss of nodulation in particular host plants (Lewin et al., 1990; Waelkens et al., 1995). In *S. meliloti*, *nodL* mutant have a delayed nodulation phenotype and the overall production of Nod factors is reduced 5- to 10-fold. Depending on the host tested, *nodL* mutants show different symbiotic defects (Luyten and Vanderleyden, 2000). Mutations in *nodIJ* result in a delay of nodulation in *R. leguminasarum* (Enavs and Downie, 1986). The *nodHPQ* genes are responsible for the formation of the sulphate group on the reducing sugar in *S. meloliti* (Roche *et al.*, 1991).

3.3. Nitrogen fixing genes

In the rhizobium-legume symbiosis, the bacterial partner differentiates into nondividing endocellular symbionts that, through the induction of nitrogenase complex, convert atmospheric N_2 to NH_3/NH_4^+, which is eventually used by the plant.

Nitrogen fixation is a highly energy-dependent process. Therefore, it is not surprising that not only the genes encoding for nitrogenase components are crucial for the nitrogen fixation, but also the genes encoding for electron transport chain proteins, for proteins of metabolic pathways and for the dicarboxilic acid transporter protein. Nitrogen fixation is directed by two sets of genes, *nif* genes and *fix* genes (Fischer, 1994; Kaminski et al., 1998).

The transcription of the rhizobial genes coding for the nitrogenase complex is dependent on the *nifA* gene encoding a regulatory protein, which is expressed under specific environmental conditions, including low O_2 tension (Batut and Boistard, 1994). *nifHDK* genes encode structural proteins of the nitrogenase enzyme, *nifENB* genes encode enzymes involved in biosynthesis of the nitrogenase Fe-Mo cofactor and *nifSWX* genes encode proteins of unknown functions that are required for full nitrogenase activity (Brencic and Winans, 2005).

The *fix* genes represent a very heterogeneous class including genes involved in the development and metabolism of bacteroids. The *fix L*, *fixJ* and *fixK* genes encode regulatory proteins. The *fix L*, *fixJ* genes belong to a family of prokaryotic two-component signal transduction system and they are responsible for the oxygen regulation in *S. meliloti* (Agron and Helinski, 1995). FixL, the sensor, activates FixJ by phosphorylation under microaerobic conditions. Phosphorylated FixJ induces the expression of the regulatory *fixK* and *nifA* genes, whose products regulate transcription of the most nitrogen fixation genes (Luyten and Vanderleyden, 2000). Mutations in *fixLJ* result in bacteroids that are impaired in their development once they reach the type II bacteroid stage (Vasse et al., 1990).

The *fixABCX* genes might code for an electron transport chain to nitrogenase. Mutations in any one of the *fixABCX* genes of *S. meliloti*, *B. japonicum*, and *A. caulinodans* completely abolish nitrogen fixation. All four *fixGHIS* gene products are predicted to be transmembrane proteins, but further biochemical analysis is required to define their function in rhizobial nitrogen fixation (Fischer, 1994). The *fixNOQP* genes encode the membrane-bound cytochrome oxidase that is required for respiration of the rhizobia in low-oxygen environments (Delgado et al., 1998; Preising et al., 1996).

4. COMPETITIVENESS AND PERSISTENCE IN THE SOIL

A large variety of bacteria, fungi, protozoa, and nematodes colonize the rhizosphere. Plant root exudates contain a great variety of different compounds, and nearly 5% to 21% of all photosynthetically fixed carbon can be transferred to the rhizosphere through the root exudates (Walker et al., 2003). Microbes compete for the utilization of the abundant, organic compounds released by the plant upon growth and for mineral elements (Mirlerau et al., 2000). Therefore, in field conditions, the ability to transport and catabolize the compounds represented in the root exudates is crucial for the survival and competitiveness of bacteria in the plant rhizosphere.

Bacteria have evolved a wide variety of metabolic strategies to cope with varied environments. Some are specialists and only able to survive in restricted environments; others are generalists and able to cope with diverse environmental conditions. Rhizobia can survive and compete for nutrients in the soil and the plant rhizosphere but can also form a beneficial symbiosis with legumes in a highly specialized plant cell environment. Different strains and species of rhizobia exhibit different catabolic capacity, this might be important in their adaptation to survival in the rhizospheres of different groups of host and non-host plants.

The metabolic diversity of rhizobia is reflected in their large, complex genome, which range in size from 6.5 Mb (*R. etli*; www.cifn.unam.mx/retlidb/), 6.7 Mb (*S. meliloti*; Galibert et al., 2001) and 7.6 Mb (*M. loti*; Kaneko et al., 2000) to 7.8 Mb (*R. leguminosarum*; www.sanger.ac.uk/Projects/R_leguminosarum/) and 9.1Mb (*B. japonicum*; Kaneko et al., 2002). Many genes are involved to transport, regulation and a wide range of catabolic systems (Boussau et al., 2004). For example, rhizobia have approximatively 170 ATP-binding cassette (ABC) transport systems (compared with 47

in *Escherichia coli*), which enable rhizobia to access a far greater range of nutrients present at low concentrations in soil and the plant rhizosphere (Prell and Poole, 2006).

Abiotic factors, such as temperature, osmotic pressure, UV light, and pH, and the relevant variation of these factors also play a role in the selection and activity of microbes in soils or at the plant surface (Savka et al., 2002). Some rhizobial strains, which are integral to legume production, seem more tolerant to abiotic stress than their host plants; under stress conditions, root-associated beneficial bacteria can help improve plant growth and nutrition. An example, in saline soils, organic matter application or inoculation of crops with tolerant symbiotic strains of *Rhizobium* may improve plant nutrition, increasing their stress tolerance (Rao, 1998). The aim of inoculation is to provide sufficient numbers of viable effective rhizobia to induce a rapid colonization of the rhizosphere so that nodulation will take place as soon as possible after germination and produce optimum yields (Catroux et al., 2001).

The microorganisms living at the plant surface are well adapted to the biological and physico-chemical constraints of the environment (Parke, 1991; Savka et al., 2002). Hence, they are poorly accessible to displacement by the introduced microbe.

Several possibilities exist to improve the competitiveness of an introduced microorganism of interest in the plant environment. One may promote its multiplication in the plant environment, impede growth of competing microorganisms, or interfere with some of the signals perceived by the microbes, provided these signals control (at least in part) the expression of functions central to microbial fitness. Since this environment is a triple interface (bacteria, plant, and soil), it is theoretically possible to modify one, two, or three of these parameters to improve microbial colonization.

Bacteria in the rhizosphere compete via production of and resistance to antibacterial compounds that can originate both from plant root system and from other soil organisms

(Savka et al., 2002). For example, the toxin mimosine, produced by the tree legume *Leucaena*, provides a nodulation competition advantage to mimosine-degrading *Rhizobium* strains (Soedarjo et al., 1994). Moreover, the production of the peptide antibiotic trifolitoxin (TFX) by *Rhizobium leguminozarum* bv. trifolii results in an increased nodule occupancy at least 20% higher than the non-producing strains in field grown plants 2 years after inoculation (Robleto et al., 1998).

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- CHAPTER 1 -

EFFECT OF SALINITY STRESS ON STRAINS OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE* AND THEIR HOST PLANTS

I.1. INTRODUCTION

One of most severe and widespread problems facing the agricultural industry is the degradation of soil quality due to desiccation and salinity. Soil salinization is increasing steadily in many parts of the world and causes major problems for the productivity of agricultural crops (Rao and Sharma, 1995). Nearly 40% of the world's land surface can be categorized as having potential salinity problems (Zahran, 1999).

In coastal regions of Mediterranean areas, summer crops are often irrigated with saline water. As a consequence, if the rainfall is not sufficient to remove them from the root-zone, salts accumulate along the soil profile. The consequences of soil salification on soil physical fertility are well documented. The saturation level of sodium is considered the main cause of clay dispersion and degradation of the aggregate stability (Varallyay, 1977; Tedeschi and Dell'Aquila, 2005), with the consequent reduction in permeability, porosity and hydraulic conductivity of soils (Frenkel et al., 1978; Amézketa, 1999). These harsh environmental conditions can have a dramatic impact on the soil bacteria (Fierer et al., 2003; Griffiths et al., 2003) and their impact on the endogenous group of proteobacteria, the rhizobia, is of particular importance for agricultural industry. The importance of nitrogen fixation for agriculture cannot be understated and is illustrated by the numerous studies of the impact of soil management on rhizobial populations in arid regions (Howieson and Ballard, 2004), as well as the isolation and characterization of desiccation- and salt-resistant strains (Chen et al., 2000; Yan et al., 2000; Jenkins, 2003). Furthermore, to enhance nodulation and nitrogen fixation efficiency, techniques that allow close contact between the bacteria and the host seed have been developed.

Several studies showed the effects of cover crop incorporation in sodic soil reclaimation, because they allowed soil physical characteristics to be improved,

including water infiltration (Cassman and Rains, 1986), aggregation and porosity (Macrae and Mehuys, 1985) and reductions in soil crust formation (Mitchell et al., 2000).

Leguminous cover crop, thanks to biological nitrogen fixation (BNF) and organic N input, may also reduce the seasonal fertilizer-N requirement (Shennan, 1992), and satisfy the nitrogen uptake of horticultural crops (de Luca et al., 2006).

Unfortunately the legume crops are known as very sensitive to saline conditions (Katerji et al., 1992) most likely because, besides the effects of salinity on soil water potential and soil physical properties, there is an additional effect of soil salinity on symbiotic N-fixation and thus on N uptake of the plant (Bernstein e Ogata, 1966; Katerji et al., 1998). The nitrogen contribution of the soil decreases as salinity increases, because salinity reduces the mineral nitrogen production by biological activity in the soil through nitrogen fixation and transformation of organic nitrogen (Van Horn et al., 2001). This effect could be related to the reduction of porosity and thus of aerobic conditions of salinized soils.

The legume crops show different salt sensitivity that could be explained by other experiments that found chickpea very resistant to drought, due to an osmotic adjustment until to -2 MPa, but more sensitive to saline stress than broadbean and soybean for a different salt tolerance of the respective rizobia strains (Katerji et al., 2003). Considering that nodule conductance to O_2 diffusion is judged a major factor of the inhibition of N_2 fixation by soil salinity, the different tolerance was associated with stability in nodule conductance under salinity of the tolerant genotypes (L'taief et al., 2007).

Information on resistance in the common vetch (*Vicia sativa* L.) and other vetch species to salinity are not available (UC SAREP, 2008). Only the salinity resistance of 30 days

plantlets of common vetch at seedling stages was evaluated with an experiment in which this species showed an increase of seedling fresh weight until to 1.35 dS/m and a decrease at 2.70 dS/m, while dry weight was not affected by salinity (Orak and Ates, 2005).

Legumes are suggested as appropriate crops for the enhancement of bioproductivity of marginal lands as well; in fact, these plants not only yield nutritious fodder, protein-rich seeds and fruits, but they also have the advantage of being able to establish symbiotic association with certain bacterial microsymbionts such as rhizobia and so to enrich soil nitrogen (Alexander, 1984). Nodulation and nitrogen fixation in legume-*Rhizobium* associations are adversely affected by salinity, which can preclude legume establishment and growth, or reduce crop yield (Mohammad et al., 1991). Some strains of Rhizobium spp., which are integral to legume production, seem more salt tolerant than their host plants; under stress conditions, root-associated beneficial bacteria can help improve plant growth and nutrition. Actually, rhizobial strains differ in their ability to response to an abiotic stress and they may use distinct mechanisms for osmotic adaptation when exposed to salt stress. After an osmotic up shift, general metabolism slows; in fact the genes involved in the tricarboxylic acid cycle, in the uptake of a carbon source (using mannitol), and in respiratory chains and ribosomal genes are repressed. Interestingly, 25% of all genes specifically downregulated by NaCl encode ribosomal proteins (Dominguez-Ferreras et al., 2006; Vriezen et al., 2007).

In stress condition, rhizobia strains can accumulate low-molecular-weight organic solutes (Miller and Wood, 1996; Zahran, 1999) or change in cell morphology and size, and modifications in the pattern of extracellular polysaccharides (Lloret et al., 1995; Lloret et al., 1998; Soussi et al., 2001). Rhizobia accumulate potassium ions (Yap and Lim, 1983), for which no new protein synthesis is required. This suggests that K^+

uptake is regulated biochemically and used as a secondary messenger. Nogales et al. (2002) reported a high-affinity K^+ uptake (Kup) system in *Rhizobium tropici* that has a homolog in *S. meliloti* 1021 (SMa1798), while a second, low-affinity Kup system can be identified (SMc00873), as well as the osmosensitive Kdp system (SMa2329, -2331, and -2333). BetS is a betaine/proline transporter also involved in the early response to osmotic stress. As with K^+ uptake activity, BetS is regulated biochemically (Pocard et al., 1997). Moreover, under growth-limiting conditions, C sources accumulate in the form of glycogen, which may assist in restoring cell volume after osmotic shock (Han et al., 2005), but accumulation of glycogen may also be a response to prevent starvation (Dominguez-Ferreras et al., 2006). After these initial reactions, stressed cells accumulate compatible solutes, which include carbohydrates, disaccharides such as sucrose and trehalose (Breedveld et al., 1993; Gouffi et al., 1999), maltose, cellobiose, turanose, gentiobiose, palatinose (Gouffi et al., 1999), and amino acids, among which mainly glutamate and proline (Hua et al., 1982; Botsford, 1984; Botsford and Lewis, 1990; Ruberg et al., 2003; Dominguez-Ferreras et al., 2006). Finally, osmotically stressed cells alter macromolecular structures, including long-chain exopolysaccharides (EPS) (Breedveld et al., 1991; Cheng and Walker, 1998; Lloret et al., 1998) and lipopolysaccharides (LPS) (Lloret et al., 1995; Bhattacharya and Das, 2003; Campbell et al., 2003).

In saline soils, organic matter application or inoculation of crops with tolerant symbiotic strains of *Rhizobium* may improve plant nutrition, increasing their stress tolerance (Rao, 1998); actually it is widely recognized that indigenous rhizobia play an important role in the dominance of Fabaceae in poor and arid soils (Zahran, 1999). The aim of inoculation is to provide sufficient numbers of viable effective rhizobia to induce a rapid colonization of the rhizosphere, so that nodulation will take place as soon as

possible after germination and produce optimum yields (Catroux et al., 2001). The legume seed is commonly inoculated with peat cultures or liquid inoculant before planting (Deaker et al., 2004).

Laboratory studies showed that salinity reduces nitrogen fixation, but no data are available for the salinity effect on nitrogen fixation for crops grown during a full season under field conditions (Cordovilla et al., 1994; Cordovilla et al., 1999).

In this study we evaluated, in open field, the effect of increasing soil salinity on the most interesting legume cover crops for the mediterranean area, broad bean and common vetch (Guiducci et al., 2004), inoculated with salt-tolerant and salt-sensitive strains of *Rhizobium leguminosarum* biovar *viciae* (*R. leg.* bv. *viciae*) showing a high symbiotic potential. The two rhizobia strains were evaluated for their ability to compete in soil containing a naturalised population of rhizobia and their potential use in sustainable agriculture projects, since biological nitrogen fixation (BNF) and his ecological role is an important process in plant nutrition.

I.2. MATERIALS and METHODS

I.2.1. BACTERIAL STRAINS and GROWTH CONDITIONS

Salt-tolerant strain *Rhizobium leguminosarum* biovar *viciae* (*R. leg.* bv. *viciae*) SAAN1 (able to grow up to 2% (w/v) NaCl) and salt-sensitive strain *Rhizobium leguminosarum* biovar *viciae* (*R. leg.* bv. *viciae*) POHY2B1 (able to grow up to 0.1% (w/v) NaCl), previously isolated in Campania region (Southern Italy) (Moschetti et al., 2005), were used for inoculation on seeds in artificial salt soils.

The strains were grown on yeast mannitol (YM) medium (10g/L mannitol, 0.4 g/L yeast extract, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4$, 0.1 g/L NaCl, pH 6.8-7.0, Vincent, 1970) at 28 °C for 5 days with rotary shaking at 150 rpm. Strain cultures were harvested at the late exponential phase of growth. Routine plating on YM agar supplemented with congo red (Graham, 1969) were performed to confirm the purity.

I. 2.2. PLANT NODULATION TESTS

The strains were evaluated for their symbiotic potential with broad bean (*Vicia faba* L. var. *major* Harz., cv. 'Dulce') and vetch (*Vicia sativa* L). Inoculation and seed treatment were performed as previously reported (Moschetti et al., 2005). The plants were cultured in a growth chamber under a constant temperature of 21 °C and 12 h d^{-1} photoperiod and watered with nitrogen-free nutrient solution (Priefer et al., 2001). Forty days after planted, the effectiveness of the nodules for nitrogen fixation was estimated from the pink of the nodules and the dark green of the leaves compared to control plants (without inoculation) (Moschetti et al., 2005). All experiments were performed in triplicate.

I.2.3. EXPERIMENTAL FIELD

In 2004 and 2005, winter experiments were carried out at the University of Naples experimental farm (latitude 40° 31' N; longitude 14° 58' E) on a field that had been irrigated since 1988 with saline water during the summer (DePascale and Barbieri, 1995). The soil was clay loam with 42% sand, 27% silt, 31% clay, trace amounts of lime, 1.57% organic matter, 0.09% N, pH of 7.1, and soil water contents at field capacity and at −1.5MPa of 34.5 and 17.5% (v/v), respectively. The effects of residual soil salinization were evaluated on broad bean (*Vicia faba* L. var. *major* Harz., cv. 'Dulce') and vetch (*Vicia sativa* L.). The irrigated summer crop, which preceded broad bean and vetch, was tomato. For this crop, the salinization treatments consisted of four salt concentrations of the irrigation water with ECw of 2.3 (S1), 4.4 (S2), 8.5 (S3) and 15.7 (S4) dSm^{-1}. Saline water was obtained by adding commercial sea salt (Na^+ 12.3, K^+ 3.8, Ca^{2+} 0.02, Mg^{2+} 0.04, Cl^- 14.4, $SO_4{}^{2-}$ 0.03 mol kg^{-1}) to the irrigation water (Na^+ 0.53, K^+ 0.05, Ca^{2+} 1.55, Mg^{2+} 0.84, Cl^- 0.38, $SO_4{}^{2-}$ 0.15, HCO^{3-} 4.73 mol m^{-3}). Furthermore, a non-salinized control (NSC, 0.5 dSm^{-1}) was included. In 1988, when we began a project aimed at evaluating the long-term effect of soil salinization on plant response to saline irrigation, the salinity treatments and relative controls were arranged in a randomized block design with three replications. Since the objective of that study was to investigate long-term effects of salinization, the salinity treatments, randomly assigned within each block in 1988, had to be reassigned to the same experimental field plots in the following years. Therefore, since 1988 each 100 m^2 experimental plot had been receiving the same EC irrigation water.

In the 2004-2005 fall/winter, the salt-tolerant strain SAAN1 (I1) and the salt sensitive strain POHY2B1 (I2) of *R. leg.* bv. *viciae*, were inoculated on seeds of broad bean and

vetch as above reported. Seeds were coated with liquid inoculants applied at 3.0 ml/kg of seed (approximately 10^5 bacteria/seed) or with a physiological solution (Negative Control, I0) before planting. After 24 h the seeds were sowed on December 7, 2004, in rows 0.8 m apart to reach 9 seeds m^{-2} for broad bean and 180 seeds m^{-2} for vetch. Two days after sowing, plots were irrigated with cell suspension (in physiological solution) of SAAN1, POHY2B1 or with physiological solution (Negative Control). To ascertain the successful of the inoculums, a total of 20 pre-treated seeds were put in greenhouse chamber and experiment was carried out as above reported.

Plants were harvested at flowering stage on May 11, 2005. Plants were cut at the soil surface, counted and weighted. Fresh and dry mass (after drying at 60 °C) yield were recorded.

Samples of plant tissues (dried and ground) were analyzed for organic nitrogen (Kjeldhal method) and nitrate contents (spectrophotometrically).

Before sowing and later, at 1-month intervals, soil samples were taken in each individual plot at 0.2 m depth increments along the 0-0.4 m soil profile for electrical conductivity (ECe) and pH measurements of the saturated-soil extract.

For each treatment, 6 individual soil samples [one per each soil depth (0–0.2 and 0.2–0.4) per three replications)] were taken monthly (6 months). Organic Nitrogen (Kjeldhal method), $N\text{-}NO_3$ and $N\text{-}NH_4$ (HACH DR/2000 spectrophotometer) were also measured on soil samples upon oven dehydration at 60°C.

Before sowing and after harvesting, soil aggregate stability was determined on soil samples collected in the 0-0.40 m soil layer. Aggregate stability was determined using the wet sieve method developed by Kemper and Rosenau (1986) and expressed as the mean weight diameter of the water-stable aggregates (MWD-WSAs). At the same time, soil bulk density (BD) was measured on undisturbed soil core collected by using 100

cm^3 metal cylinder samplers. Soil cores were oven dried at 105°C for 24 hours and weighed. The total soil porosity (TSP) was estimated from the bulk density data, using 2.65 g cm^{-3} as soil specific weight: TPS = (2.65-BD/2.65) x 100.

The data were analysed by ANOVA and the means were compared by Duncan's Multiple Range Test.

Yield response to salinity was evaluated according to the Maas and Hoffman threshold-slope model $Y = 100-s(ECe - T)$, where Y is the relative yield (%); s the slope [yield reduction (%) per unit increase in salinity above T], ECe the time-weighted average electrical conductivity of the soil saturation extract taken from the root zone (0–0.4 m soil profile) (dSm^{-1}); T the threshold, i.e. the maximum soil salinity that does not reduce yield below the one obtained under non-saline conditions (Maas and Hoffman, 1977).

I.2.4. RHIZOBIA ISOLATION

The root nodules were excised randomly from legume crops (two months old) and rhizobia were isolated from fresh nodules by the "Hotel isolation method" (Vincent, 1970). Single colonies were picked and checked for purity by repeated streaking on YM agar medium (Vincent, 1970) supplemented with Congo red in order to highlight possible contaminants (Graham, 1969). All isolates were incubated at 28 °C and kept at –20 °C in YM broth with 25% (v/v) glycerol until analysis.

All field isolates were streaked on YMA plates supplemented with NaCl at the concentration of 1%, 2%, 3%, 4% and 5% (w/v) in order to test their salt tolerance *in vitro*. The standard YMA medium with 0.1% (w/v) NaCl was used as control. Plates were incubated at 28 °C for 14d. These tests were carried out in triplicate.

I.2.5. RAPD FINGERPRINTING

Total genomic DNA from the isolates and from the 2 inoculated strains was extracted using Instagene Matrix (BioRad), following the manufacturer's instructions. RAPD-PCR was performed in a total volume of 25 μl containing 3 μl (approximatively 20 ng) of bacterial DNA, 1 × buffer (Invitrogen), 3.5 mM l^{-1} $MgCl_2$, 0.4 mM l^{-1} of each of the four dNTPs, 0.6 μM l^{-1} of primer and 2.5 U *Taq* polymerase (Invitrogen). The 10-mer random primers used for genomic strain typing were CC1 (5'-AGC AGC GTG G-3') and PRIMM239 (5'-CTG AAG CGG A-3') (Moschetti et al., 2005), with 70% and 80% G-C content, respectively. The amplifications were carried out in a PTC-100 thermocycler (M J Research Inc.) and template DNA was denatured for 1 min at 94 °C; then the PCR was carried out for 40 cycles (1 min at 94 °C, 1 min at 31 °C, 2 min at 72°C, for each cycle). Finally, a 7 min extension period at 72 °C was performed. Amplified products were resolved on a 2% and 1.5% (w/v) agarose-TBE gel electrophoresis, respectively. 1 Kb Plus DNA Ladder (Invitrogen) was used as molecular weight marker.

Bands were automatically detected by using the software Phoretix 1 advanced version 3.01 (Phoretix International Limited, Newcastle upon Tyne, England). The cluster analysis was performed by the program after band matching; the method described by Saitou and Nei (1987) was used to obtain the correlation matrix of the RAPD patterns. For each primer a similarity matrix was created, and finally joined into a single matrix, in which the respective values from each primer contributed to the mean. The resulting matrix was used in the average linkage method by the Cluster procedure of Systat 5.2.1 in order to estimate the percentage of similarity (*S*) in the RAPD fingerprints among strains.

I.3. RESULTS

I.3.1. SOIL

On September 2004, after the harvest of the tomato crop, the ECe in the 0–0.4 m soil layer ranged between 2.3 (S0) and 9.6 dSm^{-1} (S4) on the average. The ECe values decreased throughout the growing season. By the end of the growing season, rainfall was sufficient to reduce the ECe in the 0–0.4 soil layer to 1.8 and 5.8 dSm^{-1}, in S0 and S4 respectively (data not shown). After summer, the mean soil pH in the 0-0.4 m soil layer ranged between 7.1 (S0) and 8.0 (S4) and it was generally higher at increasing salinity. Soil pH remained rather stable during the growing cycle.

The diameter of water stable aggregates (MWD-WSA) strongly decreased as salinity increased, showing 39% reduction in S4 as compared to S0 (Table 1). A little increase (5% on the average) occurred from September to May, thanks to the reduction of soil salinity due to the leaching rainfalls (Fig. 1).

As a consequence of degradation of structural stability, the bulk density increased (from 1.23 to 1.38) and total porosity decreased (from 54% to 48%) when salinity increased (Table 2).

Time-weighted average electrical conductivity of the soil saturation extract taken from the root zone (0–0.4 m soil profile) was not affected by the crop and by the inoculation treatment (Table 2).

I.3.2. YIELD

The residual soil salinity affected both fresh and dry yield of non-irrigated broad bean. In contrast, the yield of vetch was significantly reduced only in the most salinized plots (S4).

Soil salinity significantly reduced the total nitrogen concentration in broad bean, whereas it remained relatively constant in vetch. Consequently, the total nitrogen (Organic N plus nitrates) accumulated by the crop decreased in broad bean at increasing soil salinity from 232 kg ha^{-1} (NSC) to 60 kg ha^{-1} (S4) (Table 3).

Plant dry matter accumulation was affected by soil salinity in both species. At harvest, plant survival was not affected by soil ECe. The fresh yield of vetch in S1, S2 and S3 treatments (30.4 t ha^{-1}, on average) was not significantly different from the non-salinized control treatment (31.0 t ha^{-1}) whereas it was 19% smaller in S4 plants as a consequence of a reduced weight (Table 3). The dry mass yield of vetch resembled the pattern observed for the fresh yield: a significant 20% decrease of the dry mass yield was observed in S4 plants relatively to the non-salinized control (Table 3). In contrast, the yield of broadbean was remarkably reduced in terms of fresh and dry mass, which were 67 and 74% smaller, respectively, in S4 compared to NSC plants (Table 3).

Salinity did not affect nitrogen concentration in both species (Table 3). The total amount of nitrogen accumulated by the plants was lower in broad bean than in vetch and, in the former, it was inversely correlated with soil salinity (Table 3).

Salinity affected the dry mass of inoculated plots: a significant decrease of the dry mass yield was observed in S1 plots inoculated with SAAN1 (I1) and POHY2B1 (I2) reference strains relatively to the non-salinized control (S0) and the non-inoculated control (I0). By contrast, the dry mass yield of non-inoculated plots (I0) did not significantly differ at increasing of salinity, remaining relatively constant (Fig. 2)

Total N uptake showed a similar trend. In fact, total nitrogen of non-inoculated plots (I0) was relatively constant at increasing of salinity and it was lower than the one of inoculated plots (I1 and I2) in non salinized control (S0), while the total N of inoculated plots (I1 and I2) showed a significant decrease in S1 relatively to the S0 (Fig. 3).

1.3.3. SALT TOLERANCE FUNCTION

Although we do not have sufficient data points in the plateau region of the Maas and Hoffman relationship to unequivocally assess when the relative yield began to decline, we estimated a threshold of 1.7 and a relative yield reduction of 16.6% per unit increase in ECe above the threshold for broad bean (Fig. 4A). The 50% yield reduction (ECe50) was 3.5 dSm^{-1}. For vetch, the salinity response curve revealed a constant yield at low and medium soil salinity and a relatively rapid decrease at higher soil salinity levels (Fig. 4B).

The relative fresh yield (*Yr*) was correlated with the soil pH, also. Approximately 80% yield reduction per unit increase in pH above a 7.20 threshold and 119% yield reduction above a 7.12 threshold were observed for vetch and broad bean, respectively.

I.3.4. PHENOTYPIC CHARACTERISTICS OF RHIZOBIAL ISOLATES

Strains previously isolated in Campania region were evaluated for their potential use as inoculants on seeds of V. faba and V. sativa in artificial salt soils. Salt-tolerant strain R. leg. bv. viciae SAAN1 and salt-sensitive strain R. leg. bv. viciae POHY2B1 were used in this study.

Visual observations of nodule formation on the host plant and of plant size and foliage colour compared to non-inoculated controls after 40 d of growth were used to evaluate their symbiotic potential with the two leguminous host plants tested. The reference strains SAAN1 and POHY2B1 showed a high ability to nodulate *V. faba* and *V. sativa* plants *in vitro* (Table 4).

Moreover, to ascertain the success of the inoculum, a total of 20 pre-treated seeds were put in greenhouse chamber and the experiment was carried out as above reported. Visual observations of nodule formation after 40 d of growth were used to evaluate the success of inoculum of seeds used for field experiment. Both reference strains used in field inoculation experiments were able to nodulate *V. faba* and *V. sativa* plants derived from seeds coated with rhizobial strains before planting and growth in hydroponic conditions (Table 5).

Thirty-three rhizobial isolates were purified from root nodules of plants of broad bean and vetch grown in artificial salt soils (0%, 0.125%, 0.25%, 0.5%, 1% NaCl) (Table 6). Salt tolerance of isolates was investigated and compared with that of the two control strains (*R. leg.* bv. *viciae* SAAN1 and *R. leg.* bv. *viciae* POHY2B1). All isolates were found to be salt-sensitive because they were able to grown only in standard culture medium (sodium chloride concentration of 0.1% (w/v)).

1.3.5. RAPD-PCR

RAPD-PCR was used to evaluate the nodule occupancy after seed inoculation of broad bean and vetch in soils with indigenous rhizobial populations. The degree of similarity among *R. leg.* bv. *viciae* isolates and reference strains was investigated by RAPD-PCR using CC1 and PRIMM239 primers. The primer CC1 produced 9 RAPD-profiles among

the 33 isolates and the 2 reference strains investigated. No rhizobial isolates produced RAPD profiles similar to the ones exhibited by inoculated strains (SAAN1 and POHY2B1) (Figg. 5a e 5b). These results were confirmed using primer PRIMM239, but the amplification patterns obtained with this primer showed a lower degree of polymorphism than those obtained with primer CC1. In fact, 6 RAPD-profiles were obtained using primer PRIMM239. Therefore, in order to obtain a result representing intraspecies relationships among all our isolates and reference strains, we combined data from RAPD-PCR patterns obtained with the two primers in a single dendrogram (Fig. 6).

A total of 12 RAPD-genotypes were obtained. Fingerprints similarity varied from 32% to 100%; patterns could be grouped into two major clusters with a similarity level of at least 65%. Cluster I (*S* 69%) grouped four genotypes (B1; B2; B3; C) containing 15 isolates; while, cluster II (*S* 71%) grouped five genotypes (D1; D2; E1; E2; F) containing 16 isolates. The isolates included in these clusters were further divided into closely related subgroups with a similarity level of at least 80%. Cluster I can be divided into two subgroups: B1, B2 and B3 made up the first subgroup and C the second subgroup; while cluster II can be divided into three subgroups: D1and D2, E1 and E2, F.

The two inoculated strains SAAN1 (genotype H) and POHY2B1 (genotype G) could not be included in any cluster. The genotype A, which included two strains isolated from the same plot, showed a similarity level of 32% with all isolates and reference strains.

Moreover, two nodules per plant were excised from *V. faba* and *V. sativa* coated with reference strains before planting and growth in hydroponic conditions as control. Total DNA was extracted and PCR-RAPD was performed in order to check the inoculated

strains. All isolates showed a RAPD pattern identical to the two inoculated strains with both primers used proving the success of inoculum.

I.4. DISCUSSION

After recurrent summer irrigations with saline waters, the residual soil salinity affected yield of non-irrigated Legume winter cover crops. Threshold (1.7 dSm^{-1}) and slope [16.6% $(dSm^{-1})^{-1}$] values, estimated according to Maas and Hoffman (1977), were close to those found by De Pascale and Barbieri (1997) in similar experimental conditions but they were lower and higher, respectively, than those reported by other authors for the same species (Katerji et al., 1992). Consequently, the ECe50 for broad bean was about 60% smaller than the corresponding value of 8.2 dSm^{-1} reported by Maas and Hofmann. It is known that environmental and cultural variables may affect plant response to salinity (Hoffman, 1990; Dalton et al., 2001; Maggio et al., 2002b). In our experimental conditions, salt-induced permanent modifications of the soil physicochemical properties may have further aggravated the effects of salinization on crop yield (Maas, 1990; Maas and Grattan, 1999). The S4 soil presented typical characteristics of alkaline–saline soil with high sodium adsorption ratio (54.7 in S4 versus 0.14 in NSC), decreased structural index and porosity, low hydraulic conductivity to water, surface seals, salts build-up along the soil profile and reduced root zone aeration (De Pascale and Barbieri, 2000). These results indicate that salinity becomes one component of a more complex scenario, which includes soil pH modifications, waterlogging and anoxia (Rhoades et al., 1992; De Pascale et al., 2003a, 2003b). All these events may seriously compromise crop growth and yield (Emerman and Dawson, 1996; Hachicha et al., 2000). Anaerobic conditions, for instance, may impair ion compartmentation and/or exclusion and consequently they may reduce plant salt tolerance (West, 1978; West and Taylor, 1984; Shannon and Grieve, 1999). Increasing soil pH as a result of saline irrigation is normally observed in alkaline soils,

where typically the high activity of Na^+ ions, relative to those of Ca^{2+} and Mg^{2+}, affects the soil chemical properties. Any excess of CO_3^{2-} or HCO_3^-, which is not precipitated by Ca^{2+} or Mg^{2+}, will increase the soil pH (Tanji, 1990). This is especially true in soils with low permeability and high exchangeable cation capacity, such as the one used in this study. In addition to species-specific pH effects on plant growth, yield reductions associated with high soil pH may be caused by nutrient imbalance (Page et al., 1990) and/or reduced soil hydraulic conductivity (Shainberg and Singer, 1990).

In contrast, soil salinity significantly reduced the plant growth and yield only at high salinity in vetch. The observed differences confirm that species within the same family may have different tolerance to salt stress (van Hoorn et al., 2001). This difference may be associated to different water and nutrient uptake in these two species, and/or alternatively to different nutrient use efficiency. Moreover, from an agronomic perspective this may have important implications in terms of legume crop selection in saline environments even under fall winter conditions.

Competition effects between different anions (Bar et al., 1997; Feigin et al., 1987; Kafkafi et al., 1982) and different cations (Subbarao et al., 1990; Izzo et al., 1991; Pérez-Alfocea et al., 1996) are known to occur in saline environment and they may result deleterious for normal plant growth (Grattan and Grieve, 1999). The presence of excess Cl- in the solution in contact with the roots was inversely correlated to nitrogen concentration (Gomez et al., 1996; Gunes et al., 1996). Consistently, we found a decreased N concentration at increasing soil salinity in broad bean. Although under saline conditions the N content in many glycophytes is severely reduced (Song and Fujiyama, 1996; Lopez and Satti, 1996), in our experimental conditions the N concentration in vetch was not significantly affected by salinity, suggesting that vetch may possess a more efficient N uptake system if compared to broad bean. In addition,

the higher efficiency in nitrogen uptake in vetch versus broad bean plants might have prevented the yield decline in the former (Adams and Ho, 1995).

Overall, in Mediterranean areas exposed to salinization, although rainfall may mitigate the deleterious effects of salinity on crop yield by leaching the excess of salts from the root zone, residual salts from the summer irrigations and salt-induced permanent modifications of the soil physical–chemical properties may affect both yield and mineral composition of non-irrigated winter cover crops such as broad bean and vetch.

An important problem in microbial ecology concerns the efficacy of rhizobial inoculants for the formation of nitrogen fixing root nodules on legume crop plants. Therefore, the present study has been mainly focused on the evaluation of nodulation competitiveness of salt-tolerant and salt-sensitive rhizobia, showing a high symbiotic potential, inoculated in artificial salt soils. R. leg. bv. viciae SAAN1 and R. leg. bv. viciae POHY2B1 strains were evaluated for their ability to compete in soils containing a naturalised population of rhizobia and their potential use in sustainable agriculture projects, since biological nitrogen fixation (BNF) and its ecological role is an important process in plant nutrition.

In our experiments the inoculated rhizobia showed a lesser competitiveness than the natural rhizobial population. In fact, 100% of nodules contained indigenous rhizobia strains since no one out of thirty-three rhizobial isolates produced RAPD profiles similar to the ones exhibited by the reference strains (SAAN1 and POHY2B1). The success of rhizobial inoculation on plant roots is often limited by several factors, including environmental conditions, the number of infective cells applied, the inoculation method and the presence of competing indigenous rhizobia (Bogino et al., 2008). Selected strains inoculated on legume seeds often fail to occupy a significant proportion of nodules when an indigenous competitor rhizobial population is

established in the soil (Dowling and Broughton, 1986; Triplett and Sadowsky, 1992; Streeter, 1994; Toro, 1996). In such soils, the better adapted indigenous populations show a higher level of infectivity than inoculants, resulting in a higher level of occupancy of the nodules formed (Van Dillewijn et al., 2001). Studies on peanut by Bogino et al., (2006) inoculated with Bradyrhizobium sp. showed limited response to new inoculation in fields where peanut was planted in prior years if compared with inoculation in areas cultivated with peanut for the first time. In the experimental farm used for this experiment, since '80s legume crops had been introduced in crop rotations, so that inoculation was not necessary for nodulation and N-fixation. Therefore, in our case, it seems that the inoculant strains were not sufficiently competitive and that the indigenous rhizobia were already maximally effective.

The presumptive failure of infection of inoculant strains of broad bean and vetch roots could be due to the inoculation technique used, even though in greenhouse the same inoculated seeds produced nodules with inside our reference strains.

Rhizobia may be introduced to legumes by inoculation of seed or soil in different methods and formulations. The selection of one of these methods depends on the seed size, fragility of cotyledons and convenience. We applied liquid inoculants to seeds and after two days to soil, so the competition problem could result from a genetic/ physiological advantage of the adapted soil population over the introduced inoculant or from a positional advantage. In fact, the natural soil population already occupies the soil profile where the roots will penetrate, while the strains artificially inoculated remain concentrated around the seeds (López-García et al., 2002).

Moreover, the dry mass and total nitrogen uptake showed a significant increase in S0 plots inoculated with reference strain SAAN1 (I1) and little lower in S0 plots inoculated with reference strain POHY2B1 (I2) relatively to the non inoculated control (I0); while

a significant decrease of the dry mass yield and total N was observed in S1 plots inoculated with SAAN1 and POHY2B1 relatively to the non-salinized control and the non-inoculated control. By contrast, dry mass and total N uptake values obtained in non-inoculated control plots remained relatively constant at increasing of salinity. The increased values in S0 plots inoculated could be due to the presence in the field of the reference strains but we were not able to isolate them because we analysed a low number of root nodules in non-salinized control plots. This hypothesis could be in according with the results obtained in plant nodulation tests that we made in vitro. In fact, the reference strain SAAN1 showed higher nodulation efficiency and consequently higher symbiotic potential in field than the reference strain POHY2B1.

Furthermore, it has been shown that Rhizobium mutants, whose adaptation to high salinity is affected, have deficiencies in their symbiotic capacity (Nogales et al., 2002). Nevertheless, response and adaptation to environmental stresses are probably complex phenomena involving many physiological and biochemical processes that may reflect changes in gene expression and in the activity of enzymes and transport proteins (Djordjevic et al., 2003; Wei et al., 2004).

We expected that the new field isolates were salt tolerant strains and were tested for their tolerance to sodium chloride in vitro. Interestingly, all the isolates were found to be salt-sensitive because they were able to grown only in standard culture medium (sodium chloride concentration of 0.1% (w/v)). Therefore, the influence of salt on survival of rhizobial strains is different in vitro experiment or in soil. A complex interaction between bacteria, plant and soil structure can determine the survival of sensitive rhizobial strains and the failure of inoculant resistant rhizobia. In fact, levels of salinity that inhibit the symbiosis between legumes and rhizobia are different from those that inhibit the growth of the individual symbionts.

These results emphasize the importance of studying the mechanisms of adaptation of rhizobia to changes in the salinity conditions of their environment.

I.5. CONCLUSIONS

Summer irrigations with saline waters damage soil physical fertility, reducing the size of soil aggregates and total porosity. Legume cover crop incorporation could be effective in sodic soil reclaimation and in improving nitrogen balance of cropping systems, but the residual soil salinity could also affect yield of non-irrigated legume winter cover crops. In this process the legume cover crops, most used for green manuring in the Mediterranean area, showed a different sensitivity to soil salinity, broadbean resulting more sensitive than common vetch. Therefore, this last species resulted more effective for nitrogen supply to cropping systems, allowing a N input higher than 200 kg ha^{-1} in the more salinized treatment, whereas broadbean reduced N input from 215 in the not-salinized treatment to 60 kg ha^{-1} in the most salinized one.

This different sensitivity to saline soils, besides the effects on soil water potential and soil physical properties, could be also related to some effects on symbiotic N-fixation.

The failure of the inoculation of the rhizobial strains in the field could be due to their not sufficient competitiveness compared to the indigenous rhizobia that were already maximally effective, because since ‘80s legume crops had been introduced in crop rotations in the experimental farm used for this experiment. Moreover the indigenous rhizobia analyzed showed high sodium chloride salt sensitivity in vitro; so they were not really tolerant to sodium chloride and not to able to help improve plant growth and nutrition in different saline soils.

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I.7. FIGURES

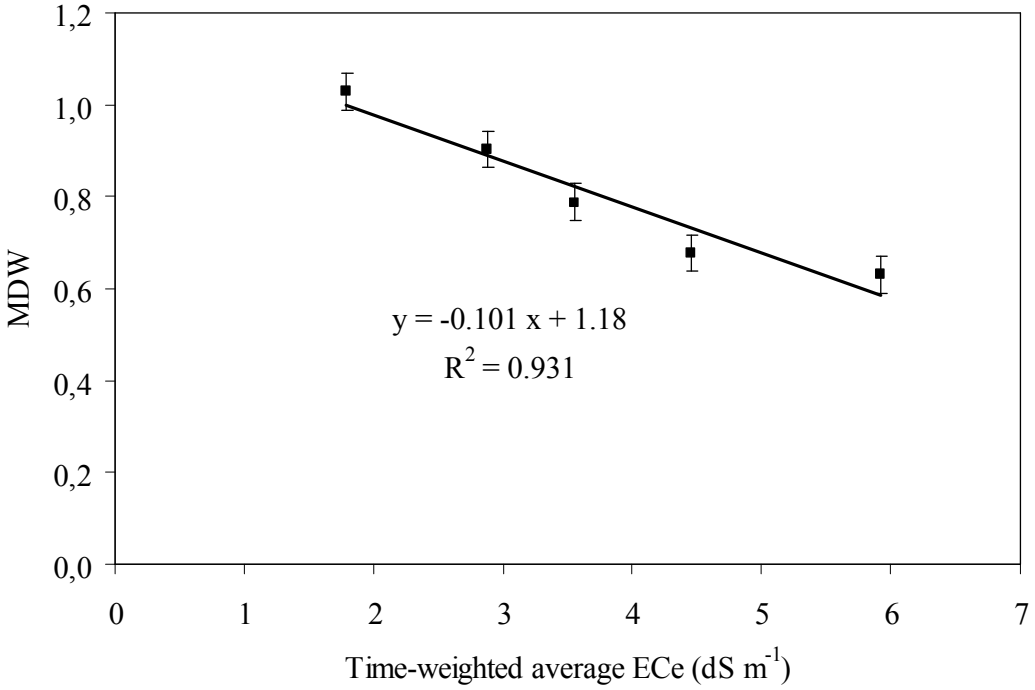

Figure 1. Mean weight diameter of water stable aggregates (MWD WSAs) at increasing soil salinity (ECe). Each point is the mean of 72 samples±standard errors.

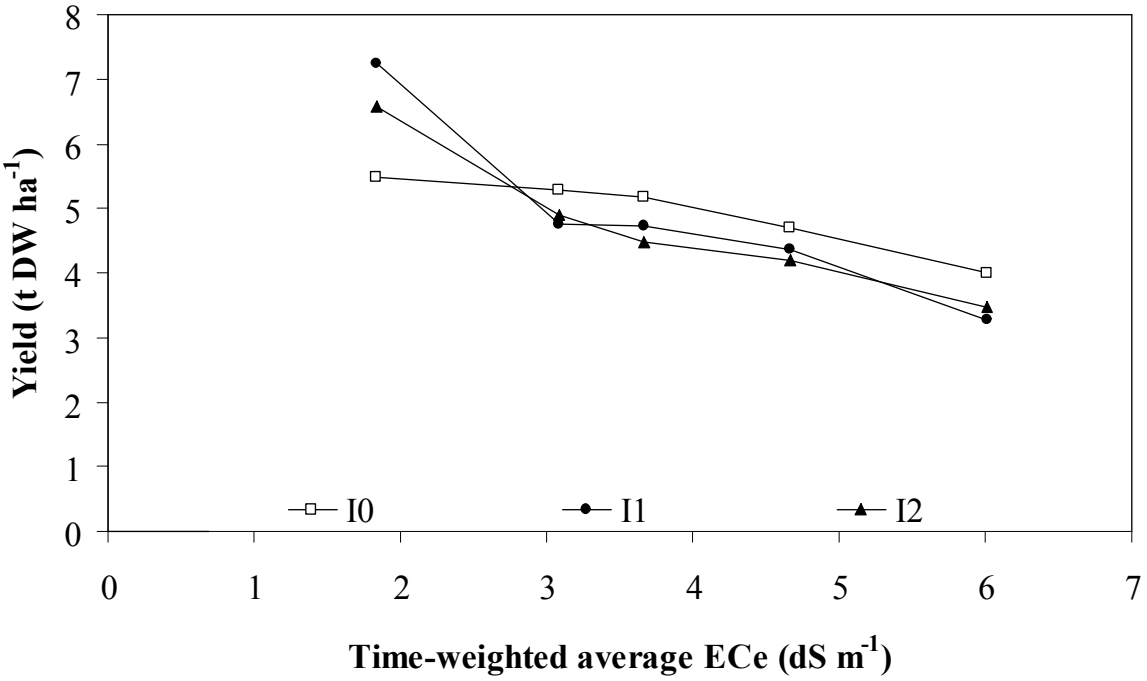

Figure 2. Dry mass yield at increasing soil salinity in response to the inoculation treatment (Negative I0; SAAN1, I1 and POHY2B1, I2).

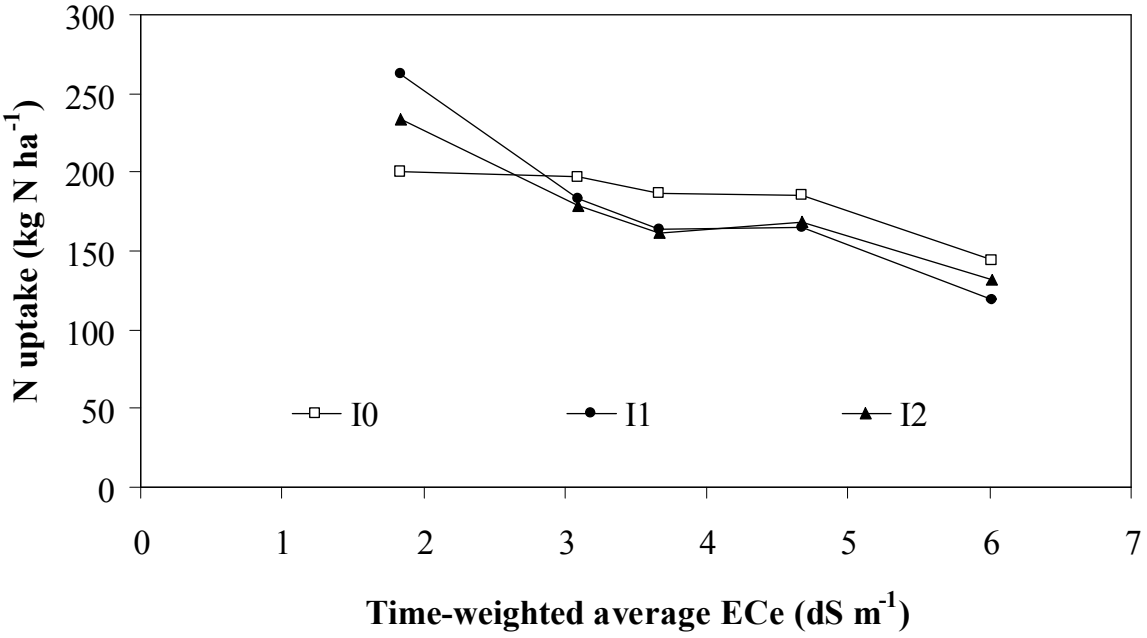

Figure 3. Total N uptake at increasing soil salinity in response to the inoculation treatment (Negative I0; SAAN1, I1 and POHY2B1, I2).

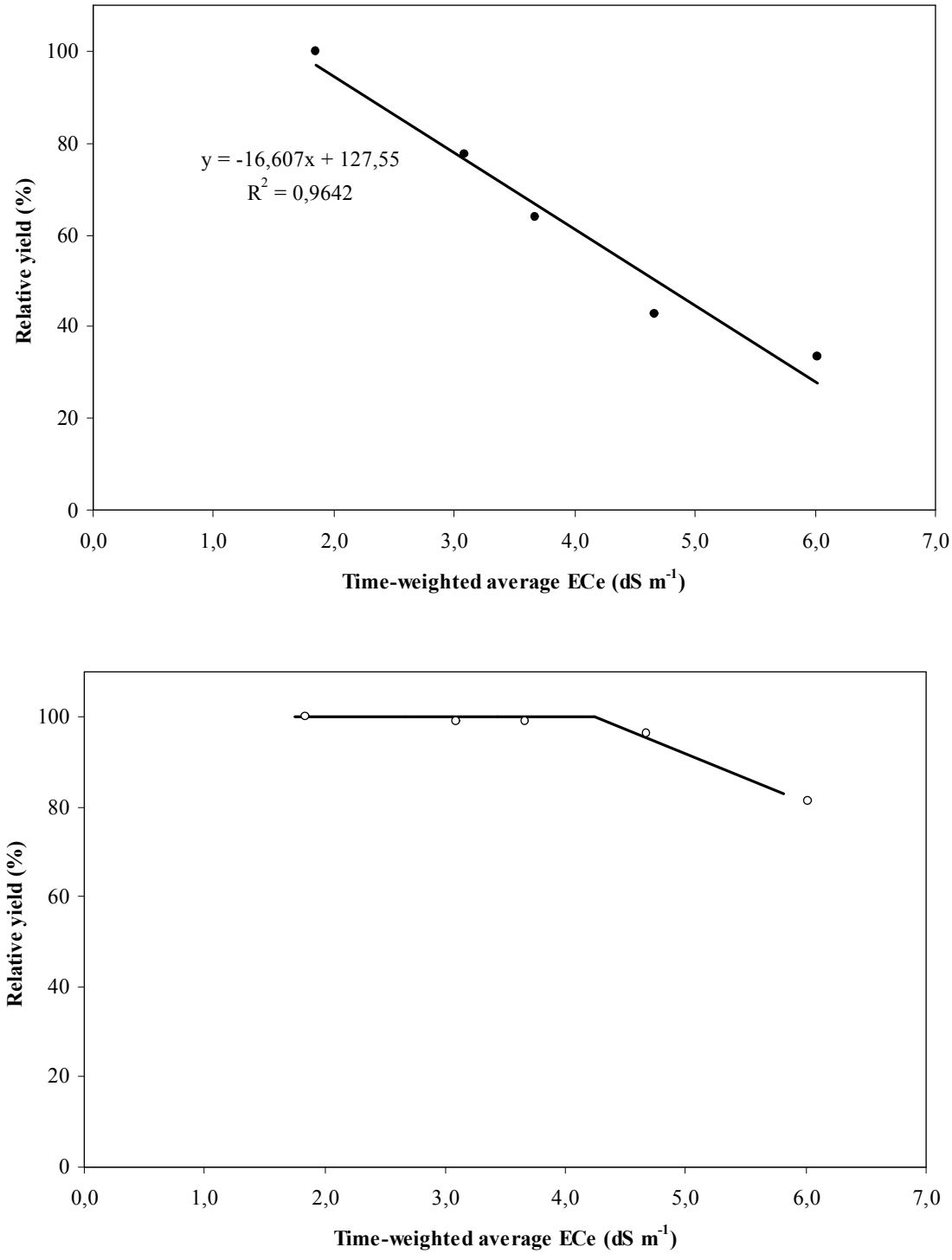

Figure 4. Relative yield (*Yr*) response at increasing soil salinity (ECe) in broad bean (A) and vetch (B).

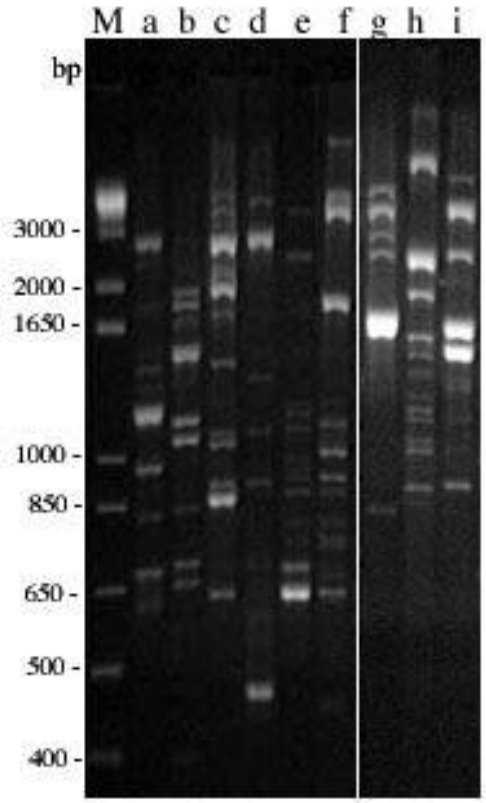

Figure 5a. Randomly amplified polymorphic DNA patterns generated by primer CC1 of 7 representative *Rhizobium* isolates and two reference strains of *Rh. leg. bv. viciae*. Lane a, reference strain SAAN1; lane b, reference strain POHY2B1; lane c, pattern D; lane d, pattern H; lane e, pattern E; lane f, pattern I; lane g, pattern C; lane h, pattern F; Lane I, pattern G. M: 1kb Plus DNA Ladder (Invitrogen, slr).

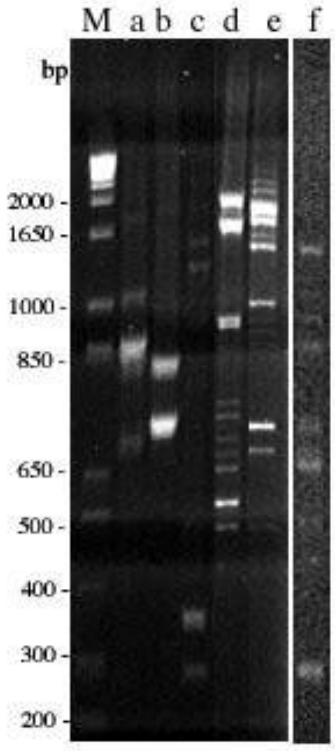

Figure 5b. Randomly amplified polymorphic DNA patterns generated by primer PRIMM239 of 4 representative *Rhizobium* isolates and two reference strains of *Rh. leg. bv. viciae*. Lane a, reference strain SAAN1; lane b, reference strain POHY2B1; lane c, pattern C; lane d, pattern E; lane e, pattern D; lane f, pattern F. M: 1kb Plus DNA Ladder (Invitrogen, slr).

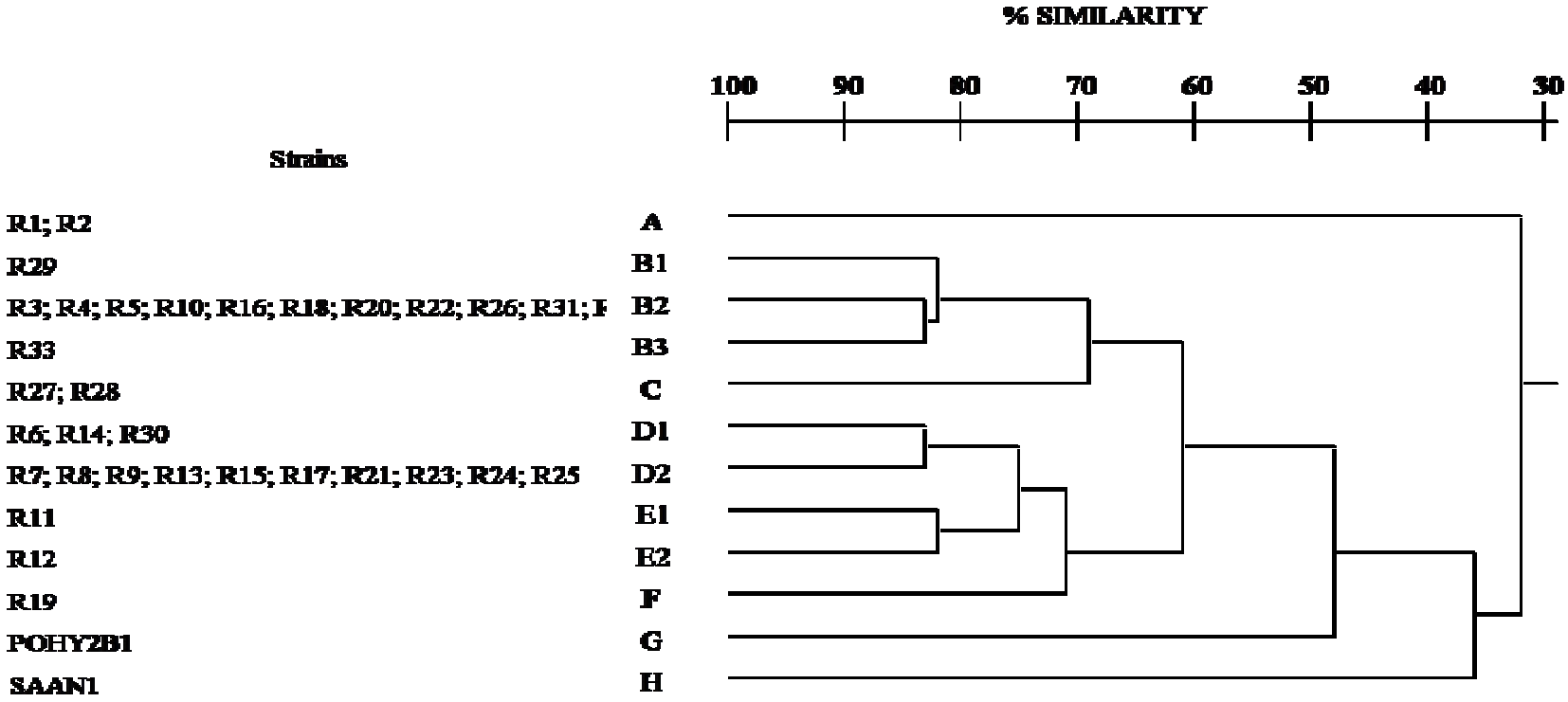

Figure 6. Dendrogram showing the degree of similarity (%) of RAPD fingerprints of all isolates investigated and two reference strains of *Rh. leg. bv. viciae* using primers CC1 and PRIMM239.

I.8. TABLES

Table 1. Mean weight diameter of water stable aggregates (MWD-WSAs) at transplanting (September 2004) and at the end of the experiment (May 2005) in the 0-0.4 m soil layer.

MWD-WSAs **(mm)**	
Time	
September 2004	0.79
May 2005	0.83*
Soil salinity	
S0	1.03
S1	0.90
S2	0.79
S3	0.68
S4	0.63
LSD	*0.133*

Table 2. Time-weighted average ECe and pH, bulk density and total N content in the 0-0.4 m soil layer (ns, *, **, non significant or significant at $P < 0.05$ or 0.01, respectively

Crop (A)	pH	ECe dS m^{-1}	Bd t/m^{-3}	Ntot ‰
Broad bean	7.36	3.86	1.30	0.99
Vetch	7.44	3.58	1.31	0.95
	ns	*ns*	*ns*	*ns*
Soil salinity (B)				
S0	6.80	1.80	1.23	0.98
S1	7.20	2.88	1.26	0.98
S2	7.32	3.55	1.31	0.98
S3	7.63	4.45	1.34	0.97
S4	8.05	5.92	1.38	0.96
LSD	*0.09*	*0.35*	*0.02*	*0.04*
Strain (C)				
I0	7.38	3.57	1.30	0.97
I1	7.41	3.78	1.31	0.96
I2	7.40	3.82	1.30	0.99
	ns	*ns*	*ns*	*ns*
Int. AxB	*ns*	*ns*	*ns*	*ns*
Int. BxC	*ns*	*ns*	*ns*	*ns*
Int. AxC	*ns*	*ns*	*ns*	*ns*
Int. AxBxC	*ns*	*ns*	*ns*	*ns*

Table 3. Fresh (FW) and dry mass (DW) yields, plant nitrogen concentration and total nitrogen uptake in broad bean and vetch (ns, *, **, non significant or significant at $P < 0.05$ or 0.01, respectively; different letters indicate significant differences by Duncan's MRT at $P < 0.05$).

CROP (A)	FW t/ha	Dry Matter %	DW t/ha	Ntot % DW	Ntot kg/ha
Broad bean	23.2	13.7	3.2	3.7	118.1
Vetch	29.5	21.9	6.4	3.7	226.1
	*	**	**	ns	*
Broad bean Soil salinity (B)	FW t/ha	Dry Matter %	DW t/ha	Ntot % DW	Ntot kg/ha
S0	36.5	11.6	6.1	3.8	215.0
S1	28.3	13.1	3.7	3.8	135.9
S2	23.3	13.6	2.7	3.8	100.9
S3	15.7	13.7	2.1	3.7	78.7
S4	12.2	16.7	1.6	3.5	60.3
LSD	6.9	4.4	1.6	0.2	54.8
Vetch					
S0	31.0	20.4	6.89	3.96	267.60
S1	30.7	22.1	6.74	3.78	249.02
S2	30.7	22.3	6.71	3.64	238.59
S3	29.9	22.3	6.30	3.60	236.51
S4	25.2	22.5	5.57	3.56	203.09
	3.5	2.7	1.2	0.3	52.2
Strain (C)					
I0	27,8	17,5	4,9	3,7	182,7
I1	25,8	18,2	4,9	3,7	178,6
I2	25,5	17,8	4,7	3,7	174,4
	ns	ns	ns	ns	ns
Int. BxC	ns	ns	*	ns	*
Int. AxBxC	ns	ns	ns	ns	ns

Table 4. Number of nodules per plants (20 plants) obtained after 40 d of growth on hydroponic conditions.

Strain	*Vicia sativa*	*Vicia faba*
SAAN1	67.0 ± 11.5	65.7 ± 9.0
POHY2B1	43.3 ± 9.6	47.6 ± 8.5

Table 5. Number of nodules per plants (20 plants) after 40 d of growth on hydroponic conditions obtained from pre-treated seeds.

Strain	*Vicia sativa*	*Vicia faba*
SAAN1	65.0 ± 9.5	63.4 ± 8.6
POHY2B1	44.0 ± 9.0	46.5 ± 8.5

Table 6. RAPD genotypes obtained using primers CC1 and PRIMM239

Strain/ isolate	[a]Plant	[b]NaCl %	Profile CC1	Profile 239	RAPD Genotype
SAAN1			A	a	H
POHY2B1			B	b	G
R1	*V. sativa*	1	C	c	A
R2	*V. sativa*	1	C	c	A
R3	*V. sativa*	1	D	d	B2
R4	*V. faba*	0.5	D	d	B2
R5	*V. sativa*	0.25	D	d	B2
R6	*V. faba*	0.125	D	e	D1
R7	*V. sativa*	0	E	e	D2
R8	*V. sativa*	1	E	e	D2
R9	*V. faba*	0.5	E	e	D2
R10	*V. faba*	1	D	d	B2
R11	*V. sativa*	0.5	F	e	E1
R12	*V. faba*	0.25	G	e	E2
R13	*V. sativa*	0.125	E	e	D2
R14	*V. faba*	1	D	e	D1
R15	*V. faba*	1	E	e	D2
R16	*V. faba*	1	D	d	B2
R17	*V. faba*	1	E	e	D2
R18	*V. sativa*	0.5	D	d	B2
R19	*V. sativa*	0.5	H	e	F
R20	*V. sativa*	0.125	D	d	B2
R21	*V. faba*	0	E	e	D2
R22	*V. faba*	1	D	d	B2
R23	*V. sativa*	1	E	e	D2
R24	*V. sativa*	1	E	e	D2
R25	*V. sativa*	1	E	e	D2
R26	*V. sativa*	1	D	d	B2
R27	*V. faba*	0.5	D	f	C
R28	*V. sativa*	0.25	D	f	C
R29	*V. faba*	0.125	I	d	B1
R30	*V. sativa*	0	D	e	D1
R31	*V. faba*	0.5	D	d	B2
R32	*V. sativa*	1	D	d	B2
R33	*V. faba*	0.5	E	d	B3

[a] Plant the nodule was excised from

[b] Residual salinity percentage in plot

- CHAPTER 2 -

CORRELATION BETWEEN KANAMYCIN RESISTANCE AND "PUTATIVE AMINOGLYCOSIDE 3'-O-PHOSPHOTRANSFERASE" GENE IN *SINORHIZOBIUM MELILOTI* STRAIN 1021

II.1. INTRODUCTION

Soil shows a high diversity of bacterial species; it has been estimated that half the known bacterial genera contain species, which can be considered as soil bacteria.

Soil is a long term sink for the group of potentially toxic elements. While these elements display a range of properties in soils, including difference in mobility and bioavailability, leaching losses and plant uptake are usually relatively small when compared with the total quantities entering soils from different diffuse and agricultural sources. As a consequence, these potentially toxic elements slowly accumulate in the soil profile over long periods of time. This could have long term implications for the quality of agricultural soils, including the maintenance of soil microbial processes, phytotoxicity at high concentrations, and the transfer of zootoxic elements into the human diet from increased crop uptake or soil ingestion by grazing.

The development of bacterial resistance to antibiotics is one of the best documented cases of contemporary biological evolution. The huge consumption of these compounds, able to inhibit bacterial growth at very low concentration, has resulted in the emergence and spread of a great amount of antibiotic resistance determinants among bacterial populations.

Antibiotic resistance is a public health concern of great urgency because of the growing ineffectiveness of antimicrobial agents in treating infectious diseases. This is mainly caused by the propagation of antibiotic resistance genes, which is exacerbated by the overuse of antimicrobials in humans and the intensive use of antibiotics in animal agriculture (Mollen et al., 2001; Rysz and Alvarez 2004).

The analysis of completed microbial genomes has indicated that horizontal gene transfer (HGT) continues to be an important factor contributing to the rearrangement of

microbial genomes (Beiko et al., 2005; Gogarten and Townsend 2005). HGT driven by mobile genetic elements, such as plasmids (Frost et al., 2005), insertion sequences (Mahillon and Chandler 1998), integrons (Nemergut et al., 2004), transposons (Pearson et al., 1996) and phages (Canchaya et al., 2003), has been shown to provide microbes with a wide variety of adaptive traits for microbial survival and proliferation (e.g. antibiotic and heavy metal resistance and diverse metabolic capabilities, including xenobiotic compound degradation and virulence). While point mutations contribute to microbial adaptation, horizontal dissemination of genes has proved to be more critical in promoting rapid genomic flexibility and microbial evolution (Thomas and Nielsen 2005).

The knowledge of the possibility of horizontal gene transfer is necessary, in view of the possibility of deliberate release of a variety of nonrecombinant microorganisms into the environment for such agricultural purposes as nitrogen fixation (*Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Frankia*), phosphate solubilization (*Burkholderia, Erwinia*), plant growth stimulation (*Rhizobium, Pseudomonas, Azospirillium, Agrobacterium*), control of phytopathogenic fungi and bacteria (*Pseudomonas, Erwinia*), insect control (*Bacillus thuringiensis*), weed control (phytopathogenic fungi), bioremediation of xenobiotic polluted sites (*Pseudomonas, Alcaligenes, Burkholderia, Comamonas*), and denitrification (*Pseudomonas, Alcaligenes, Comamonas*) (Davison, 1988; Wilson and Lindow, 1993; Davison, 1999). Indeed, data are also needed concerning the persistence, survival, competition, nutrition, stress, and physiological state of the introduced bacteria (van Veen et al., 1997). For example, it was shown, under optimized laboratory conditions, that a kanamycin resistance gene integrated in the DNA of a transgenic plant could transform *Acinetobacter* sp. to Km^R (Gebhard and Smalla, 1998).

II.2. AMINOGLYCOSIDE ANTIBIOTICS

The aminoglycoside/aminoclyclitol antibiotics include many important drugs such as kanamycin, gentamicin, amikacin, tobramycin, and streptomycin. These antibiotics constitute a large family of amino-compounds which exhibit broad antibacterial and antiprotozoal activity. The aminoglycosides target the bacterial ribosome, and in particular footprint to the 16S rRNA, where they are thought to interfere with translation, often resulting in incorrect reading of the mRNA, which results in a variety of downstream effects. Unlike other antibiotics which interfere with bacterial translation such as tetracycline and chloramphenicol, most aminoglycosides are bactericidal rather than bacteriostatic. This property makes aminoglycosides highly desirable antiinfective agents (Wright and Thompson, 1999).

One of the more significant consequences associated with translational errors caused by many aminoglycosides is membrane damage (Davis et al., 1986). This results in a breach of membrane integrity and a disruption of ion gradients, which precipitates cell death. Aminoglycosides that bind to the ribosome but do not bring about mistranslation, such as hygromycin or spectinomycin, do not result in membrane damage and, as a consequence, are bacteriostatic (Bakker, 1992).

All aminoglycosides contain a six-membered aminocyclitol ring (a cyclohexane group to which amino and hydroxyl groups are attached) and carbohydrate moieties, many of which are aminosugars. Thus aminoglycosides are water soluble, basic in nature, and generally positively charged at physiological pH. The aminocyclitol ring is generally derived from glucose-6-phosphate, either through the synthesis of *myo*-inositol followed by oxidation and transamination, to give *scyllo* inosamine in the case of streptamine containing aminoglycosides e.g. streptomycin, or through 2-deoxyscyllo-inosamine

required for the synthesis of 2-deoxystreptamine containing aminoglycosides such as kanamycin, gentamicin, and neomycin (Piepersberg, 1997; Wright and Thompson, 1999).

Resistance to the aminoglycoside antibiotics can show itself in three manners: 1) mutations in target ribosomal RNA or proteins, 2) altered uptake of the molecules, or 3) the expression of resistance enzymes.

Enzymatic resistance reveals itself in two forms: enzymes which modify the target rRNA, and enzymes which modify the aminoglycosides directly. Modifying enzymes include acetyl CoAdependent *N*-acetyltransferases, ATP-dependent *O*-adenyltransferases, and ATP-dependent *O*-phosphoryltransferases. Modified aminoglycosides lose their capacity to bind ribosomes in such a way which impairs their biological activity, and thus the cells exhibit a drug-resistance phenotype.

The aminoglycoside phosphotransferases (APH) family of enzymes include several members which are differentiated on the basis of three criteria: 1) substrate specificity or resistance phenotype, 2) regiospecificity of phosphoryl transfer, and 3) protein/gene sequence.

The largest family of APHs includes the enzymes that modify kanamycin and related compounds at the 3'-hydroxyl group (Fig. I-1).

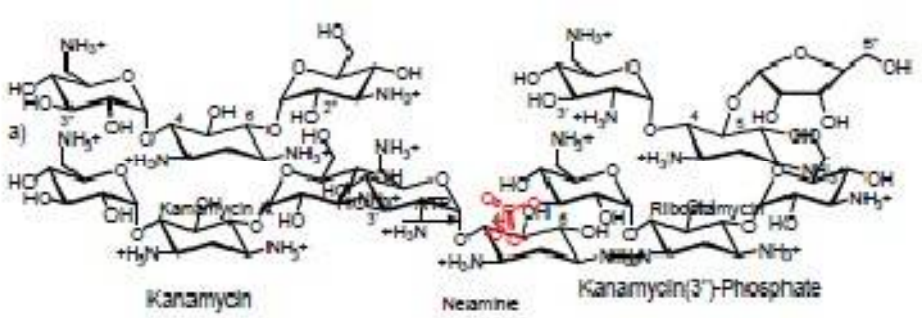

Fig. I-1. Reactions catalyzed by APH(3'). a) 3'-phosphorylation of kanamycin A (Wright and Thompson, 1999).

II.3. SINORHIZOBIUM MELILOTI STRAIN 1021

Sinorhizobium meliloti is a model organism for studies of plant-microbe interactions. This Gram negative soil bacterium can enter an endosymbiosis with alfalfa plants through the formation of nitrogen-fixing nodules.

S. meliloti strain 1021 is a nitrogen-fixing symbiotic bacterium selected as streptomycin-resistant derivates of the natural isolate *S. meliloti* SU47 (Meade and Signer, 1977; Casse et al., 1979).

S. meliloti 1021 has been the subject of extensive genetic, biochemical, and metabolic research; this knowledge provides a solid foundation for genomic experimentation. In fact, the complete genome sequence of strain 1021 is now known (Galibert et al., 2001) and this has provided a basis for different approaches of functional genomics to this bacterium. The complete genome is tripartite and comprises a 3.65-Mb chromosome, and 1.35-Mb pSymA and 1.68-Mb pSymB megaplasmids. Moreover, the genome sequence is useful in understanding the dynamics of inter-kingdom associations and of life in soil environments.

Antibiotic resistance has been frequently used in ecological studies as well as in distinguishing the introduced inoculants strain from indigenous rhizobia and monitoring their survival and occupancy of legume nodules (Josey et al., 1979; Bromfield et al., 1985; Simon and Kalalova, 1996). Studies carried out on *Rhizobium* strains from different host infective groups have shown that most rhizobial strains exhibit multiple antibiotic resistance (Josey et al., 1979; Dadarwal et al., 1987; Xavier et al., 1998). However, some of the rhizobial strains possess no detectable antibiotic markers (Dadarwal et al., 1987; Sharma et al., 1991). In such strains antibiotic markers could be introduced either by isolating spontaneous mutants or transposition (Schwinghamer, 1967; Ramirez et al., 1998).

Variation in competitiveness for nodule formation and ability to fix dinitrogen has been found for antibiotic-resistant mutants of *Rhizobium* or *Bradyrhizobium* strains nodulating alfalfa (Lewis et al., 1987), clover (Rynne et al., 1992), and *Desmodium intortum* (Date and Hurse, 1992).

Changes in symbiotic effectiveness have been reported to vary with the type of antibiotic resistance (Sindhu and Dadarwal, 2001). Therefore, the antibiotic- resistant mutants should be analyzed for their symbiotic effectiveness before using these mutants for ecological studies or nodulation competitiveness.

S. meliloti 1021 is known as a kanamycin sensitive strain (30 µg/ml). A spontaneous kanamycin-resistant mutant (GM42) (200 µg/ml) was isolated from *S. meliloti* strain 1021 (Ventorino et al., 2006). Capela and co-workers (2001) identified a "putative aminoglycoside 3'-O-phosphotransferase" as the hypothetical product obtained by coding the *SMc03094* chromosomal gene.

In this study the GM42 mutant strain was investigated by phenotypic and molecular characterization and the correlation between *SMc03094* gene and kanamycin resistance phenotype in GM42 was evaluated.

II.2. MATERIALS and METHODS

II.2.1. GROWTH MEDIA

All the media, if another not indicated, were dissolved by distilled water and autoclaved.

LB-medium (Luria-Bertrani Broth), (Sambrook et al., 1989)), used for *E. coli* strains

10 g/l Tryptone
5 g/l Yeast Extract
5 g/l NaCl
pH 7.0

TY-medium (Beringer, 1974), used for rhizobial strains

5 g/l Tryptone
3 g/l Yeast extract
0.4 g/l $CaCl_2$
pH 7.0

Modified Vincent minimal-medium (Becker et al., 2004; Vincent, 1970)

Stock Solution A:
2.56 g/l K_2HPO_4
1.56 g/l KH_2PO_4
0.246 g/l $MgSO_4 \times 7\ H_2O$
1 g/l NH_4Cl
Ingredients were dissolved in 800 ml Millipore water and the pH value was set to 7.0 by KOH or H_3PO4. Then, Millipore water was added to fill 1 liter and the stock solution was autoclaved.

Stock Solution B: 1 mg/ml Biotine
Dissolved by 0.1 N NaOH and sterile filtered

Stock Solution C: 67 g/l $CaCl_2$
Autoclaved

Stock Solution D: 10 g/l $FeCl_3 \times 6\ H_2O$
Sterile filtered

Stock Solution E: 3 g/l H_3BO_3
2.23 g/l $MnSO_4 \times 4\ H_2O$
0.287 g/l $ZnSO_4 \times 7\ H_2O$
0.125 g/l $CuSO_4 \times 5\ H_2O$
0.065 g/l $CoCl_2 \times 6\ H_2O$
0.12 g/l $NaMoO_4 \times 2\ H_2O$
Sterile filtered

For full strength medium, 10 ml of solution B, 1 ml of each solution C, D, and E, and 10 ml of a solution 1 M of mannitol or maltose (Dissolved in Millipore water and sterile filtered) were added one by one to 980 ml of solution A.

SOB Medium (Inoue et al., 1990), used for preparation of the competent *E. coli* cells

Stock Solution A: 20 g Tryptone
5 g Yeast extract
0.58 g NaCl
dissolved in 980 ml H_2O

Stock Solution B: 2.5 mM KCl

Stock Solution C: 10 mM $MgCl_2 \times 6\ H_2O$

Stock Solution D: 10 mM $MgSO_4 \times 7\ H_2O$

10ml of the stock solution B were added to the stock solution A before autoclaving. Solutions C and D were autoclaved separately, and 5 ml of each were added to the autoclaved and cooled mixture of A and B solutions.

SOC medium (Inoue et al., 1990), used for recovery of the transformed *E. coli* cells

SOB medium supplemented with 20 ml 180 g/l solution of glucose

Plant medium for hydroponic condition (Priefer et al., 2001)

Stock Solution A: 0.75 g/l KCl

Stock Solution B: 0.1875 g/l $MgSO_4 \times 7H_2O$

Stock Solution C: 0.1875 g/l $CaSO_4 \times 2H_2O$

Stock Solution D: 0.1875 g/l $Ca_3(PO_4)_2$

Stock Solution E: 9.6 g/l FeEDTA

Stock solution F: 0.57 g/l H_3BO_3
0.31 g/l $MnSO_4 \times 2H_2O$
0.09 g/l $ZnSO_4 \times H_2O$
0.08 g/l $CuSO_4 \times 5H_2O$
0.016 g/l MoO_3
0.0008 g/l $CoCl_3 \times 6H_2O$

After stirring, pH value was measured and set to pH 7.0.

II.2.2. MOLECULAR IDENTIFICATION

To ascertain the identification of GM42 at species and strain level, RFLP-16S rDNA, RAPD-PCR, and REA-PFGE were performed. For the polymerase chain reaction assays, total DNA was extracted using Instagene Matrix (BioRad). The amplifications were carried out in a PTC-100 thermocycler (M J Research Inc.).

II.2.2.1. RFLP-16S rDNA

FD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and RD1 (5'-AAG GAG GTG ATC CAG CAG CC-3') primers described by Weisburg et al. (1991) which correspond to *Escherichia coli* 16S rRNA gene positions 8-27 and 1524-1540, respectively, were used (approximately 1.5 kb). PCR was performed in a total volume of 50 µl containing 20 ng of bacterial DNA, 1 × buffer (Invitrogen), 2.5 mM l^{-1} $MgCl_2$, 250 µM l^{-1} of each of the four dNTPs, 0.2 µM l^{-1} of each primer and 2.5 U *Taq* polymerase (Invitrogen). (Blaiotta et al. 2002). PCR conditions consisted of initial denaturing step at 95°C for 3 min, 30 cycles (94°C for 1 min, 54°C for 45 s and 72°C for 2 min) and an additional final chain elongation step at 72°C for 7 min. The presence of PCR products was ascertained by agarose (1% w/v) gel electrophoresis, at 100V for 1 h.

Restriction endonucleases *Cfo*I, *Hinf*I, *Msp*I and *Nde*II (Promega), as recommended by Laguerre et al. (1994), were used separately to digest 30 µl of PCR products. Restricted DNA was analyzed by electrophoresis in 2% agarose at 120V for 4 h. *E. coli* S17-1 and *R. phaseoli* 163 were used as controls.

II.2.2.2. RAPD-PCR

RAPD-PCR was performed in the total volume of 25 μl containing 20 ng of bacterial DNA, 1 × buffer (Invitrogen), 3.5 mM l^{-1} $MgCl_2$, 0.4 mM l^{-1} of each of the four dNTPs, 0.6 μM l^{-1} of primer and 2.5 U *Taq* polymerase (Invitrogen). The 10-mer random primer used for genomic strain typing was CC1 (5'-AGC AGC GTG G-3') (Moschetti et al., 2005), with 70% G-C content. The template DNA was denatured for 1 min at 94°C; then the PCR was carried out for 40 cycles (1 min at 94°C, 1 min at 31°C, 2 min at 72°C, for each cycle). Finally, a 7 min extension period at 72°C was performed. Amplified products were resolved on a 2% (w/v) agarose-TBE gel electrophoresis. *R. phaseoli* 163 was used as control. 1 Kb Plus DNA Ladder (Invitrogen) was used as molecular weight marker.

II.2.2.3. REA-PFGE

Samples were prepared according to Corich et al. (1991) with some modifications. The pellet from 10 mL of broth culture was washed with 5 mL of SE buffer (75 mM NaCl, 25 mM sodium EDTA, pH 7.4) and resuspended in SE in order to obtain a final concentration of 30 mg/mL (McClelland et al., 1987). A small slice, about 1–2 mm long of plug, was placed in a Petri dish (5 cm Ø) containing 6 mL of Tris (10 mM) for 15–30 min. Finally, the enzymatic digestion with XbaI was performed as reported by Corich et al. (1991).

Electrophoresis of the digested DNA was performed by the CHEF system (Bio-Rad) with 1% (w/v) agarose gel and 0.5 × tris borate EDTA (TBE) as running buffer, at 10°C. 8-48- kb ladder in agarose plugs (BioRad) was used as molecular size marker. Restriction fragments were resolved at a constant voltage of 6 V/cm, with the following

pulse times: 5 s for 6 h, 10 s for 6 h, and 15 s for 9 h. (Ventorino et al., 2007). E. coli S17-1and R. phaseoli 163 were used as controls.

II.2.3. PHENOTYPIC CHARACTERIZATION

Metabolic fingerprints of the strains 1021 and GM42 were obtained by measuring their ability to metabolize different carbon sources.

Moreover, the nodulation efficiency of the two strains was evaluated too.

II.2.3.1. METABOLIC FINGERPRINTS

The strains were grown on TY media at 30°C for 24 h. These cells in inoculating fluid (OD_{600} = 0.3 ± 0.05) (absorbance was assessed by software) were used to inoculate the Biolog GN2 microplates (150 µl per well). Microplates were incubated at 30°C for 24 h. After incubation the microplates were placed in the MicroStation Reader for analysis and the metabolic fingerprints was obtained. All experiments were performed in triplicate.

II.2.3.2. GROWTH CURVES

The strains were grown on TY media at 30°C for 24 h. A single colony was picked and inoculated in 5 ml TY and grown overnight. The day after the OD_{600} of each strain was measured. An equal cell number was centrifuged to eliminate TY medium and the cells were inoculated in 50 ml of minimal medium with mannitol or maltose as carbon

source. The samples were collected and OD_{600} was measured every two hours. All experiments were performed in triplicate.

The growth curves were fitted by Gompertz function using Curve Expert 1.3 software, and growth rate and asymptotic value were calculated for each fit. When applied to microbial growth curve, Gompertz's function is : y = a*exp[- exp(b-cx)], where a is the asymptote, defined as the maximum number of microorganisms that can be reached (N_{max}); b is the lag time, defined as the x-axis intercept of the tangent in the inflection point; c, is the maximum specific growth rate, defined as the tangent in the inflection point (μ_{max}).

II.2.3.3. PLANT NODULATION TESTS

The strains were evaluated for their symbiotic efficiency with alfalfa plants. Inoculation and seed treatment were performed as previously reported (Moschetti et al., 2005). The plants were cultured in a growth chamber under a constant temperature of 21°C and 12 h d^{-1} photoperiod and watered with nitrogen-free nutrient solution (Priefer et al., 2001). Forty days after planting, the effectiveness of the nodules for nitrogen fixation was estimated from the pink of the nodules and the dark green of the leaves compared to control plants (without inoculation) (Moschetti et al., 2005). All experiments were performed in triplicate.

II.2.4. CORRELATION BETWEEN *SMC03094* GENE AND KM^R PHENOTYPE

Different approaches were performed to find a gene or genes determining the kanamycin resistant phenotype in GM42.

II.2.4.1. RT-PCR of *SMc03094* GENE

S. meliloti 1021 and GM42 were grown on TY media at 30°C for 24 h. A single colony was picked and inoculated in 50 ml TY. The cells were grown overnight and harvested by centrifugation of 1 ml of the culture (OD600 = 0.6). Total RNA was extracted using NucleoBond RNA/DNA (Macherey-Nagel) following the manufactures'instructions.

PAP-Forw (5' –GCT TCG AGA GGG ACG CAC TG -3') and PAP-Rev (5' –GCA GCC GGT AAA AGG CGA GC -3') primers were used to amplify Smc03094 gene. RT-PCR was performed in a total volume of 50 µl containing 1 µg of bacterial RNA (previously treated with DnaseI), 1 × Reaction Mix (Invitrogen), 0.2 µM l-1 of each primer and 1 µl RT/Platinum Taq Mix (Invitrogen). PCR conditions consisted of an initial cycle at 50°C for 25 min for cDNA synthesis and one step at 94°C for 2 min for pre-denaturation, followed by 35 cycles (94°C for 30 s, 56°C for 30 s and 72°C for 1 min) and an additional final chain extension step at 72°C for 10 min. RT-PCRs of nodL gene and of 16S rDNA were performed as positive controls.

II.2.4.2 WESTERN-BLOTTING

The strains were grown on TY media at 30°C for 24 h. A single colony was picked and inoculated in 50 ml TY and grown overnight. 0.1 g of cells and 0.2 g of glass beads were resuspended into 1 ml lysis buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue) and were crushed in Ribolyser, power 6.5 for 30 s. Then, the samples were boiled at 100°C for 10 min, centrifuged at 13000 rpm at 4°C for 10 min and the supernatant was recovered.

Electrophoresis was carried out using SDS-PAGE gel (12% polyacrylamide) and the Tris–glycine–SDS buffer (25 mM Tris, 198 mM glycine, and 0.1% SDS) on a Mini-

Protean system (BioRad) at 11 mA/gel until the dye front reached the bottom edge of the gel. Precision plus protein standard Dual Color (BioRad) was used. Then, the proteins were transferred on a nitrocellulose filter (0.2 μm) using Tris-glycine-methanol buffer (25 mM Tris, 198 mM glycine, and 20% methanol) on a Mini Trans-Blot Cell (BioRad). The blocking was performed using 5% dry milk dissolved in TBS buffer and shaking at RT for 30 min. The filter was washed in TBS buffer with shaking at RT for 15 min.

The first hybridization was performed using rabbit polyclonal primary antibody to Neomycin Phosphotransferase II (1:250) (abcam) in 2.5% dry milk dissolved in TBS buffer and incubating at 4°C overnight with shaking. The filter was washed three times using TBS (10 min each wash step). The second hybridization was performed using goat polyclonal to rabbit IgG (conjugated to HRP) antibody in 2.5% dry milk dissolved in TBS buffer and incubating at RT for 3 h with shaking. Three wash steps were performed as above reported.

The signal detection was performed using HRP-color (Bio-rad) and hydrogen peroxide. Proteins extracted from *E. coli* S17-1 pSup 1021 (containing *neomycin phosphotransferase*II gene) were used as positive control; proteins extracted from *E. coli* DH5α were used as negative control.

II.2.4.3 CLONING of *SMc03094* GENE in EXPRESSION VECTOR

II.2.4.3.1. Preparation of electrocompetent cells of *E. coli* TOP10

1. Inoculate a single colony of *E. coli* into 5 ml LB medium and grow at 37°C overnight with moderate shaking

2. Inoculate 0.5 ml of the culture into 50 ml LB medium and grow at 37°C with shaking to an OD_{600} of 0.6
3. Chill cells on ice for 15 min and transfer to a pre-cooled centrifuge tube
4. Centrifuge cells 20 min at 4200 rpm at 2°C
5. Pour off supernatant and resuspend the pellet in 50 ml ice-cold water. Centrifuge cells as in step 4
6. Pour off supernatant immediately and resuspend the pellet by swirling in remaining liquid
7. Add 50 ml of ice-cold water, mix well, and centrifuge cells as in step 4
8. Pour off supernatant immediately and resuspend the pellet by swirling in remaining liquid
9. Add 5 ml ice-cold 10% glycerol and mix well. Centrifuge cells at 4200 rpm for 10 min at 2°C
10. Aliquot 50 µl cells into pre-cooled microcentrifuge tubes and freeze on dry ice. Store at -80°C.

II.2.4.3.2. Preparation of insert and vector

SMc03094 gene was amplified using PAPHF-EcoRI (5' –GAG GGA ATT CAT GGA CGT GAG AGA ATT G- 3') and PAPHR-PstI (5' –CCA ACT GCA GTC AGA AAA ATT CGT CCA G -3') primers. PCR was performed in a total volume of 50 µl containing 20 ng of bacterial DNA, 1 × buffer (Invitrogen), 2.5 mM l^{-1} $MgCl_2$, 250 µM l^{-1} of each of the four dNTPs, 0.2 µM l^{-1} of each primer and 2.5 U *Taq* polymerase (Invitrogen). PCR conditions consisted of initial denaturing step at 94°C for 3 min, 5 cycles (94°C for 45 s, 53 °C for 45 s and 72°C for 1 min), 30 cycles (94°C for 45 s,

57°C for 45 s and 72°C for 1 min), and an additional final chain elongation step at 72°C for 7 min. The presence of PCR products was ascertained by agarose (1% w/v) gel electrophoresis, at 100V for 1 h.

Restriction endonucleases *EcoR*I and *Pst*I (Promega) were used to digest PCR product (insert) and pKK223-3 prokaryotic expression vector (Pharmacia Biotech).

II.2.4.3.3. Ligation

The digested DNA fragments with compatible "sticky" ends can be joined together using the enzyme T4-Ligase. DNA ligation involves creating a phosphodiester bond between the 3´ hydroxyl of one nucleotide and the 5´ phosphate of another.

Ligation reaction was performed in a total volume of 10 µl containing 50 ng plasmid, 30 ng insert, 1 × ligation buffer, 1 µl T4-Ligase (Invitrogen). The ligation mixture was incubated overnight at 4°C.

II.2.4.3.4. Transformation of *E. coli*

Transformation of *E. coli* TOP10 was performed by electroporation.

- Add ligation product to 50 µl electrocompetent cells and mix
- Transfer the DNA and cells into a pre-cooled cuvette
- Place the cuvette into the sample chamber
- Apply the pulse (2.5 kV, 25 µF, 200 ohms)
- Remove the cuvette. Add immediately 1 ml SOC medium and transfer to a sterile tube. Incubate 60 min at 37°C with moderate shaking
- Plate the transformed cells (10 – 100 µl) on LB plates containing the antibiotic for selection.

II.2.4.3.5. Plasmid extraction and sequencing

Plasmid DNA from transformed cells of *E. coli* was isolated using QIAprep Spin Miniprep kit (Qiagen) following the manufactures' instruction.

To ascertain the presence and the identity of the insert, plasmid DNA was sequenced using two primers (4505F: 5' –TGA TTT AAT CTG TAT CAG GCT G -3'; and 47R: 5' –AAT GTG TGG AAT TGT GAG CGG -3') synthesized by Primm based on pKK223-3 sequence (GenBank accession number M77749).

II.2.5. DNA-MICROARRAYS

Transcriptional profiles of *S. meliloti* 1021 and GM42 were compared by DNA-microarrays.

II.2.5.1 RNA isolation

Cells (OD_{600}=0.8) were briefly centrifuged (13000 rpm, 1 min, 4°C) and cell pellets were immediately frozen in liquid nitrogen. Total RNA was purified using the RNeasy Mini Kit (Qiagen). Cells were disrupted in RLT buffer provided with the kit in Fast Protein tubes (Qbiogene) using the Ribolyser (Hybaid, Heidelberg, Germany) (30 s, level 6.5) before spinnig column purification according to the RNeasy Mini Kit RNA purification protocol (Rüberg et al., 2003).

II.2.5.2. Sm14KOligo microarrays

Each array contained 6208 70mer oligonucleotides directed against protein-coding orfs of *S. meliloti* and 8080 50mer to 70mer oligonucleotides directed against intergenic

regions in two replicates; a set of 70mer stringency control oligonucleotides (70 % to 90 % identity); 6 alien 70mer oligonucleotides as negative control; and spotting buffer and empty position controls. Each spot was in duplicate.

II.2.5.3. Hybridization and image acquisition

Hybridization and image acquisition were performed as described by Rüberg et al., 2003.

Image processing was performed with ImaGene (version 6.0.1). For each spot the background corrected spot intensities were calculated using the means of all chosen pixels for background and signal. Negative spots or spots that were flagged as empty or having bad quality were removed.

The mean intensity (A-value) was calculated for each spot using the standard formula $A_i=\log_2(R_iG_i)^{0.5}$. $R_i=I_{ch1i}-B_{gch1i}$ and $G_i= I_{ch2i}-B_{gch2i}$, where I_{ch1i} or I_{ch2i} is the intensity of a spot in channel 1 or channel 2 and B_{gch1i} or B_{gch2i} is the background intensity of a spot in channel 1 or channel 2, respectively. The logarithm to the base 2 of the ratio of intensities (the M-value) was calculated for each spot using the formula $M_i=\log_2(R_i/G_i)$ (Becker et al., 2004). Normalization and t-statistics were carried out using the EMMA 1.1 microarray data analysis software developed at the Bioinformatics Resource Facility, Center for Biotechnology, Bielefeld University) (http://www.genetik.uni-bielefeld.de/ EMMA/) (Dondrup et al. 2003). Genes were regarded as differentially expressed if $p \leq 0.05$ and $M \geq 1$ or $M \leq -1$.

II.2.6. PROMOTER ACTIVITY of *SMC03094* and *SMC03095* GENES

Smc03094Forw-Kpn (5' -GAC AGG TAC CAT CAT GAA CCC ACT TGC GG -3') and Smc03094Rev-Bam (5' -ACT CGG ATC CTG GCA GGT CCA ATT CTC TCA -3') primers were used to amplify the intergenic region upstream the *SMc03094* gene; while Smc03095Forw-Kpn (5' -GAC AGG TAC CCG GAG GCA TCT TCT TCG AGA -3') and Smc03095Rev-Bam (5' -ACT CGG ATC CAT CTG CTC GGT CAT GTC GTA -3') primers were used to amplify the intergenic region upstream the *SMc03095* gene. PCR was performed in a total volume of 50 µl containing 20 ng of bacterial DNA, 1 × buffer (Invitrogen), 2.5 mM l^{-1} $MgCl_2$, 250 µM l^{-1} of each of the four dNTPs, 0.2 µM l^{-1} of each primer and 2.5 U *Taq* polymerase (Invitrogen). PCR conditions consisted of initial denaturing step at 95°C for 3 min, 30 cycles (95°C for 30 s, 52°C for 30 s and 72°C for 30 s), and an additional final chain elongation step at 72°C for 7 min. The presence of PCR products was checked by agarose (2% w/v) gel electrophoresis, at 80V for 1 h.

Restriction endonucleases *Kpn*I and *BamH*I (Fermentas) were used to digest purified PCR product.

II.2.6.1. Construction of promoter probe vector pGvig

Plasmid pGvig carrying the *exoP-EGFP* cassette was constructed based on the vector pG18mob2 (Kirchner and Tauch, 2003). The cassette derived from plasmid pKmig and contained *exoP* gene for the insertion in the *S. meliloti* genome and promoterless *EGFP* gene to evaluate the activity of hypothetical promoter region.

Linear plasmid pG18mob2 obtained by using restriction endonucleases *EcoR*I and *Hind*III (Fermentas) was used for the ligation reaction with the *exoP-EGFP* cassette.

The promoter fragments to be tested were inserted into the resulting pGvig vector by using the *Kpn*I, and *BamH*I restriction sites located upstream of the promoterless *EGFP* gene.

Plasmids obtained by ligation reaction were cloned in *E. coli* DH5α (high efficient strain for transformation), and the plasmid isolated from DH5α transformed cells was cloned into *E. coli* S17-1 (high efficient strain for conjugation experiment with *S. meliloti*).

II.2.6.2. Promoter probe vector pSRPP18

The hypothetical promoter region was inserted into the plasmid pSRPP18 (Bahlavane et al., 2008) by using the KpnI, and BamHI restriction sites located upstream of the promoterless LacZ gene.

The aacC1 gene (gentamicin resistance gene), derived from plasmid pMS255 (Becker et al., 1995), was inserted in antisense orientation upstream of the insert by using restriction endonucleases KpnI.

Plasmids obtained by ligation reaction were cloned in E. coli DH5α (high efficient strain for transformation), and the plasmid extracted from DH5α transformed cells was cloned into E. coli S17-1 (high efficient strain for conjugation experiment with S. meliloti).

II.2.6.3. Preparation of competent cells of *E. coli* DH5α and S17-1

In this work a highly efficient chemical method of competent cell preparation was used (Inoue et al., 1990):

1. Pick 7 - 10 large *E.coli* colonies from the LB-agar plate and resuspend them in 3 ml of SOB. Use approximately 1 ml of the cell suspension to inoculate 150 ml of SOB. Incubate the flask in a shaker (150 rpm) overnight at the room temperature (18 – 22°C). Harvest the culture at the $O.D._{600}$ of 0.5 – 0.6.
2. Transfer the culture to centrifuge tubes and cool them 10 min on ice
3. Centrifuge for 10 min, at 3000 rpm, 4°C, discard the supernatant
4. Add 50 ml ice-cold TB buffer (10 mM HEPES, 55 mM $MnCl_2 \times 2H_2O$, 15 mM $CaCl_2 \times 2H_2O$, 250 mM KCl) and incubate for 10 min on ice
5. Centrifuge as step 3
6. Resuspend the pellets gently in 10 ml of ice-cold TB buffer. Add 0.7 ml of DMSO and incubate on ice for 10 min more
7. Aliquot the cells (200 µl) using pre-cooled microcentrifuge tubes and plastic tips.
8. Freeze the aliquots in liquid nitrogen and store at -80°C.

II.2.6.4. Transformation of competent cells by DNA

1. Thaw 200 µl of competent cells at room temperature. Add DNA in the volume of 10 µl or less. Incubate the cells together with DNA on ice for 30 min
2. Heat-shock the cells by placing in 42°C water bath for 30 s and later cooling them on ice for 5 min
3. Add 750 µl of SOC medium to the cells and incubate at 37°C for 1 h
4. Plate the transformed cells (100 µl) on LB plates containing the antibiotic for selection.

II.2.6.5. Conjugation

- Resuspend in 1 ml of TY a little colony from one day old *S. meliloti* culture on TY agar and from one to three day old *E. coli* S17-1 culture on LB agar containing Km (50 µg/ml) and mix (OD_{600} = 0.2)
- Drop 30 µl on TY agar without antibiotics and incubate overnight at 30°C
- Resuspend a tiny amount of the mixed strain colony in 1 ml of TY (OD_{600} = 0.4)
- Plate 100 µl of the suspension on TY medium containing Nx (10 µg/ml) and Gm (40 µg/ml). Incubate at 30°C for three days

To ascertain that the colonies obtained were S. meliloti, PCR was performed by using ExoP2683-H-for (5' -CCC AAG CTT TAC GCG ATC TGC TCC ACT C-3') and LacZ2rev (5' -TGA GGG GAC GAC GAC AGT ATC-3') primers in a total volume of 10 µl. PCR conditions consisted of initial denaturing step at 95°C for 5 min, 30 cycles (92°C for 30 s, 55°C for 30 s and 72°C for 1 min), and an additional final chain elongation step at 72°C for 5 min. The presence of PCR products was checked by agarose (2% w/v) gel electrophoresis, at 80V for 1 h.

II.2.7. FUNCTIONAL ANALYSIS of *SMC03094* and *SMC03095* GENES

To test the correlation between kanamycin resistant phenotype and the chromosomal region comprising *SMc03094* and *SMc03095* genes, cloning vectors for gene distruption of these genes in GM42 was constructed.

II.2.7.1. *SMc03094* gene

Smc03094-HindIIIForwUp (5' -CGC TAA GCT TAG CTT GGA ACC CTT TCG C -3') and 03094-PstI-Rev-Up (5' -CGC TGC AGG GTG GCA GGT CCA ATT CTC -3') primers were used to amplify the upstream region of the *SMc03094* gene, comprising 50 bp of the gene and 450 bp of the upstream region; while 660-PstI-For (5' -CGC TGC AGA TCA CCC ACA ACC TGG GAG A -3') and 1180-BamHI-Rev (5' -GCG GAT CCA TTC TGA CGA GCG CCG AT -3') primers were used to amplify the downstream region of the *SMc03094* gene, comprising 50 bp of the gene and 450 bp of the downstream region. PCR was performed in a total volume of 50 µl containing 20 ng of bacterial DNA, 1 × buffer (Invitrogen), 2.5 mM l^{-1} $MgCl_2$, 250 µM l^{-1} of each of the four dNTPs, 0.2 µM l^{-1} of each primer and 2.5 U *Taq* polymerase (Invitrogen). PCR conditions consisted of initial denaturing step at 95°C for 3 min, 30 cycles (95°C for 30 s, 53°C for 30 s and 72°C for 30 s), and an additional final chain elongation step at 72°C for 7 min. The presence of PCR products was checked by agarose (2% w/v) gel electrophoresis, at 80V for 1 h.

Restriction endonucleases *Hind*III and *Pst*I (Fermentas) were used to digest purified PCR products obtained by using Smc03094-HindIIIForwUp and 03094-PstI-Rev-Up primers; while *Pst*I and *BamH*I (Fermentas) were used to digest purified PCR products obtained by using 660-PstI-For and 1180-BamHI-Rev primers.

II.2.7.2. *SMc03095* gene

Smc03095-UP-Hin-For (5' -CAG AAG CTT ATC TTC AAC CGG TCG CTG AA -3') and Smc03095-UP-PstI-Rev (5' -CGC TGC AGA ATT CGA TCG CCT GCC TCA -3') primers were used to amplify the upstream region of the *SMc03095* gene,

comprising 50 bp of the gene and 500 bp of the upstream region; while Smc03095-do-PstI-For (5' -CGC TGC AGA CAG ATC ATG AAC CCA CTT GC -3') and Smc03095-do-Bam-Rev (5' -GCG GAT CCT TTT GCC GCA GCA ATG CT -3') primers were used to amplify the downstream region of the *SMc03095* gene, comprising 50 bp of the gene and 500 bp of the downstream region. PCR conditions were performed as above reported.

Restriction endonucleases *Hind*III and *Pst*I (Fermentas) were used to digest purified PCR products obtained by using Smc03095-UP-Hin-For and Smc03095-UP-PstI-Rev primers; while *Pst*I and *BamH*I (Fermentas) were used to digest purified PCR products obtained by using Smc03095-do-PstI-For and Smc03095-do-Bam-Rev primers.

II.2.7.3. Plasmid cloning vector

Plasmid pK18mobsac (Schäfer et al., 1994) was used to transform GM42. The experimental procedures for the construction of the plasmid cloning vectors used for gene distruption of the *SMc03094* and *SMc03095* genes were the same for both of them. Ligation reaction, transformation of competent cells of *E. coli*, and selection of transformed cells were performed as above reported.

- Upstream gene region insert (insert I) and plasmid pK18mobsac were cut by using restriction endonucleases enzymes *Hind*III and *Pst*I
- Ligation reaction was performed by using T4-ligase enzyme
- Competent cells of *E. coli* DH5α were transformed with ligation product I
- Transformed cells were selected and plasmid was extract by using QIAprep Spin Miniprep kit (Qiagen)

- Plasmid was sequenced by using pQE-FP primer (5’ -CGG ATA ACA ATT TCA CAC AG -3’) to verify the orientation of the insert into the plasmid
- Downstream gene region insert (insert II) and plasmid pK18mobsac were cut by using restriction endonucleases enzymes *Pst*I and *BamH*I
- Ligation reaction was performed by using T4-ligase enzyme
- Competent cells of *E. coli* DH5α were transformed with ligation product II
- Transformed cells were selected and plasmid was extract
- Plasmid was sequenced by using pM13-FP primer (5’ -TGT AAA ACG ACG GCC AGT -3’) to verify the orientation of the insert into the plasmid
- *LacZ-aacC1* cassette (insert III), derived from plasmid pAB2001 (Becker et al., 1995), and plasmid pK18mobsac were cut by using restriction endonucleases enzyme *Pst*I
- Dephosforylation of DNA 5’-termini of the linear plasmid was performed by using shrimp alkaline phosphatase (SAP) (Invitrogen). SAP was added directly to the restriction mixture of the plasmid after restriction reaction. 1 unit of SAP was added per 1 picomole of DNA 5´-termini and the mixture was incubated at 37°C for 1 h. The reaction was stopped by heating at 65°C for 15 min
- Ligation reaction was performed by using T4-ligase enzyme
- Competent cells of *E. coli* DH5α were transformed with ligation product III
- Transformed cells were selected and plasmid was extract
- Plasmid was sequenced by using pM13-FP (5’ -TGT AAA ACG ACG GCC AGT -3’) and pQE-FP (5’ -CGG ATA ACA ATT TCA CAC AG -3’) primers to verify the orientation of the insert into the plasmid
- Purified plasmid cloning vector was used to transform competent cells of *E. coli* S17-1.

II.3. RESULTS

II.3.1. MOLECULAR IDENTIFICATION

To ascertain the identification of GM42 three different molecular approaches were performed. The genomic DNAs of *S. meliloti* 1021 and GM42 were compared by using restriction fragment length polymorphism analysis of the 16S rDNA (RFLP-16S rDNA) for species-specific differentiation, randomly amplified polymorphic DNA-PCR (RAPD-PCR) analysis for intra-specific differentiation, and restriction endonucleases analysis combined with pulsed-field gel electrophoresis (REA-PFGE). Comparing fingerprints obtained there were no differences in all cases (Fig. 1, 2, and 3).

II.3.2. PHENOTYPIC CHARACTERIZATION

II.3.2.1. METABOLIC FINGERPRINTS

The MicroLog System Biolog brings a high level of accuracy to the rapid characterization of microbial organisms. Biolog's microplates incorporates 95 pre-selected carbon sources (including carboxylic acids, amino acids, and peptides) in a pre-filled microtiter format. The ability of strains to metabolize each carbon source is measured by the presence or absence of a purple hue in the wells. Tetrazolium violet, a redox dye, forms a purple color when oxidized by cellular respiration of the microorganism. Therefore, data obtained provide an enormous amount of discriminating biochemical characterization information.

S. meliloti strain 1021 and GM42 showed a different metabolic fingerprint. The ability of GM42 to metabolize the N-Acetyl-D-glucosamine involved in the glycoprotein synthesis and in the transport mediated by the phosphotransferase system proved to be interesting. The different carbon sources metabolized by *S. meliloti* 1021 or GM42 are listed in Table 1.

II.3.2.2. GROWTH CURVE

To confirm the results obtained with metabolic analysis using MicroLog Biolog, we performed exponential growth analysis of the strain 1021 and GM42.

The strains were inoculated in minimal medium supplemented with mannitol or maltose as unique carbon source. The growth curves generated were fitted by Gompertz function.

GM42 was not able to metabolize mannitol (Table 2). *S. meliloti* 1021 showed an asymptotic value (N_{max}) equal to 1.67 OD_{600} and a maximum specific growth rate value (μ_{max}) equal to 0.89 OD_{600} h^{-1}. By contrast, there no was significant variation of the growth in GM42 (N_{max} = 0.47 OD_{600}; μ_{max} = 0.03 OD_{600} h^{-1}).

Moreover, GM42 showed a slow growth in presence of maltose. In fact, *S. meliloti* 1021 showed a μ_{max} value equal to 0.2 OD_{600} h^{-1}, while GM42 showed a μ_{max} value equal to 0.06 OD_{600} h^{-1}. In addition, the N_{max} value was 1.52 and 1.29 OD_{600} in *S. meliloti* 1021 and GM42, respectively (Table 2).

II.3.2.3. PLANT NODULATION TESTS

Seeds of alfalfa were inoculated with *S. meliloti* 1021 and GM42 to verify the nodulation efficiency of the kanamycin-resistant strain. Visual observations of nodule

formation on the host plant and of plant size and foliage colour compared to non-inoculated controls after 40 days of growth were used to evaluate their symbiotic effectiveness. GM42 did not lose its nodulation efficiency; in fact, no significant difference, comparing the number of nodules obtained inoculating alfalfa with 1021 or GM42, was found (Table 3).

II.3.3. CORRELATION BETWEEN *SMC03094* GENE AND KM^R PHENOTYPE

Capela and co-workers (2001) identified a "putative aminoglycoside 3'-O-phosphotransferase" as the hypothetical product obtained by coding the *SMc03094* chromosomal gene. Therefore, different approaches were used to verify the relationship between *SMc03094* gene and the kanamycin resistance in *S. meliloti* strain 1021.

II.3.3.1. RT-PCR of *SMc03094* GENE

RT-PCR of the *SMc03094* gene was performed to check transcriptional difference in this chromosomal region between *S. meliloti* 1021 and GM42.

RT-PCR was performed using the total RNA extracted from *S. meliloti* 1021 and GM42. After amplification it was possible to see the difference in the transcription of *SMc03094* gene comparing the two strains. In fact, *S. meliloti* 1021 did not produced any RT-PCR product, while GM42 showed a single RT-PCR product at around 700 bp (nucleotide size of *SMc03094* gene) (Fig. 4). Moreover, RT-PCRs of the *nodL* gene and of 16S rDNA were performed to confirm the presence of RNA extracted from *S. meliloti* 1021 in the amplification reactions (positive controls) (Fig. 4).

This result confirmed that there might be a correlation between coding *SMc03094* gene and resistance to aminoglycoside antibiotics.

II.3.3.2. WESTERN-BLOTTING

Western-blotting on the total proteins extracted from *S. meliloti* 1021 and GM42 was performed to check the presence of an amynoglicoside phosphotransferase enzyme in GM42. Protein extractions were performed from three independent cultures of the two strains.

Anti-Neomycin Phosphotransferase II (NPTII) antibody was used as primary antibody.

The results confirmed that in GM42 there might be a protein that conferred resistance to aminoglycoside antibiotics. In fact, *S. meliloti* 1021 did not produce any signal after hybridization, while GM42 showed a single signal corresponding to the presence of a protein of about 25 kD (Fig. 5). Moreover, total proteins extracted from *E. coli* S17-1 pSup 1021 (containing the plasmid pSup1021 carrying the *NPT*II gene) were used as positive control while total proteins extracted from *E. coli* DH5α were used as negative control (Fig. 5). In *E. coli* S17-1 pSup 1021 Anti-Neomycin Phosphotransferase II antibody recognized a protein of about 25 kD just like in GM42.

II.3.3.3. EXPRESSION of *SMc03094* GENE in *E. coli*

SMc03094 gene from *S. meliloti* 1021 was cloned in the high-expression vector pKK223-3, derived from plasmid pBR322. In this construct the putative *aminoglycoside phosfotransferase* (*APH*) gene was placed under the control of the strong *tac* promoter.

The construct was used to transform electrocompetent cells of *E. coli* TOP10. Transformed cells were selected and tested for their kanamycin resistance.

Transformed cells showed a kanamycin-resistant phenotype; in fact, they were able to grow on LB medium containing 500 μg/ml kanamycin. Moreover, the insert was sequenced by using two oligonuclotide based on the plasmid sequence, to ascertain the presence and the identity of the insert. The sequences obtained were compared with the NCBI sequence (GenBank accession number NC003047). No oligonucletide difference was found.

This result proved that the expression of *SMc03094* gene in *E. coli* conferred resistance to kanamycin.

II.3.4. TRANSCRIPTIONAL DIFFERENCES in *S. MELILOTI* 1021 and GM42

DNA microarrays are used to measure the levels of abundance of defined mRNAs in the experimental conditions. The known DNAs (*probe*), corresponding to the mRNAs of the studied organism, are affixed to a support at the pre defined spots. The mRNAs, isolated from the sample, are reverse transcribed and labelled with two different fluorofores (Cyanine 5 and Cyanine 3). The samples (*targets*) are mixed and hybridized to the array.

An oligonucleotide-based whole-genome *S. meliloti* 1021 was used to analyze and to compare the transcriptional profiles of GM42 and *S. meliloti* 1021.

Results showed a few expression differences in GM42. In fact, comparing the expression of the open reading frame sequences, only seven genes were up-regulated and six genes were down-regulated in GM42 (Fig. 6). The genes up-regulated were: *SMc03095* (hypothetical unknown protein), *SMc00183* (conserved hypothetical protein), *SMc01217* (major facilitator superfamily-transport protein), *SMc01609*

(putative 6,7-dimethyl-8-ribityllumazine synthase) on the chromosome; *SMa0232* (conserved hypothetical protein) and *SMa0329* (short chain alcohol dehydrogenase-related dehydrogenase) on the pSymA; *SMb20647* (hypothetical protein) on the pSymB. The genes down-regulated were: *SMc00784* (bacterial extracellular solute-binding protein), and *SMc01714* (hypothetical transmembrane protein) on the chromosome; *SMa0983* (hypothetical protein) on the pSymA; *SMb21293* (putative guanine deaminase), *SMb20040* (hypothetical protein transmembrane), and *SMb20684* (conserved hypothetical protein) on the pSymB. *SMc03094* was up-regulated in GM42, but its M-value was 0.89. Therefore, *SMc03094* was considered not differential expressed in the data analysis. It turned out to be very interesting the up-regulation of *SMc03095*. This gene is located on the chromosomal region upstream of *SMc03094* gene and, besides, it has the same transcriptional orientation of *SMc03094*; therefore, these two genes might be under the control of the same promoter.

If we consider, in the data analysis, the intergenic region in addition to the genes, many transcriptional differences were obtained (Fig. 7). It proved interesting the up-regulation of intergenic region 4934 that showed a M-value equal to 2.33, the highest value obtained. This region is the intergenic region that is located on the chromosome between *SMc03095* and *SMc03094*. Since the intergenic region 4934 is upstream of *SMc03094* gene, it might be the promoter region for coding this gene.

II.3.5. PROMOTER ACTIVITY

To check the promoter region that regulate the coding of *SMc03095* and *SMc03094* genes, the intergenic regions located upstream of these two genes were cloned in the promoter probe pGvig.

The plasmid pGvig derived from pGmob2, which already contained the RP4 *mob* region, necessary for the transfer into *S. meliloti* from *E. coli* S17-1 cells, which have a chromosomally integrated copy of RP4 able to supply the transfer functions *in trans*. Furthermore, pG18Mob2 is a suicide vector since it is able to replicate in *E. coli*, but not in *S.meliloti*.

Plasmid pGvig (Fig. 8) was constructed inserting the *exoP-EGFP* cassette. The *exoP* partial gene derived from *S.meliloti* 1021 and it is necessary for the insertion of the fragment in the *S. meliloti* genome by recombination. The *enhanced green fluorescent protein* (*EGFP*) gene is promoterless and was used to evaluate the activity of hypothetical promoter region. The promoter fragments to be tested were inserted into the pGvig vector by using the *Kpn*I, and *BamH*I restriction sites located upstream of the promoterless *EGFP* gene.

The intergenic regions upstream *SMc03095* and *SMc03094* genes and deriving from *S. meliloti* 1021 and GM42 were cloned in pGvig and used to transform *S. meliloti* 1021.

The fluorescence emitted by EGFP in *S. meliloti* 1021 transformed with the four plasmids was measured by using a spectrofluorimeter. The empty plasmid (pGvig without intergenic region) in *E. coli* S17-1 was used as negative control.

The experiment failed because there was a mistake at the beginning of the nucleotide sequence in the *EGFP* gene used. In fact, the empty plasmid emitted a high fluorescence and thus it was impossible to measure the effective fluorescence emitted by the activity of promoter regions inside the transformed cells of *S. meliloti* 1021. Therefore, another promoter probe vector (pSRPP18) was used.

Plasmid pSRPP18 derived from pK18mob which, like pGmob2, is a suicide vector and contains the RP4 *mob*. Plasmid pSRPP18 carried the *exoP* partial gene (SMb20961) for the insertion of the fragment in the *S. meliloti* genome and a proterless *LacZ* gene. The

promoter fragments were inserted upstream of the *LacZ* gene. Therefore, in this case, it is possible to evaluate the promoter activity by β-Galactosidase assays of *S. meliloti* strains carrying the integrated promoter probe vector pSRPP18 with promoter fragment inserts.

Moreover, since pSRPP18 carried the kanamycin resistance gene, it was necessary to clone the *aacC1* gene, which confers gentamicin resistance, in the promoter vector.

The promoter probe vectors pSRPP18 with promoter region were obtained (Fig. 9), and the next step will be the transformation of *S. meliloti* 1021.

II.3.6. FUNCTIONAL ANALYSIS of *SMC03094* and *SMC03095* GENES

DNA-microarrays results showed the up-regulation of *SMc03095* gene. Therefore functional analysis of *SMc03095* and *SMc03094* genes was performed by gene destruction of these genes in GM42, to ascertain their involving in kanamycin- resistant phenotype.

Plasmid pK18mobsac was used as vector. This plasmid derives from pK18mob, therefore it is a suicide vector and contains the RP4 *mob*. In addition, pK18mobsac carries a genetically modified *sacB* gene which confers sucrose-sensitivity to Gram-negative bacteria as additional selection.

Two pK18mobsacV plasmids (Fig. 10) were obtained by cloning three inserts in the multi-cloning site (MCS) of pK18mobsac.

Plasmid pK18mobsacV-94 carried: two inserts containing the upstream chromosomal region of *SMc03094* gene and 5'-end (50 bp) of *SMc03094* gene (insert I), and the 3'-end (50 bp) of *SMc03094* gene and downstream chromosomal region of *SMc03094*

gene (insert II) for the crossing-over; one insert containing *LacZ-aacC1* cassette (insert III) for both the selection of *S. meliloti* strains carrying the integrated vector pK18mobsacV-94 and destruption and replacement of *SMc03094* gene.

Plasmid pK18mobsacV-95 carried: two inserts containing the upstream chromosomal region of *SMc03095* gene and 5'-end (50 bp) of *SMc03095* gene (insert I), and the 3'-end (50 bp) of *SMc03095* gene and downstream chromosomal region of *SMc03095* gene (insert II) for the crossing-over; and one insert comprising *LacZ-aacC1*cassette (insert III).

Actually, the vectors pK18V were obtained, and the next step will be the transformation of *S. meliloti* 1021.

II.4. DISCUSSION

II.4.1. PHENOTYPE CHARACTERIZATION of GM42

S. meliloti 1021 is known as a kanamycin sensitive strain (30 µg/ml). A spontaneous kanamycin-resistant mutant (GM42) (200 µg/ml) was isolated from *S. meliloti* strain 1021.

It is reported that variation exists in effectiveness of nodule formation, competitiveness for nodule formation, and ability to fix nitrogen in antibiotic-resistant mutant of rhizobial strains (Date and Hurse, 1992; Simon and Kalalova, 1996). Therefore the kanamycin- resistant mutant strain GM42 was evaluated for its symbiotic effectiveness in alfalfa and compared with *S. meliloti* 1021.

Sindhu and Dadarwal (2001) tested the nodulation ability and symbiotic effectiveness of spontaneous streptomycin - resistant mutants. Their results showed that acquisition of streptomycin resistance in *Rhizobium* sp. *Cicer* strains is associated with decreased symbiotic effectiveness in chickpea. In fact, some mutants showed Nod^- phenotype, some showed Nod^+ fix^- phenotype, and others showed decreased nodule number. By contrast, GM42 was not found symbiotically inferior in comparison with parent strain, owing to the absence of variation in the nodule formation of GM42 in alfalfa. In fact, no significant difference, comparing the number of nodules obtained inoculating alfalfa seeds with 1021 or GM42, was found. This result showed that the antibiotic-resistant mutated phenotype is not always associated with variation in effectiveness of nodule formation according to Pankhurst (1977), Lewis et al. (1987), and Rynne et al. (1991). They found little or no change in symbiotic effectiveness in antibiotic resistant mutants of *Lotus* rhizobia, *R. meliloti*,and *R.leguminosarum* bv. *trifolii*.

Moreover, the metabolic characterization of GM42, on the basis of its capability to metabolize different carbon sources, was performed. GM42 and *S. Meliloti* 1021 showed a very different metabolic fingerprint. It turned out interesting to notice the inability of GM42 to metabolize mannitol, a typical carbon source that rhizobial strains are able to metabolize. In order to confirm these results, exponential growth analysis of the strain 1021 and GM42 was performed. GM42 was not able to grow in minimal medium with mannitol as sole carbon source. In fact, *S. meliloti* 1021 showed an asymptotic value equal to 1.67 OD_{600} and a maximum specific growth rate value equal to 0.89 OD_{600} h^{-1}. By contrast, there no was significant variation of the growth in GM42 (N_{max} = 0.47 OD_{600}; μ_{max} = 0.03 OD_{600} h^{-1}), confirming the result obtained by using MicroLog Biolog.

II.4.2. KANAMYCIN RESISTANCE DETERMINANTS

Different molecular approaches were performed to identify gene or genes determining the kanamycin-resistant phenotype in GM42. We analyzed the chromosomal region comprising *SMc03094* and *SMc03095* genes.

Capela and co-workers (2001) identified a "putative aminoglycoside 3'-O-phosphotransferase" as the hypothetical product obtained by the transcription of the *SMc03094* chromosomal gene. Moreover, Alexandre and co-workers (2006) found congruence of results obtained clustering different isolates by the use of intrinsic antibiotic resistance (IAR) and 16S rDNA RFLP/sequence isolates, which suggested a chromosomal location for antibiotic resistance genes. This hypothesis is supported by the identification of two antibiotic resistance genes, coding for kanamycin and

streptomycin resistance proteins, annotated in the rhizobial chromosome of *M. loti* strain MAFF303099 (Kaneko et al., 2000).

RT-PCR of the *SMc03094* gene showed the expression of this gene in GM42 but not in *S. meliloti* 1021. Moreover, the western-blotting, performed by using anti-Neomycin Phosphotransferase II (NPTII) antibody as primary antibody, showed the presence of an aminoglycoside phosphotransferase protein in GM42. On the contrary, no signal corresponding to a protein was found in *S. meliloti* 1021.

In addition, the *SMc03094* gene was cloned and expressed in *E. coli* cells. In this case, the transformed cells showed kanamycin-resistant phenotype, thus confirming the previous results.

In order to have a major vision of the transcriptional differences between GM42 and *S. meliloti* 1021, DNA-microarrays were performed. An oligonucleotide-based whole-genome *S. meliloti* 1021 was used and the transcriptional profiles of GM42 and *S. meliloti* 1021 were analyzed and compared.

As we expected, results showed a few transcriptional differences in GM42. In fact, with this approach it is possible to analyze partially the genome sequence.

Comparing the expression of the open reading frame sequences obtained from GM42 and *S. meliloti* 1021, only seven genes were up-regulated and six genes were down-regulated in GM42, and most of them coded for unknown proteins. Interestingly, the *SMc03095* gene was up-regulated (M-value = 1.93). By contrast the *SMc03094* gene was clustered with genes that showed standard expression level. *SMc03094* gene showed M-value equal to 0.89; therefore this gene is up-regulated if compared with *S. meliloti* 1021, but the statistical analysis used regarded as differentially expressed the genes with $M \geq 1$ or $M \leq -1$. These results confirmed that correlation might be between kanamycin resistance and the chromosomal region analyzed.

Therefore, in order to ascertain the involvement or not of *SMc03094* and *SMc03095* genes in kanamycin- resistant phenotype, functional analysis of these genes was performed in GM42. Two plasmids for the destruption and replacement of *SMc03094* and *SMc03095* genes in *S. meliloti* 1021 were constructed. Because of the difficulty to clone the *LacZ-aacC1* cassette (about 4.5 kb), transformation experiments of GM42 are in progress at the moment.

The microarrays analysis showed many regulation differences if intergenic regions were compared. Interestingly, the intergenic region 4934, which is located on the chromosome between *SMc03095* and *SMc03094*, was up-regulated, showing a M-value equal to 2.33, the highest value obtained. Since the intergenic region 4934 is upstream of *SMc03094* gene, it might be the promoter region for the transcription of this gene.

Therefore, to check the promoter region that regulates the transcription of *SMc03095* and *SMc03094* genes, the intergenic regions located upstream of these two genes were cloned in the constructed promoter probe pGvig. The activity of the hypothetical promoter region was measured by the luminescence emitted from the prometerless *EGFP* gene located downstream of the cloned insert. Unfortunately, the experiment failed because there was a mistake at the beginning of the nucleotide sequence in the *EGFP* gene used. In fact, the empty plasmid emitted a high fluorescence and thus it was impossible to measure the real fluorescence emitted by the activity of promoter regions inside the transformed cells of *S. meliloti* 1021.

Therefore, another promoter probe vector (pSRPP18) was used. In this case, the hypothetical promoter regions were cloned upstream of the *LacZ* gene. The activity of the promoter will be measured by β-Galactosidase assays of *S. meliloti* strains carrying the integrated promoter probe vector pSRPP18 with promoter fragment inserts.

The promoter probe vectors pSRPP18v were obtained and, at the moment, transformation experiments of *S. meliloti* 1021 are in progress.

The results obtained confirm that the *SMc03094* gene codes for a kanamycin resistant protein when transcripted in *E. coli*. Moreover, the transcription of *SMc03094* and *SMc03095* genes occurs in GM42 but not in *S. meliloti* 1021, and an amynoglycoside phosphotransferase protein is coded in GM42 but not in the parental strain. Therefore, correlation might be between kanamycin-resistant phenotype and the chromosomal region analyzed in GM42.

Further analysis will be performed to understand metabolic and transcriptional differences in GM42.

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II.6. FIGURES

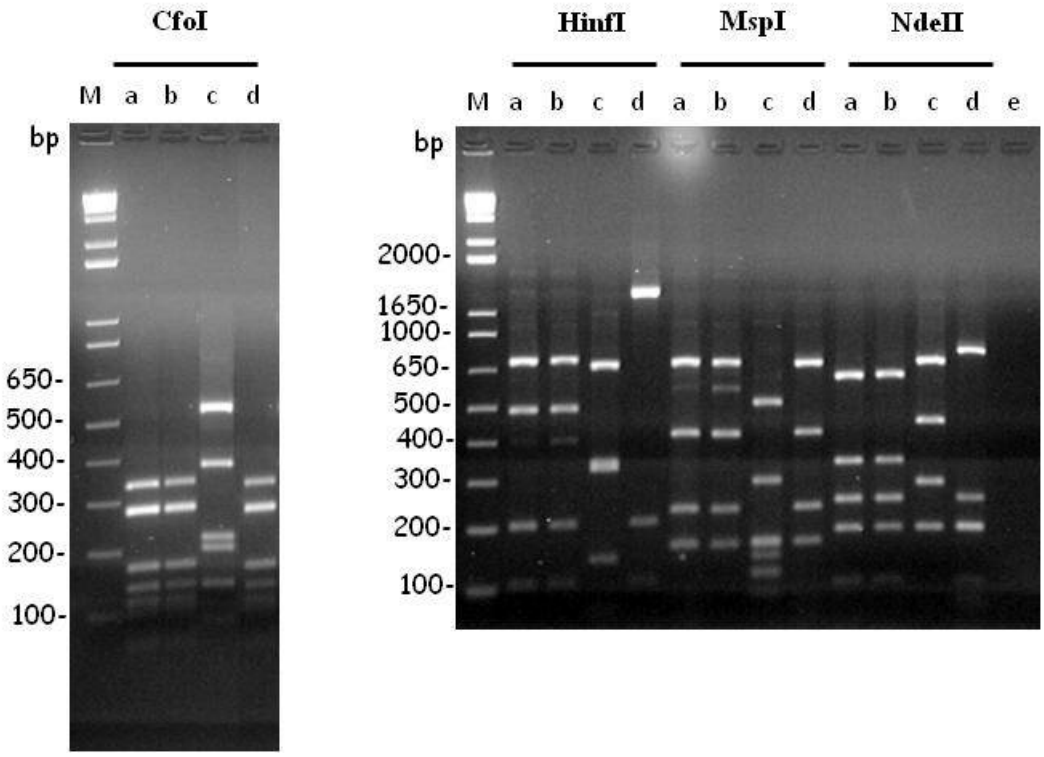

Figure 1. Agarose gel electrophoresis of 16S rDNA-RFLP products by using *Cfo*I, *Hinf*I, *Msp*I, and *Nde*II endonucleases restriction enzymes. (a) *S. meliloti* 1021, (b) GM42, (c) *E. coli* S17-1, (d) *R. phaseoli* 163, (e) negative control. M, 1 kb DNA Ladder Plus (Invitrogen) used as molecular weight marker.

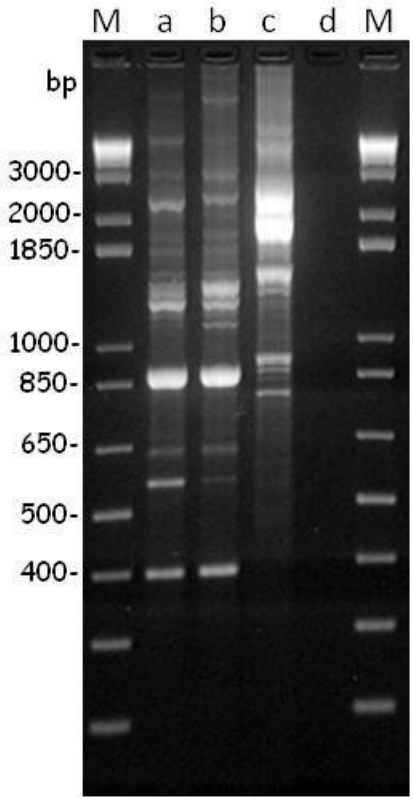

Figure 2. Agarose gel electrophoresis of RAPD-PCR products by using CC1 primer. (a) *S. meliloti* 1021, (b) GM42, (c) *R. phaseoli* 163, (d) negative control. M, 1 kb DNA Ladder Plus (Invitrogen) used as molecular weight marker.

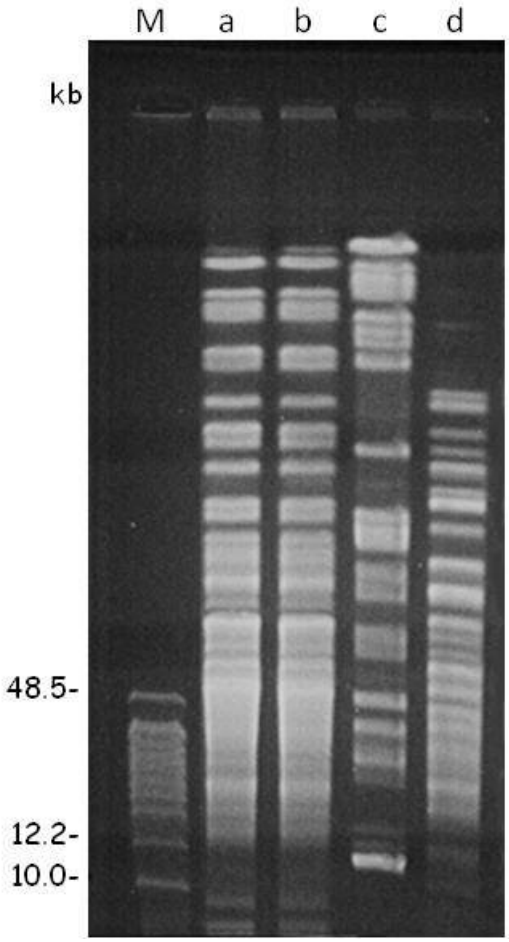

Figure 3. REA-PFGE using *XbaI* endonucleases restriction enzyme. (a) *S. meliloti* 1021, (b) GM42, (c) *E. coli* S17-1, (d) *R. phaseoli* 163. M, 8-48 kb DNA Ladder (Bio-Rad) used as molecular weight marker.

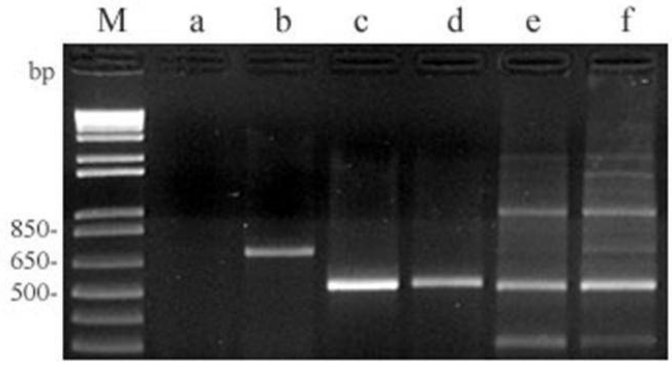

Figure 4. Agarose gel electrophoresis of: *smc03094* RT-PCR-products from *S. meliloti* 1021 (a) and GM42 (b); *nodL* gene RT-PCR-products from *S. meliloti* 1021 (c) and GM42 (d); 16S rDNA gene RT-PCR-products from *S. meliloti* 1021 (e) and GM42 (f). M, 1 kb DNA Ladder Plus (Invitrogen) used as molecular weight marker.

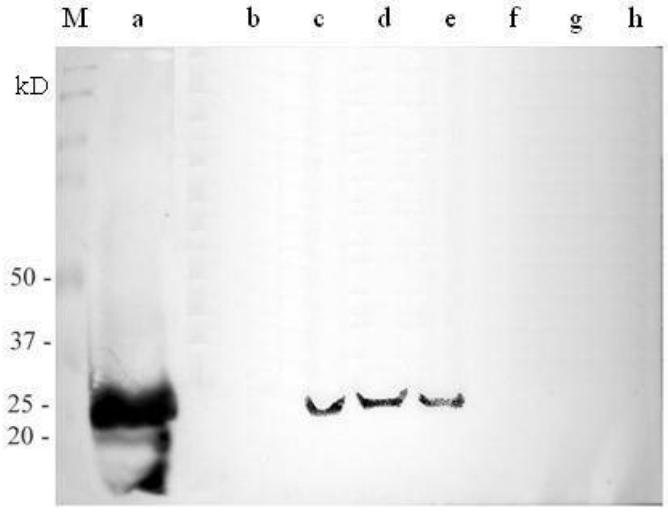

Figure 5. Western-blotting. The anti-Neomycin Phosphotransferase II was used as primary antibody. a, *E. coli* S17-1 pSup 1021; b, *E. coli* DH5α; c-e, GM42; f-h, *S. meliloti* 1021. M, precision plus protein standard Dual Color (BioRad).

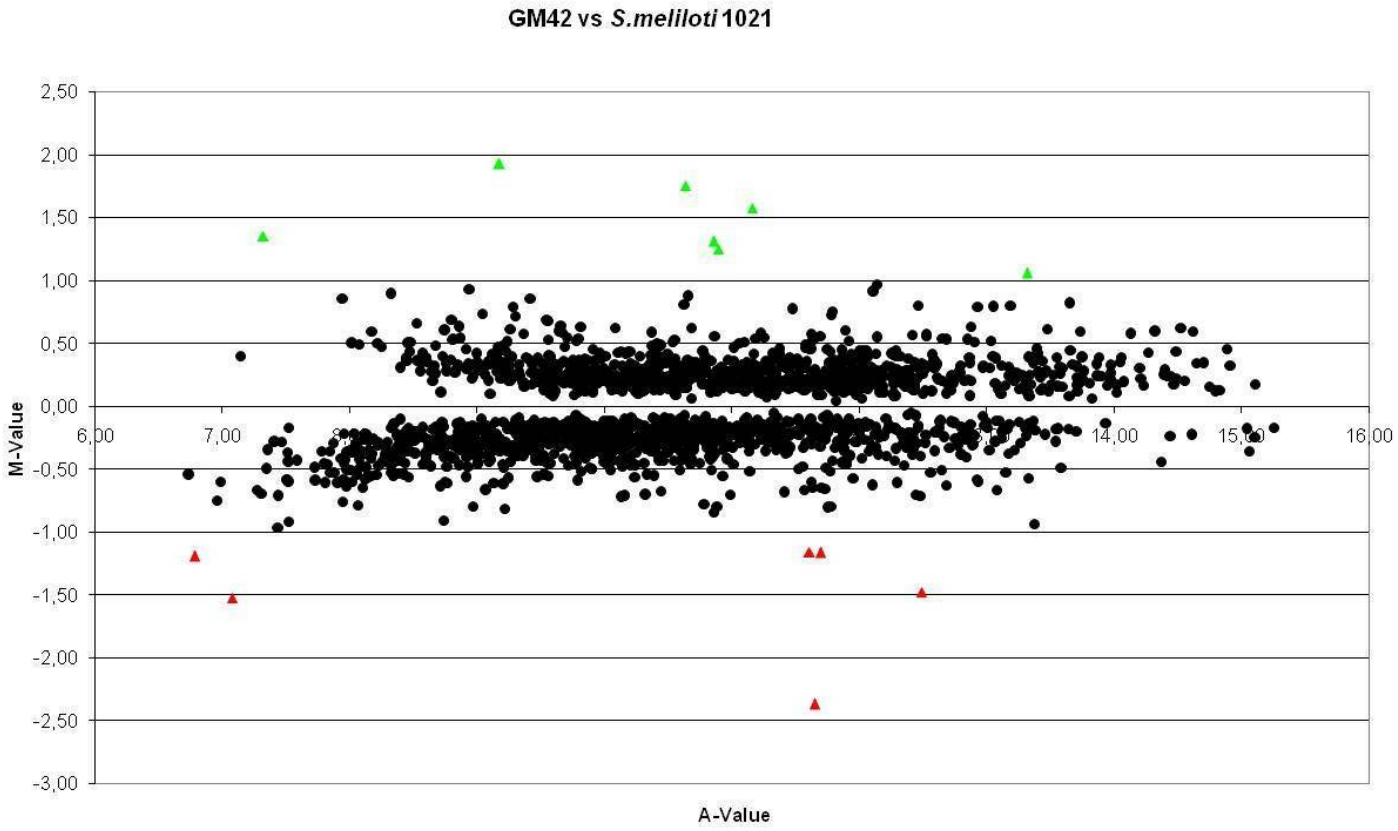

Figure 6. DNA-microarrays analysis. Expression profiles of the open reading frame sequences in GM42 versus *S. meliloti* 1021. Green spot, up-regulated gene; black spot, wild type expression level; red spot, down-regulated gene.

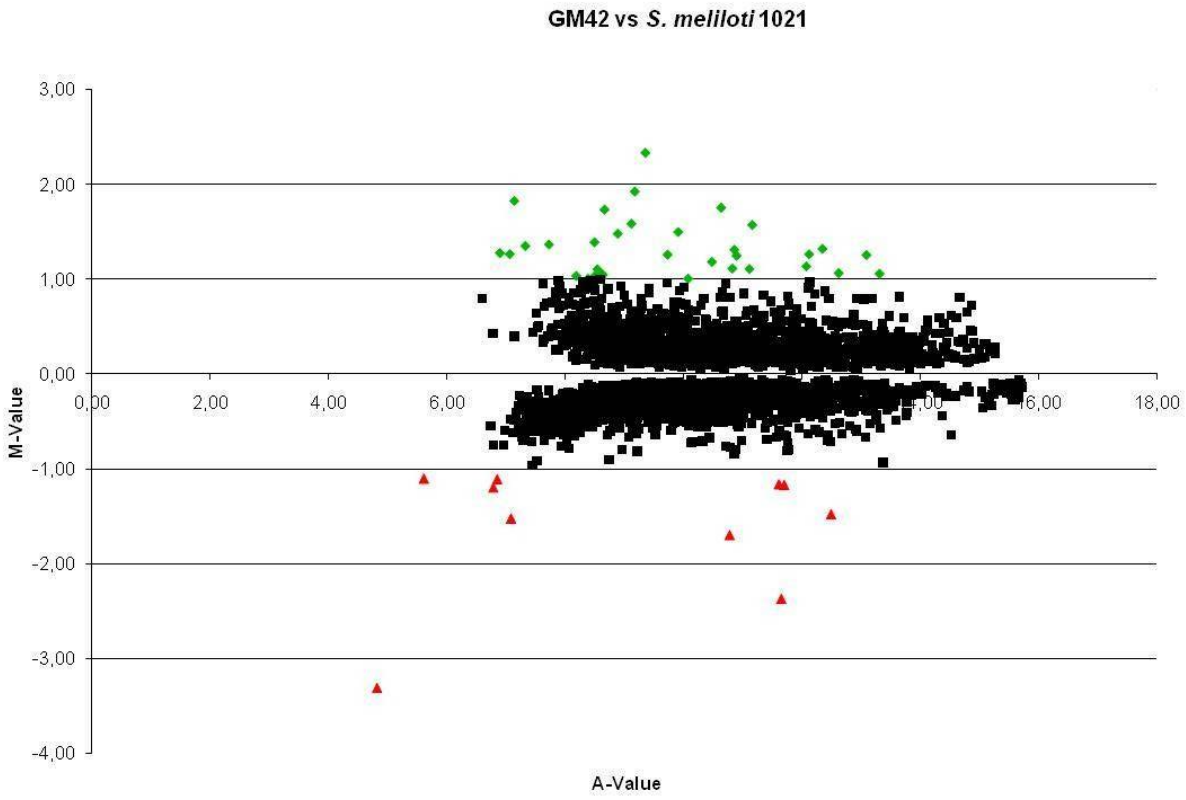

Figure 7. DNA-microarrays analysis. Expression profiles of the open reading frame sequences and intergenic regions in GM42 versus *S. meliloti* 1021. Green spot, up-regulated gene; black spot, wild type expression level; red spot, down-regulated gene.

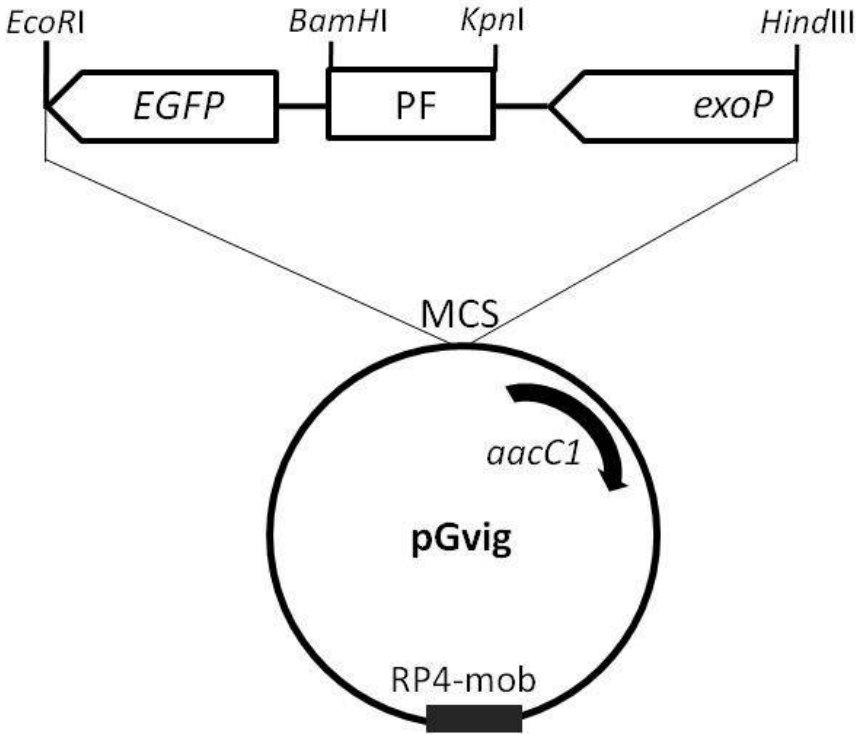

Figure 8. Promoter probe vector pGvig. PF, promoter fragment.

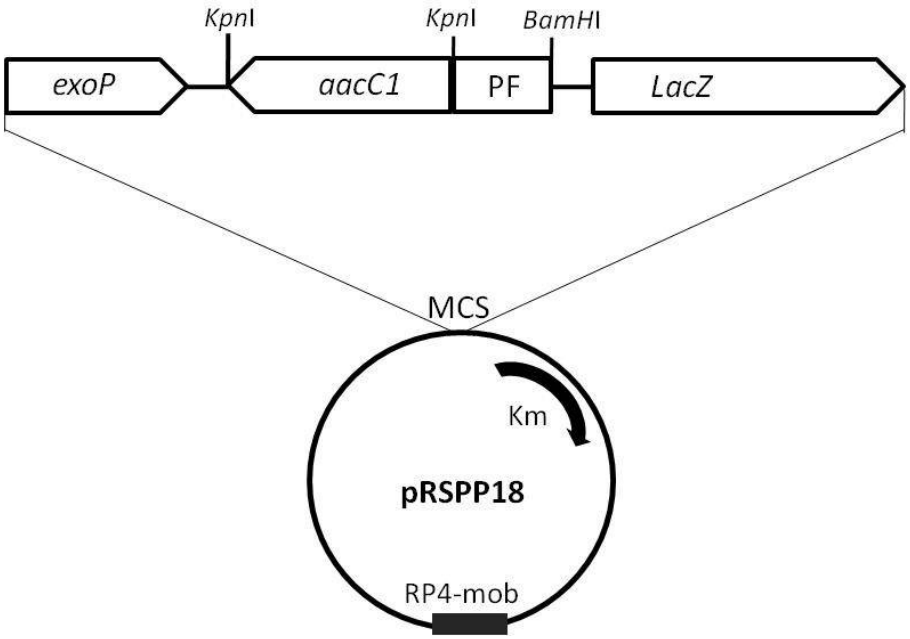

Figure 9. Promoter probe vector pRSPP18. PF, promoter fragment; *aacC1*, gentamicin resistance gene.

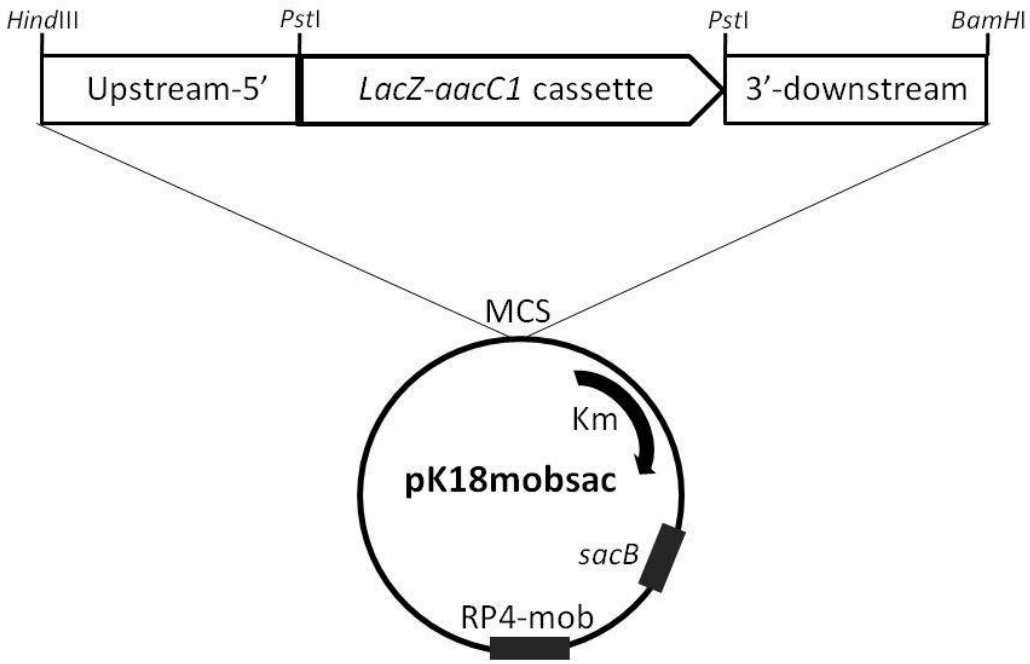

Figure 10. Plasmid vector pK18mobsac. Upstream-5', upstream chromosomal region of gene and 5'-end of gene (insert I), 3'-downstream, 3'-end of gene and downstream chromosomal region of gene (insert II).

II.7. TABLES

Table 1. Metabolic fingerprints of *S. meliloti* 1021 and GM42. The + and - symbols correspond to the ability or not to metabolize a particular carbon source, respectively. Only the differences are reported.

Carbon Source	S. meliloti 1021	GM42
Dextrin	-	+
N-Acetyl-D-glucosamine	-	+
D-Arabitol	-	+
D-Fructose	-	+
L-Fucose	-	+
Gentiobiose	-	+
α-D-Glucose	-	+
α-D-Lactose	-	+
Maltose	+	-
D-Mannitol	+	-
Xylitol	-	+
Formic Acid	-	+
D-Galactonic-Acid Lactone	+	-
D-Gluconic Acid	+	-
α-Hydroxy Butyric Acid	-	+
β- Hydroxy Butyric Acid	-	+
Itaconic Acid	+	-
α-Keto Valeric Acid	-	+
D,L-Lactic Acid	-	+
Propionic Acid	-	+
Quinic Acid	+	-
L-Phenylalanine	+	-
L-Pyroglutamic Acid	+	-
D,L-Carnitine	+	-
Inosine	+	-
Uridine	+	-
Thymidine	+	-
Phenythylamine	+	-
D,L-α-Glycerol Phosphate	+	-

Table 2. N_{max} and μ_{max} values measured by Gompertz's function in *S. meliloti* 1021 and GM42 grown in minimal medium with mannitol or maltose as carbon source.

STRAIN / CARBON SOURCE	N_{max} (OD)	μ_{max} (h^{-1})
S.meliloti 1021 / Mannitol	1.67	0.89
GM42 / Mannitol	0.47	0.03
S.meliloti 1021 / Maltose	1.52	0.2
GM42 / Maltose	1.29	0.06

Table 3. Number of nodules per plants (20 plants) obtained after 40 d of growth on hydroponic conditions.

Strain	Number nodules
S.meliloti 1021	57.0 ± 9.5
GM42	55.3 ± 8.6