Rhizobacteria Associated to *Vitis vinifera* and their Effect on the Control of *Xiphinema index*, *Meloidogyne ethiopica* and *Vitis* Growth

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Cover: Roots of grapevines parasitised by *Xiphinema index* (left) and healthy (right)
(photo: E. Aballay)

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Abstract

Plant-parasitic nematodes are one of the most important pests affecting the growth of vineyards due to the destruction of new roots. Several species are associated with this problem, the most important being the ectoparasite *Xiphinema index* and the endoparasite *Meloidogyne ethiopica*. The search for new pest and disease control methods based on bioantagonist microorganisms is an important aspect of modern agriculture and the development of tools based on the use of rhizobacteria is becoming a widely evaluated alternative.

The present study on suppressive soils started with a survey undertaken in productive Chilean vineyards to explore the younger roots of grapevines and identify the presence of rhizobacteria. More than 1800 soils were surveyed and a set of 11 vineyards were selected and considered suitable for bacteria isolation, as they showed low densities of plant-parasitic nematodes.

A total of 400 bacterial isolates in 25 genera were obtained using tryptic soy broth agar and identified with fatty acid profiling. Two of the most frequently isolated species were *Pseudomonas putida* (35.1%) and *P. fluorescens* (6.1%). The effect of these isolates on the parasitism and reproduction of *X. index* was assessed through assays using potted vine plants (cv. Thompson Seedless) and a bacterial suspension containing 1×10^6 CFU/mL. Some isolates from *Bacillus megaterium, B. brevis, B. mycoides, B. sphaericus, B. thuringiensis, Pseudomonas corrugata, P. putida, P. alcaligenes, P. savastanoi, P. fluorescens, P. pseudoalcaligenes, P. viridiflava, Stenotrophomonas maltophilia, Serratia plymuthica, Cytophaga johnsonae, Rahnella aquatilis, Stenotrophomonas* sp., *Variovorax paradoxus* and *Curtobacterium flaccumfaciens* reduced root damage and suppressed populations.

Isolates of *B. brevis, Comamonas acidovorans, B. megaterium, Pantoea agglomerans* and *P. savastanoi* increased plant growth or root weight, but did not control nematodes.

Most of the culture filtrates obtained from isolates from four vineyards were effective in killing *X. index* and decreasing egg hatching, which was not related to damage or population control. It was estimated that 89, 32 and 16% of the isolates were effective against *X. index* under supernatant conditions, in potted plants growing in sterile substrate and in potted plants growing in field soil, respectively.

Sixteen bacterial isolates previously assessed were also evaluated on *M. ethiopica* in vine cv. Chardonnay. Seven isolates (*Serratia marcescens, C. acidovorans, P. agglomerans, Sphingobacterium spiritivorum, B. mycoides, Alcaligenes piechaudii* and *S. plymuthica*) decreased damage or reproduction, showing that different species of nematodes can respond differently to a particular isolate.

Keywords: Xiphinema index, rhizobacteria, plant-parasitic nematodes, *Meloidogyne ethiopica*, vineyards, control.

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Dedication

To the members of my big family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Aballay, E., Persson, P. & Mårtensson, A. (2009). Plant-parasitic nematodes in Chilean vineyards. *Nematropica* 39 (1), 85-97.

II Aballay, E., Mårtensson, A. & Persson, P. (2011). Screening of rhizosphere bacteria from grapevine for their suppressive effect on *Xiphinema index* Thorne & Allen on *in vitro* grape plants. *Plant and Soil* 347, 313-325.

III Aballay, E., Prodan, S., Mårtensson, A. & Persson, P. (2012). Assessment of rhizobacteria from grapevine for their suppressive effect on the parasitic nematode *Xiphinema index*. *Crop Protection* (in press).

IV Aballay, E., Ordenes, P., Mårtensson, A. & Persson, P. (2012). Effects of rhizobacteria on parasitism by *Meloidogyne ethiopica* on grapevines. *European Journal of Plant Pathology* (on line).

The contribution of Erwin Aballay to the papers included in this thesis was as follows:

I Planning of study. The survey and management of the information of nematode populations. Preparation of manuscript reviewed by P. Persson and A. Mårtensson.

II Planning of study with the co-authors. Rhizobacteria extractions and identification using FAME along with by Dr. Paula Person. Assays development, analysis of the information. Preparation of manuscript reviewed by P. Persson and A. Mårtensson.

III Planning of study with the co-authors. Development of assay with potted plants, preparation of mixtures of soils, extraction an inoculation with nematodes, analysis of data. Preparation of manuscript, reviewed by P. Persson.

IV Planning of study with the co-authors. Preparation of plants and inoculation of nematodes. Rhizobacteria inoculation along with P. Ordenes. Preparation of manuscript, reviewed by P. Persson.

Abbreviations

CFU	Colony forming units
FAME	Fatty acid methyl esters
PBS	Phosphate buffered saline
PGPR	Plant growth promoting rhizobacteria
PPN	Plant-parasitic nematodes
SI	Similarity index
TSBA	Tryptic soy broth agar

1 Introduction

Plant-parasitic nematodes (PPN) are one of the groups of organisms that cause great damage to crops worldwide. The majority are associated with roots and a fraction is characterised by parasitising aerial parts, *i.e.* leaves, stems and buds.

In soil, damage can be caused by ectoparasitic nematodes destroying meristematic tissue, mainly in root tips, or by endoparasites attached to tissues near the vascular or cortical cells. By feeding on the roots and destroying tissue, they allow other soil microorganisms (mainly fungi and bacteria) to enter the roots, colonise the tissues and increase the damage and cause plant death (Hussey & McGuire, 1987; Sikora & Carter, 1987). Moreover, some nematodes are capable of transmitting virus diseases in many economically important crops, including grapes (*Vitis vinifera* L.) (Taylor & Brown, 1997).

Control of this pest has basically been achieved through the use of chemical nematicides, fumigants and non-fumigant organophosphates and carbamates, which are applied directly to soil at planting and are used throughout the crop cultivation for many years (Hague & Gowen, 1987). Although non-fumigant chemical nematicides are highly toxic products, their effectiveness to control these pests in the fields is low for several reasons and their use has not been able to decrease the problem (Bunt, 1987).

During the past 30 years, a new area of research has been under development, focusing on the effect of bacteria from the rhizosphere of plants. There have been many studies assessing their effect on root diseases, mainly in annual crops, solubilisation of phosphorus, nitrogen fixation, synthesis of plant hormones and other processes, including the impact on plant-parasitic nematode parasitism (Kerry, 2000; Buchenauer, 1998; Weller, 1988). There are many reports in different countries indicating that some rhizobacterial isolates show suppressive effects on the development of nematode populations and/or on the incidence of damage caused by these organisms. Most of these reports have focused on the action of nematodes on annual crops, such as tomatoes,

potatoes, beet or cereals (Johansson *et al*., 2003; Nejad & Johnson, 2000; Howie & Echandi, 1983). However, few studies have examined their presence and impact on perennial crops, including grapevine, which is a crop of high economic importance in Chile and many other countries (West *et al*., 2010; Kluepfel *et al*., 1993). Some studies on suppressive soils demonstrate that bacteria may provide effective control of nematodes in intensive agriculture (Dong & Zhang, 2006; Kokalis-Burelle *et al*., 2002), but there is little experience of nematodes particularly affecting vineyards.

1.1 Aims and thesis outline

The overall aims of this thesis were to isolate and identify rhizobacteria associated to the complex *Vitis vinifera* roots and plant-parasitic nematodes and assess the nematicidal activity of some isolates, especially on *Xiphinema index* and *Meloidogyne ethiopica*, two important pests of grape root systems in Chile.

Specific objectives were to:

1. Isolate and identify rhizobacteria from grape roots of both table and wine grape varieties grown on soils with either low or high densities of *Xiphinema index* but good root growth.
2. Study the nematicidal effects of selected rhizobacteria against *X. index* through *in vitro* and *in vivo* tests.
3. Assess the isolates selected in the previous test against another important species of nematode with a different mode of parasitism (*Meloidogyne ethiopica*).

1.2 Hypothesis

The starting hypothesis for the research presented in this thesis was that the presence of grapevine root systems with a low presence of the nematode *Xiphinema index*, or displaying good growth despite higher populations of this nematode, is due to the presence of rhizobacteria that can act either as a suppressant against nematodes or a growth promoter for vine roots.

The different research steps, details and corresponding paper are presented in Figure 1.

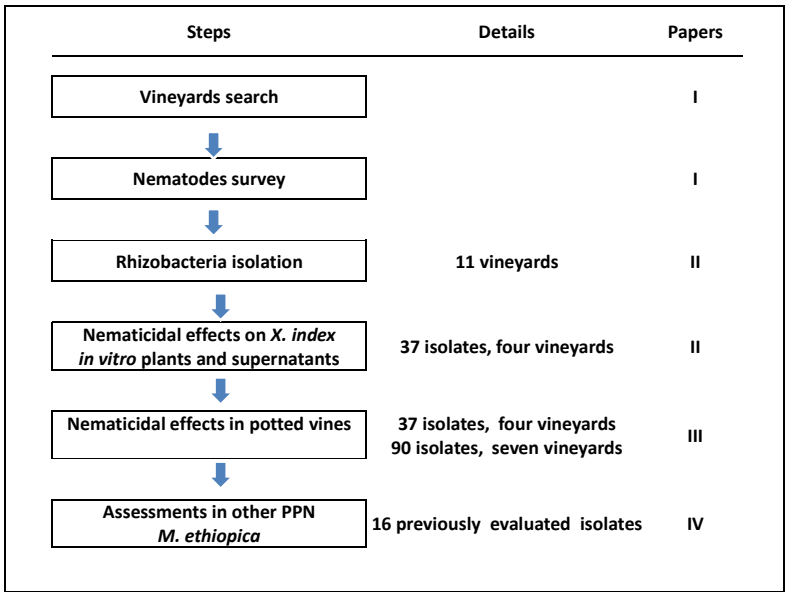

Figure 1. Steps in this thesis with corresponding papers.

2 Importance of PPN in vineyards

2.1 Damage

Vitis vinifera is a crop grown in many countries around the world to produce wine, fresh table grapes and liquors. Like many intensively grown crops, it suffers attacks by different pests and microorganisms, affecting leaves, stems, vines, berries and roots. Plant-parasitic nematodes are an important group of root-affecting agents, which are present in most of the countries where grapes are cultivated under an intensive management system and, remarkably, the most aggressive genera and species are widespread, being frequent in most grape-growing countries (Brown *et al.*, 1993; Mullins *et al.*, 1992). Plant-parasitic nematodes are commonly found in vineyards in all regions of the world and are often associated with areas of low vine vigour (Pinkerton *et al.*, 1999; Ferris & Mckenry, 1975).

The main plant-parasitic nematodes known to be associated with vineyards in Chile are *Meloidogyne* spp. (root-knot nematode), *Tylenchulus semipenetrans* (citrus nematode), *Mesocriconema xenoplax* (ring nematode) and the virus vector nematode (dagger nematode) *Xiphinema index* and other *Xiphinema* species (Valenzuela *et al.*, 1992; Allen *et al.*, 1971). The ectoparasitic nematode *Xiphinema index* is the most important nematode in grapevine in the country, mainly in table grapevine, given its ability to reproduce and reduce plant growth along with transmitting the *grapevine fan leaf virus* (GFLV). *Xiphinema index* is present in an extensive area between the centre-north and centre-south areas of the country, mainly in northern vineyards on light soils. A similar situation occurs with the *X. americanum* group. All *V. vinifera* cultivars are sensitive to this genus, but some, such as

Sultana, Red Globe, Perlette and Superior, show a more intensive growth reduction when some *Xiphinema* sp. is present in high levels.

On the other hand, *Meloidogyne* species, endoparasitic nematodes associated with many agricultural crops, vegetables, fruit trees, ornamentals and weeds, are also very frequently found associated to root systems of grape plants, being especially harmful in wine grape cultivars such as Chardonnay, Cabernet Sauvignon, Merlot and Shiraz. At least six *Meloidogyne* species may be present in Chile, but the most frequently reported is *M. ethiopica* (Carneiro *et al.*, 2007).

Damage to grapevines caused by plant-parasitic nematodes, often in association with plant pathogenic fungi and bacteria (Valenzuela & Aballay, 1996), consists of loss of plant vigour and quality and even plant death in sensitive cultivars such as Chardonnay.

The presence of some important fungal root disease in grapes in California and Chile has been attributed to the presence of nematodes. These include black-foot disease, caused by species of the fungus *Cylindrocarpon* (Scheck *et al.*, 1998).

In Chile, most of the grape cultivars are not grafted since Chile is free from grape aphid (*Daktulosphaira vitifoliae*), which is an important pest of roots in most countries (Mullins *et al.*, 1992). The use of rootstocks is being introduced only recently to overcome problems associated with *e.g.* plant-parasitic nematodes, salty soils and complex replant situations.

Mesocriconema xenoplax and *Tylenchulus semipenetrans* are also frequently reported and may in the future present more restrictions, since most rootstocks are not tolerant to their parasitism (Edwards, 1988), as may other nematode species less frequently found, such as *Pratylenchus vulnus* (Chitambar & Raski, 1984).

2.2 Control methods

The high density of these parasites, particularly in light and irrigated soils, is a permanent problem for farmers and almost all known control methods have been used at some time, *e.g.* shallow, tolerant rootstocks, soil fumigation in replant situations, soil solarisation and others. Application of nematicides prior to planting and in established vineyards may be required to maintain the productivity of vines growing in nematode-infested soils (González, 2007; Pinkerton *et al.*, 1999).

For a number of years, the use of water-soluble chemical nematicides, organophosphates and carbamates, was standard practice in many countries in

situations where soil fumigants were inappropriate or too expensive (Stirling, 1991). Application technologies, *e.g*. through the irrigation system, injection or other mechanisms, were improved and most of the problems caused by plant-parasitic nematodes were thought to be solved with these products.

After 1975, the use of these chemicals began to be more restricted, owing to their adverse effects on the environment, such as groundwater contamination, death of wild birds, high mammalian toxicity, negative effects on human health and the risk of residues in food (Stirling, 1991). Most of the technologically advanced countries have now restricted or banned their use, including fumigants such as methyl bromide and ethylene dibromide (EDB) (Dong & Zhang, 2006) and new control strategies are required (Walker & Stirling, 2008).

However, the use of soil fumigants and non-fumigant nematicides is still a permanent strategy in many countries where the problems caused by nematodes result in significant reductions in the yield and quality of the crops (Walker & Stirling, 2008; Pinkerton *et al*., 1999) and when no other options are successful. Chile has a wide variety of crops growing in a wide diversity of soils and climates, which has contributed to the incidence of significant damage to crops by PPN and the use of chemical alternatives as the main control method.

The concerns about chemical nematicides as regards their significant health and environmental risks mean that research efforts are being increased to find control alternatives. Non-chemical alternatives are always being evaluated, considering the possibilities of large-scale applications, costs and effectiveness. Alternative strategies to control nematodes include crop rotation, resistant varieties and organic amendments (Walker & Stirling, 2008; Rodriguez-Kabana *et al*., 1987). In perennial crops such as vineyards, the plants may last 25 or more years if no extreme disease problem appears or market changes become critical. This means that the possibilities for pest management are restricted to after-planting measures. For example, fresh or composted organic materials are frequently used in vineyards in Chile to improve the development of roots. However, the nematicidal effect of these materials is not very clear and the costs may be as high as those of chemical treatments. Manure releases some organic compounds (*e.g*. butyric and propyonic acid) and ammonia, which may have nematicidal activity (Kaplan & Noe, 1992). However, the results obtained to date have been extremely variable, as the effects of organic amendments on nematode populations depend on the source of the materials, their chemical composition, the elaboration process, the nematode species present and the time of application (McSorley & Gallaher, 1996).

The use of different rootstocks is a good alternative, especially under replant conditions, but is restricted to new plantations. Furthermore, most of these rootstocks are tolerant only to one or two nematode genera and sensitive to the other PPN present in soil and may also display high variability in different fields. Their tolerance may change in different places (Téliz *et al*., 2007; Edwards, 1989) due to the presence of nematode populations that are able to break the resistance (Anwar *et al*., 2002).

2.3 Biological control

An alternative not well evaluated for nematode management in vineyards is the use of microorganisms antagonistic to nematodes. There is a great need for information on nematode interactions with other organisms in soil and particularly the rhizosphere. In soil, there are many interactions between PPN and microbial organisms, some of which act as suppressors. Bacteria and other prokaryotes may parasitise nematodes directly, the most well-known being *Pasteuria penetrans*, which has been reported to affect more than 200 nematode species (Stirling, 1991). Nematode-trapping fungi, such as species of *Arthrobotrys*, can form mycelial structures that destroy nematodes, while other fungi such as *Paecilomyces* spp. can penetrate and destroy eggs, females or cysts (Putten *et al*., 2006).

Some other soil organisms that are antagonistic to nematodes include microartropods, other nematodes and protozoa (Guerena, 2006; Rodríguez-Kábana, 1991).

In spite of all the alternatives, efforts to inoculate soil with microbial species antagonistic to phytonematodes have not been successful for agriculture for many reasons, such as specific host nematode, bad adaptation to different soil conditions or agriculture management (Dong & Zhang, 2006; Compant *et al*., 2005). The addition of organic amendments to soil can stimulate or enhance microbial activities detrimental to plant-parasitic nematodes, *e.g*. the use of chitinous materials (Rodríguez-Kábana, 1991).

Bacteria also play an important role in this association, especially bacteria associated with the rhizosphere (rhizobacteria), which are one of the most abundant microorganisms in the rootzone (Germida *et al*., 1988). Their presence can significantly modify the rhizosphere environment and directly or indirectly affect the nematode or the host-parasite interrelationship, including the antagonistic effect of some genera of rhizobacteria such as *Bacillus* or *Pseudomonas* against fungi, soilborne bacteria and nematodes. As a result, they are referred to as plant growth promoting rhizobacteria (PGPR) (Dong & Zhang, 2006; Compant *et al*., 2005).

2.4 Rhizobacteria and biological control of PPN

The presence of bacteria other than *Pasteuria penetrans* that may have a direct effect on PPN is an important issue to study. Understanding how nematode populations are influenced by their host plants and their associated bacteria is essential to the development of management strategies for nematodes (Garbeva *et al*., 2004).

Increasing attention is being paid to the biocontrol potential of rhizosphere bacteria from several plant species against various plant pathogens, including nematodes and viruses (Zehnder *et al*., 2000; Kloepper *et al*., 1999).

According to Kloepper *et al*. (1992), rhizobacteria are rootzone bacteria that colonise roots in the presence of indigenous soil microflora and can exert beneficial effects on plant development through growth promotion and/or biological control (PGPR).

Most development stages of PPN commonly occur in the rhizosphere, where they may be in intimate contact with their microbial antagonists (Insunza *et al*., 2002; Sikora, 1997). The rhizosphere, the zone of soil around roots, differs from the bulk of soil in its biological and chemical properties and supports much greater microbial activity than the rest of soil, since carbon-rich compounds are more abundant, allowing many more bacteria and fungi to be associated to this thin layer (Compant *et al*., 2009). All PPN are obligate parasites and must enter this habitat to reach their host (Kerry, 2000), which means different interactions in a complex system (Sikora, 1997).

Antagonistic activity of rhizobacteria against several PPN has been demonstrated, mainly endoparasitic nematodes (Burkett-Cadena *et al*., 2008; Mendoza *et al*., 2008; Ali *et al*., 2002). These bacteria have the ability to multiply and spread in the rhizosphere environment, where most of the development stages of nematodes commonly occur, and they may colonise potential infection sites on the root, or they may act by direct contact with the pathogens (Sikora, 1997). Bacteria have been shown to affect nematodes by a variety of mechanisms, including production of specific enzymes, compounds toxic to nematodes, such as ammonia, cyanide, hydrogen sulphide and volatile fatty acids (Kerry, 2000), and/or some antibiotics. Chitinases can attack nematode eggshells, while proteases can harm external structures of nematode eggs or cuticular structures due their protein collagen nature (Kerry, 2000; Rodriguez-Kabana *et al*., 1987).

Some possible modes of action of these bacteria have been demonstrated. These include direct effects on egg hatch and nematode mobility and indirect effects such as alteration of root exudates and induced resistance, which makes roots less attractive.

PGPR strains have repeatedly been reported to reduce the damage caused by a number of fungal plant pathogens (Johansson *et al*., 2003; Weller *et al*., 2000), but fewer studies refer to biocontrol of PPN. Both rhizobacteria and endophytic bacteria seem to have the potential to reduce plant damage due to nematodes, mainly the endoparasitic nematodes, *i.e*. species of *Meloidogyne*, *Heterodera*, *Globodera* and *Pratylenchus* (Mendoza *et al*., 2008; Siddiqui & Mahmood, 1999; Kloepper *et al*., 1991). Only a few investigations have examined ectoparasitic nematodes, *e.g*. *Criconemella xenoplax* (Kluepfel *et al*., 1993) or trichodorids (Insunza *et al*., 2002).

3 Materials and Methods

Within the scope of this thesis work, a number of different studies were performed, from a soil survey to greenhouse experiments.

3.1 Survey of vineyards in Chile

A soil survey was undertaken to cover a wide area cultivated with grapes from semi-arid to temperate climatic regions, comprising about 40,000 km^2 (Paper I).

Soil and root samples were taken from productive vineyards that were at least four years old. Selection of the sampling sites was made on the basis of vine age and grape cultivar, excluding those showing serious root problems due to fungus, bacteria or insect damage.

Sampling was made with a shovel to 25-35 cm depth, in the rootzone of the plants. About 25 subsamples were taken at random to make an approx. 2-kg sample covering up to 4 hectares when the soil was from the same soil series. Each subsample was taken from a different plant, selecting those that were in similar conditions, and that represented the average of the sampled crop. Nematodes were extracted from a 250 cm^3 volume of soil by combining the sieving and decanting method with Baermann's funnel (Hooper & Evans, 1993; Southey, 1986), using sieves of 710, 250, 150 and 45 μm mesh size. For better recovery of adults and the fourth juvenile stage of *Xiphinema* spp., the soil water suspension was sieved through the 750 and 250 μm mesh sieves and then filtered on a nylon sieve of 90 μm mesh size for 24 hours (Brown & Boag, 1988). Genera and species identification and counting were carried out with a dissection microscope (Carl Zeiss, Stemi 2000 C) at 50-90 magnification.

Those vineyards where grapes were grafted onto rootstocks were not considered, since these are not representative of the normal cropping system in Chile. Ten cultivars were considered for nematode evaluations, the five most

commonly cultivated table grapes and the five most commonly cultivated wine grapes.

3.2 Rhizobacteria isolation and identification.

Eleven vineyards were chosen for root sampling, based on low populations of plant-parasitic nematodes or the presence of vine plants with good growth in spite of higher populations of *Xiphinema index*. Plants selected were older than seven years, ungrafted, cultivated under replant conditions and with similar agricultural management.

Roots and soil from different plants were collected with a shovel to a depth of 15-25 cm during summer, including new feeder roots.

During the spring of 2007 four vineyards were sampled and the other seven were surveyed during the following year.

To isolate rhizobacteria, pieces of feeding roots (10 cm length) were separated from the soil, washed, gently crushed on a sterile watch-glass, placed in sterile phosphate buffer saline (PBS) at pH 7.4 and shaken on a rotary shaker at 250 revolutions per minute (rpm) for 30 minutes, to allow extraction of rhizobacteria that inhabit the rhizoplane and endorhizosphere (Kloepper & Beauchamp, 1992). Dilutions of the PBS solution were plated onto half-strength tryptic soy broth agar (TSBA, Oxoid Ltd, UK), and incubated for 48 h in the dark at 22 °C. Pure cultures were subsequently transferred to fresh TSBA medium, grown for 24-48 hours, suspended in sterile freeze medium (8 g nutrient broth in 1000 mL distilled water mixed 1:1 with glycerol solution) and frozen at -80°C until further use. About 50 bacterial isolates from each soil were considered for further bioassays.

For bacterial identification, isolates were grown on TSBA and incubated at +28 ºC for 24 h. Approximately 50 mg fresh weight of cells was harvested and the fatty acid methyl esters (FAMEs) were extracted as described by Sasser (1990). After extraction, FAMEs were separated by a Hewlett Packard 5890 series II gas chromatograph. Individual FAMEs were identified and quantified by the peak-naming table component of the Microbial Identification System (MIS, Microbial ID, US).

3.3 Suppressive activity of the bacterial isolates on *Xiphinema index*

The nematicidal activity of rhizobacteria was assessed by three experimental procedures: 1) Evaluation of the effect of the bacterial filtrates on the survival of mobile stages of nematodes; 2) growth of assays in glasshouses using *in*

vitro plants to determine the plant-rhizobacteria-nematode interaction; and 3) evaluation of the effect of the rhizobacteria under more natural conditions, working with potted grape plants established in substrates made from unsterilised agricultural soil.

Most of the evaluations were performed on the ectoparasitic nematode *X. index*, but considering that under natural conditions plants can also be infested with other plant-parasitic nematodes, a set of assessments was carried out on the endoparasitic nematode *Meloidogyne ethiopica*, also an important pest in vineyards.

3.4 Culture filtrates

This activity was performed with the rhizobacteria isolated from the first four of the 11 vineyard soils sampled. Isolates were cultivated on half-strength TSBA (15 g/L), inoculated into Erlenmeyer flasks containing 50 mL sterile half-strength TSB and grown at 22 ºC with rotary shaking at 180 rpm for 48 h. Cell suspension concentration of all isolates was adjusted to 10^6 CFU/mL.

After two times centrifugation at 4000 rpm for 20 minutes, the supernatant was collected. Aliquots of 0.5 mL of nematode suspension in sterile tap water containing 50 *X. index* were placed into sterile glass Petri plates of 32 mm diameter with 1.5 mL of culture supernatant. These plates were maintained at 22-23 ºC and the number of dead-like nematodes was counted for each treatment under dissecting microscope at 16-18 h, *i.e.* enough time to evaluate mortality. Dead-like nematodes were those that remained immobile when gently and repeatedly touched with a needle. Percentage mortality was calculated for each replicate.

To verify the nematostatic and nematoxic effect of the culture supernatants, at the end of the exposure time the immobile nematodes were transferred to sterile tap water for 48 h, to observe whether recovery occurred.

3.5 Tests using *in vitro* plants

In order to assess whether the set of rhizobacteria isolated from the first four soils had any effect on *X. index* populations or infections and on the growth of grapes, a greenhouse assay using *in vitro* grape plants was performed using 37 isolates.

Excised new shoot tips of virus-free grape plants, cv. Thompson Seedless, were selected and established *in vitro* on Murashige and Skoog medium (Murashige & Skoog, 1962). After *in vitro* shoot production, multiplication and rooting phases, propagules were selected to be transferred to peat-moss and

perlite substrate, moistened with half-strength autoclaved Hoagland nutrient solution and grown in a 500-mL plastic pots.

After four to six weeks, when the plants had a developed root system, they were transferred to a substrate adequate for nematode activity, which consisted of a sterile mixture of sand and loamy soil (3:1 v/v).

After 14 days, bacterisation of the grape plants was performed. For this, selected rhizobacterial isolates maintained at -80 ºC were streaked to multiply in half-strength TSB and incubated for 24 h on a rotary shaker (160 rpm). After incubation, the bacteria were pelleted (15 minutes at 3000 × g). Pellets were washed twice, suspended in an isotonic solution of $MgSO_4$ (Johansson *et al*., 2003) and adjusted to a final concentration of 10^6 CFU/mL (Kluepfel *et al*., 1993). Grapevine plants were removed from the pots and their root system was washed with sterile distilled water and immersed for 20 minutes in the bacterial suspension.

After inoculation, the vines were planted in 500-mL plastic pots containing fresh sterile substrate and 50 mL of bacterial inoculum were added to the soil in each pot, around the root zone, as suggested by Insunza *et al*. (2002) and Kluepfel *et al*. (1993). Fourteen days after bacterisation, the soil was inoculated with approx. 400 specimens of *X. index*, 70% adult females and 30% juveniles of different stages, extracted from the roots of *Ficus carica* by the Cobb's sieving and decanting method, modified according to Brown & Boag (1988). Two controls were used, a set of non-bacterised plants infested with nematodes and a set of plants free from bacteria and nematodes.

After 16 weeks, the *X. index* population, vegetative growth and root damage were recorded.

3.6 Experiments with potted plants

This set of experiments was developed to evaluate the effect of rhizobacteria in the protection of roots of plants grown under more natural soil conditions against plant-parasitic nematodes.

The bacterial isolates were prepared as described previously and were separated into two groups, according to information obtained in the previous studies. The first group comprised 49 rhizobacterial isolates from sites 1-4, most of them previously used in the other experiments. Two experiments were replicated in two separate seasons, in spring 2008 and spring 2009 (assays 1 and 2, respectively). Each assay lasted one growing season, comprising 6 months of growth until the plants entered dormancy.

The second group comprised 90 isolates from grapes growing in the remaining seven vineyards (soils 5-11) not previously assessed. With these

bacteria, only one experiment was carried out, but lasting two seasons. Plants were inoculated with these bacteria at the same time as assay 2 on the first group.

The plant material used consisted of Thompson Seedless grape plants obtained by propagation of cuttings from virus-free vines, in a steamed growth medium consisting of 50% sand and 50% peat moss by volume. Two-month-old plants were used for the greenhouse assays.

For bacterial inoculations, plants were removed from the propagation medium and the root system was soaked for 20 minutes in bacterial suspension. The plants were immediately replanted in new growth medium using 3-L pots filled with a steamed mixture of sand, loamy field soil and composted organic matter (2:1:1 by volume). An additional 100 mL of the bacterial suspension was added to the growth substrate.

On day 14 after bacterial inoculation, the soil was infested with specimens of *X. index* by pipetting the nematodes suspended in sterile tap water into the rootzone. For assay 1 on the first group of bacteria, 200 nematodes per pot were added, while for assay 2 and the second group of bacteria 400 nematodes per pot were added.

Once inoculated, plants were grown in a shaded 10 m x 20 m greenhouse covered by a rashell mesh, which intercepted 30% of sunlight and prevented overheating of plants and pots. The maximum and minimum temperature outside the greenhouse in mid-summer was approx. 34 °C and 15 °C, respectively, and that within the greenhouse was 28 °C and 15 °C, respectively.

The effect of the isolates was evaluated at the end of the growing season, in early autumn, by determining nematode populations and damage associated with nematode feeding.

3.7 Effects of rhizobacteria on *Meloidogyne ethiopica*

A set of 16 rhizobacterial isolates previously used in experiments to assess their effect on parasitism by *X. index* were evaluated. The first experiment was performed to determine the effect of culture filtrates prepared as indicated previously, on hatching of *M. ethiopica* eggs.

Nematode eggs were extracted from grapevine roots infested with *M. ethiopica* according to the method described by Hussey & Barker (1973). Then 0.5 mL aliquots of suspension in sterile tap water containing approximately 50 eggs, with about 30% containing second-stage juveniles (J2) and 70% different embryonic stages, were placed in 35-mm diameter sterile glass Petri dishes. A 2-mL portion of culture filtrate was added to each plate and all plates were kept at 26 ºC for 24 hours (Siddiqui *et al*., 2007). Hatching was determined by

counting the second stage juveniles hatched in a Baermann funnel over a period of 10 days, with counts every two days. Two control treatments were used, TSB and the chemical organophosphate nematicide fenamiphos (1.5 µL/ml water).

The effect of bacteria on nematode parasitism was assessed in plants of cv. Chardonnay obtained from grapevine cuttings and rooted in steamed substrate. The bacterial inoculum was prepared as indicated previously for the assay experiments with *X. index*.

Two-month-old plants, with two leaves, were removed, washed with sterile water and the roots soaked in bacterial suspension for 20 minutes. Inoculated plants were planted in 3-L pots filled with a sterile substrate composed of sand:agricultural soil in proportions of 2:1. An additional volume of 100 mL per pot of the bacterial suspension was added to each pot.

Fifteen days after bacterial inoculation, 1000 eggs of *M. ethiopica* were applied per pot.

Once inoculated, plants were grown in a shaded 10 m x 20 m greenhouse, in the same way as plants inoculated with *X. index*.

Three controls were used, a chemical nematicide fenamiphos (0.5 mL/pot), a solution containing only the nematodes, and an isotonic solution.

The treatments were evaluated six months after inoculation, once plants had entered dormancy. Numbers of galls, eggs and second-stage juveniles were recorded, as well as fresh weight of aerial parts and roots. Soil was processed according to the soil sieving and Baermann funnel method, using 250 cm^3 of the substrate for J2 extraction (Christie & Perry, 1951).

3.8 Experimental design and statistical analysis

For Paper I, most of the information was analysed with descriptive statistics, which were useful for observations of nematode distribution and nematodes densities. The relationship between nematodes per taxon and associations between them without or with environmental influences were determined by correspondence analysis (CA) and canonical correspondence analysis (CAA), respectively, using CANOCO software 4.5 (Leps & Smilahuer, 1999). For Paper II, multivariant analysis was performed to determine relationships between rhizobacteria and environmental variables associated to grape root growth, through redundancy analysis (RDA).

For greenhouse experiments and *in vitro* tests (Papers II, III and IV), rhizobacteria isolates were compared with two or three controls distributed according to a completely randomised design. The data obtained were subjected to one-way analysis of variances (ANOVA) and treatments were

compared with Dunnett's test at $p<0.05$ or $p<0.01$ depending on the experiment using Minitab Statistical Software for Windows, release 13.

When necessary, nematode numbers were transformed to log (x+1) prior to application of multivariate analysis (Paper I). Prior to Dunnett's test, the data were transformed to arcsine of percentage data (Papers II and IV).

4 Results and discussions

4.1 Distribution of plant-parasitic nematodes in Chilean vineyards (Paper I)

The survey carried out covered 1818 soil samples and 12 nematode genera were extracted, but only four of these were considered be highly pathogenic to the root system of *Vitis* in Chile (Table 1). The most frequent genera occurring in large populations were *Xiphinema* (*X. index*, *X. americanum sensu lato*), *Meloidogyne* (three species, with *M. ethiopica* the most frequent), *Mesocriconema* (*M. xenoplax*) and *Tylenchulus* (*T. semipenetrans*). Species of *Xiphinema* were present in 71% of the sampled area and none of the samples was free of PPN.

The citrus nematode, *Tylenchulus semipenetrans*, had been detected in previous surveys, mainly in grapes following citrus (Aballay & Navarro, 2005). Based on recent reports of *Meloidogyne* spp., the populations detected correspond to *M. ethiopica* (Carneiro *et al*., 2007; Carneiro *et al*., 2003), a species that in the past was misidentified as *M. incognita*. This has caused confusion in the choice of cultivars, with some of them having been selected because they were known to be tolerant to *M. incognita*. *Mesocriconema xenoplax* was abundant and its importance is increasing in Chile. Currently the use of nematicides is the only method used to control this nematode, as no rootstock has been reported to be tolerant or resistant to it in Chile.

The genus *Xiphinema* is widespread and has become the main root pest of grapes, as it is represented by *X. index* and *X. americanum sensu lato* which are known to transmit the *grape fan leaf virus* (GFLV) and *tomato ring spot virus* (TomRSV) respectively, both present in Chile (Auger *et al*., 1992). This is of concern for farmers and nurserymen as regards the implementation of a virus–free plant production programme. Only 29% of the fields studied in our survey were free from *Xiphinema* spp. In most cases these fields were in new

production areas. Several samples with a low number of specimens were from areas with previous old vineyards, replanted with different cultivars of *V. vinifera* and in which no nematode increase or symptoms of replant problems were observed (McKenry, 1999), which may be due to some biotic or abiotic factor (Kerry, 2000).

The population density of a determined taxon was fairly variable, as shown by the parameters determined (Table 1). At least one of the species was present in high density in every sample. Maximum soil population densities per species indicate the possible maximum infestation degree, which is even more complicated considering that the vine may support more than one type of parasitism.

The CCA between cultivars and nematode taxa showed a narrower relationship between *Meloidogyne* spp. and wine grape cultivars, mainly with the cvs. Chardonnay and Cabernet Sauvignon. Meanwhile *Xiphinema* spp., *M. xenoplax* and *T. semipenetrans* were more associated with table grape cultivars. Soil texture was not a strong environmental vector, with a low influence over nematode distribution, meaning that most genera showed a weak association with it (Paper I).

The influence of the soil environment on the dynamics of plant feeders is considered the second most important factor after the host plant (Cadet *et al*., 2004; Norton, 1989). However, in Paper I there was only a low influence of soil texture over nematode populations, as high nematode population densities were detected in both clayey and sandy soils. Some soil management activities that affect soil porosity, such as the wide use of agricultural lime (Ca_2SO_4) or activities such as tillage, also produce a looser soil, increasing the soil pore spaces.

Cultivar had the largest influence on the size of nematode populations, since all cultivars were sensitive to nematode infections. However, there was a narrower association between *Meloidogyne* spp. and two wine cultivars and also between *Xiphinema* spp., *M. xenoplax* and *T. semipenetrans* and table grape cultivars.

The explanatory variables used were not able to explain most of the nematode incidence in samples. Thus other factors must determine the presence of plant-feeding nematodes and identification of these factors is necessary before new management programmes can be devised.

Table 1. *Population densities and statistical parameters for the five main taxa of plant-parasitic nematodes per 250 cm^3 soil associated with Vitis vinifera* L. *along the major productive zone in Chile (n = 1818)*

Parameter	*X. index*	*X. americanum s.l.*	*Meloidogyne* spp.(J2)*	*Mesocriconema xenoplax*	*Tylenchulus semipenetrans* (J2)
Mean	160	67	149	49	266
S.E.	380	213	466	144	2224
Range	0-3850	0-3780	0-6816	0-1860	0-41350
Infested samples (%)	48	48	45	49	13

* *Meloidogyne ethiopica* was the most abundant species.
J2, second stage juvenile.

4.2 Screening of rhizosphere bacteria from grapevine for their suppressive effect on *Xiphinema index* on grape plants grown *in vitro* (Paper II)

4.2.1 Bacterial isolated from grape roots, identification and distribution

More than 400 bacterial isolates were obtained from the 11 grapevine rhizosphere sites. Isolates with a Similarity Index (SI) lower than 0.5 were considered not reliably identified at genus level (Weller *et al.*, 2000), while 209 isolates were correctly identified and belonged to 25 different genera, comprising endophytic and epiphytic rhizobacteria, considering the way the roots were processed for the bacterial isolation The most commonly occurring genera were *Pseudomonas* (n=104) and *Bacillus* (n=29), accounting for about 49.8 and 13.9% of the root-associated populations identified, respectively.

The isolates with SI higher than 0.7 were considered correctly identified at species level according to Weller *et al.* (2000) and are presented in Table 2 in Paper II. The most frequent were *Pseudomonas putida* (35.1%) and *Pseudomonas fluorescens* (6.1%). Human harmful bacteria like *Escherichia coli* were also frequently (7.6%) found. These bacteria were however not considered as potential bio control agents and not included in the succeeding tests. The composition of the microbial communities showed that some species were strongly correlated to the origin of the irrigation water, *e*.g. *Pantoea agglomerans* and *E. coli* were found only in roots irrigated with river water, which contains more organic matter than the underground water from deep wells.

Fatty acid content is reported to be a reliable method that allows accurate differentiation at species, subspecies and sometimes biovar and pathovar level (Scortichini *et al.*, 2005; Weller *et al.*, 2000; Farag *et al.*, 1999; Persson & Sletten, 1995), although pathovars in some species, *e.g. P. syringae*, may not

be well differentiated (Weller *et al*., 2000). This means that in the future, pathovar identification must be confirmed with other methods.

4.2.2 Assessment of suppressive activity of rhizobacteria against *Xiphinema index*

The first screening was performed with some isolates selected from the first four soils sampled. Grapes produced *in vitro* inoculated with the isolates *Stenotrophomonas* sp. 158, *Bacillus brevis* 37 and *Comamonans acidovorans* 49 had significantly higher shoot weights than control plants infested only with nematodes. The same bacterial isolates, plus *Pseudomonas putida* isolate 139, also significantly increased root weight of inoculated plants compared with control plants not inoculated with the nematode.

Considering root damage, most of the plants inoculated with bacteria showed lower levels of nematode damage and the observed differences were highly significant ($p<0.01$) with the isolates *B. brevis* 200, *Bacillus cereus* 146, *Bacillus megaterium* 185, *Pseudomonas corrugata* 216, *P. savastanoi* pv. *fraxinus* 86, *P. syringae pv. syringae* 199 and *Serratia plymuthica* 213. Significant differences ($p<0.05$) were also observed with the isolates *B. brevis* 37, *B. megaterium* 133, *Pantoea agglomerans* 3600, *P. syringae glicinae* 30 and *Stenotrophomonas maltophilia* 168.

On immersion of nematodes in two assays with the culture filtrate, most of the bacteria exuded or released some elements or substances that caused the death of the nematodes (Table 4 in Paper II), confirming results obtained with *in vitro* plants (Table 3 in Paper II). Considering both assays with the supernatants, 19 isolates showed mortality levels of up to 50%, of which *B. cereus* 146, *P. agglomerans* 3600, *P. corrugata* 216, *P. savastanoi* 86, *P. syringae* 199 and *S. plymuthica* 213 also caused low damage to roots.

Final nematode populations showed a variation greater than that of the root damage and ranged from 78 to more than 3300 nematode specimens per pot (Table 3 in Paper II). Nematode populations in soils of plants bacterised with the isolates *B. megaterium* 185, *P. corrugata* 216, *P. savastanoi* pv. *fraxinus* 86 and *S. plymuthica* 213 were significantly lower ($p<0.01$) than those in the control, whereas *B. brevis* isolate 200 and *S. maltophilia* isolate 168 reduced nematode population density ($p<0.05$).

Few studies have been performed on grapevine and none with rhizobacteria isolated from grapevine roots except those by Barka *et al*. (2000) and Kose *et al*. (2003). Those authors worked on grapes with bacteria isolated from other sources to promote growth and rooting of plants. The plant response to rhizobacteria assessed in Paper II indicates that certain bacterial isolates have growth stimulating activity. The isolates *B. brevis* 37, *C. acidovorans* 49 and

Stenotrophomonas sp. 158 increased both shoot and root weight, while *P. putida* isolate 139 increased only root weight. Several mechanisms have been reported through which bacteria may increase growth of plants, independently of disease control. These include fixing atmospheric nitrogen, synthesis of hormones (indoleacetic acid) or enzymes that degrade ethylene precursor, 1-aminocyclopropane-1-carboxylate (ACC), among others (Glick *et al.*, 1998).

In this investigation, the stimulation of plant growth was not directly associated with reduced infection by *X. index*, as only with *B. brevis* isolate 37 was plant growth stimulation accompanied by significantly low levels of root damage (Table 3 in Paper II). In addition, a decrease in nematode populations or root damage did not significantly increase the fresh weight of either roots or canopy, except in the case of the bacterial isolate *B. brevis* 37. Nonetheless, after 4 months of plant growth, most of the growth variables of bacterised plants were larger than those of non-bacterised control plants infested with nematodes.

Most of the rhizobacteria evaluated were associated with a certain level of decrease in the population of *X. index*, although nematode populations were significantly different from the control for only six of these. Four of these rhizobacteria showed significantly lower nematode populations than the control ($p<0.01$), thus suggesting that the effect on nematodes is due to bacterial presence. However, most of the other rhizobacteria, for which the observed differences were not significant, also reduced numbers of galls on the roots compared with the control. This may suggest a certain nematostatic rather than nematoxic effect of the rhizobacteria.

The data on the supernatants help to explain some of the effects seen in the assay with the potted *in vitro* plants. Some isolates showed low damage to roots and also low numbers of nematodes, *e.g. B. megaterium* 185 (Table 4 in Paper II). Nevertheless, the mortality rate of *X. index* caused by filtrates was less than 50%, which would be an indicator of a low short-term lethal effect and some long-term nematostatic effect occurring under sublethal concentrations.

Stenotrophomonas maltophilia isolates have previously been reported to be an antagonist of ectoparasitic nematodes of the family Trichodoridae (Insunza *et al.*, 2002) and of the pine wood nematode *Bursaphelenchus xylophilus* (Gu *et al.*, 2007).

In Paper II, *S. maltophilia* isolate 168 reduced root damage by *X. index* and population size, while *S. plymuthica* isolate 213 also showed suppression of *X. index* ($p<0.01$). These data on the interaction of *Stenotrophomonas* spp. with plant-parasitic nematodes are a novel finding.

4.3 Assessment of rhizobacteria from grapevine for their suppressive effect on *Xiphinema index* (Paper III)

The use of potted plants was valuable in evaluating the performance of rhizobacteria under more natural conditions, working with agricultural soil.

Of the 139 isolates bacteria assessed, *i.e*. from soils 1-4 (group 1) and 5-11 (group 2), 17 isolates (12%) were shown to effectively decrease damage to the root system caused by the nematodes (Tables 1 and 2 in Paper III).

The damage level observed during assays 1 and 2 for the first set of bacteria, which were carried out in different seasons, showed that some of the isolates were able to reduce root damage to the plants. In assay 1, 23 isolates resulted in less damage to the root system than the control inoculated with *X. index* ($p<0.05$), while 11 isolates had this effect in assay 2.

The greater number of isolates showing efficacy against the parasitic activity of the nematode in assay 1 (23) compared with assay 2 (11) is most likely due to the lower initial population in assay 1, which also resulted in a lower level of damage (Table 1 in Paper III). The initial population may be particularly important for the degree of damage, considering that only a few nematodes feeding on a root apex can destroy it and cause the typical gall symptoms to appear (Wyss, 1978). Under field conditions, the fact that a more extensive and rapid galling response occurs when more nematodes are present is most likely one of the reasons why some chemical and biological nematicides show varying results in the control of root damage.

Considering the effects of the bacterial isolates of the first set during assays 1 and 2, seven were found to be effective in decreasing the damage in both studies ($p<0.05$); *Bacillus brevis* isolate 37, *B. megaterium* 69 and 133, *Cytophaga johnsonae* 135, *Pseudomonas fluorescens* 55, *Rahnella aquatilis* 203 and *Stenotrophomonas maltophilia* 66. The remaining isolates showed more variability between assays, with some being different from the control in assay 1 but not in assay 2, or *vice versa*. Furthermore, some isolates did not induce any decrease in the damage level in either season, despite the fact that other isolates from the same species were fairly effective in both assays. This finding, which was observed in isolates such as *B. brevis* 200 and *B. megaterium* 185, indicates that important differences can occur between isolates obtained from the same soils.

Studies on the second group of rhizobacteria, from soils 5-11, showed that some of these isolates had good activity in suppressing the parasitic activity of *X. index*. Ten isolates resulted in gall numbers that were significantly different from the control. Most of the isolates caused a lower number of galls/g root than the control plants, although for some their population increases were greater, *e.g*. *Agrobacterium radiobacter* 617, *B. brevis* 716 and *Burkholderia*

cepacia 526. In this assay too, different isolates of the same species resulted in different degrees of root damage, *e.g*. *Bacillus mycoides* 820 and 603 and *Bacillus pumilus* 502 and 1005 (Table 2 in Paper III).

Most of the plant roots treated with isolates of *Pseudomonas putida* (7 of a total of 9), showed a lower damage index than the control.

Among the 10 most effective isolates, *Variovorax paradoxus* 1105 and *Pseudomonas pseudoalcaligenes* 515 showed the lowest numbers of galls/g root. The other eight effective isolates were *B. mycoides* 820 and 530, *Bacillus sphaericus 925*, *B. thuringiensis 833*, *Curtobacterium flaccumfaciens 1115*, *Pseudomonas putida* 805, *Pseudomonas alcaligenes* 635 and *P. viridiflava* 1020.

A total of eight isolates, including two isolates of *Agrobacterium radiobacter* and *Flavobacterium odoratum*, showed lower root growth than the control in spite of the fact the number of galls was not significantly different from the control. With the exception of *P. alcaligenes* 635, not all of the isolates that were able to reduce the level of root damage caused by *X. index* showed significant differences in root growth compared with the control.

Among the seven isolates from the first group shown to be able to decrease root damage significantly in potted plants (Table 1 in Paper III), only *B. brevis* 37 and *B. megaterium* 133 displayed good nematode control and also reduced root damage in Paper II. These isolates therefore had good activity under different sets of conditions, *i.e*. in *in vitro* tests using rhizobacteria supernatants, in small pots with *in vitro* plants and with large pots using a mixture of field soil and plants from cuttings.

Considering the 17 isolates from both groups of bacteria that were effective in decreasing root damage, seven were isolates of *Bacillus* and five of *Pseudomonas*. This confirms findings by Tian *et al.* (2007) and Siddiqui *et al.* (2005), who concluded that *Bacillus* and *Pseudomonas* are the main genera opposing nematodes in the rhizosphere. Their action is probably linked to increased chitinase and peroxidase activity or the production of secondary metabolites or cuticle-degrading proteases, *i.e*. serine proteases (Lian *et al.*, 2007). According to Huang *et al*. (2010), some strains of *B. megaterium* produce nematicidal volatiles, which are active against juveniles and inhibit the hatch of eggs of *Meloidogyne incognita*. Most previous studies referring to the modes of action of these bacteria point out that these are associated with a direct nematotoxic effect, rather than a nematostatic effect inhibiting feeding, movement or reproduction (Huang *et al.*, 2010; Gu *et al.*, 2007; Lian *et al.*, 2007).

Stenotrophomonas maltophilia has previously been reported to be an antagonist of PPN and in Paper II *S. maltophilia* isolate 168 was found to

reduce root damage by *X. index in vitro*. It was also able to reduce the root damage to the potted plants in field soil in Paper III.

Three strains of *V. paradoxus* were evaluated in Paper III and *V. paradoxus* 1105 was found to be effective in suppressing damage to the vine root system (Table 2 in Paper III). In studies with *Heterodera schachtii*, *V. paradoxus* inhibited hatching of juveniles *in vitro* by 100%, but had little effect on J2 infection of mustard roots (Neipp & Becker, 1999).

In Paper III, the bacterial isolates tested did not prove to have an important effect in stimulating root growth. For the first group of bacteria (soils 1- 4), there were no differences between any of the isolates and the controls (data not shown), while for the second group (soils 5-11), some strains showed differences from the control but these were negative, *i.e*. eight of the isolates reduced root growth significantly (Table 2 in Paper III), particularly *F. odoratum* 827. In Paper II, of the 37 isolates used in assays 1 and 2, a few showed growth-stimulating activity, but that study was shorter and was performed with *in vitro* plants under more controlled conditions.

4.4 Rhizobacteria performance under the three assessments conditions

In all studies with the first group of bacteria (soils 1- 4) using adult and J4 specimens of *X. index*, 89% of the bacterial isolates killed the nematodes after 16 hours of exposure to supernatant. Overall, 32% of the strains were effective in protecting the roots when inoculated into the *in vitro*-produced plants in small pots with sterile substrate and 16% were able to decrease the damage to the potted plants grown for 6 months in field soils. This means that under more natural conditions, their effects may be altered by the soil, environment and agricultural practices, which might affect root colonisation (Figure 2).

The seven isolates from the first group of bacteria and the 10 from the second group obtained under potted conditions form a good set of microorganisms to be evaluated in the next step, field studies in productive vineyards experiencing nematode attacks, mainly by *X. index*. *Bacillus brevis* 37 and *B.megaterium* 133 showed nematicidal activity in all our different experiments, from *in vitro* tests to field soils, and are thus suitable material for further studies.

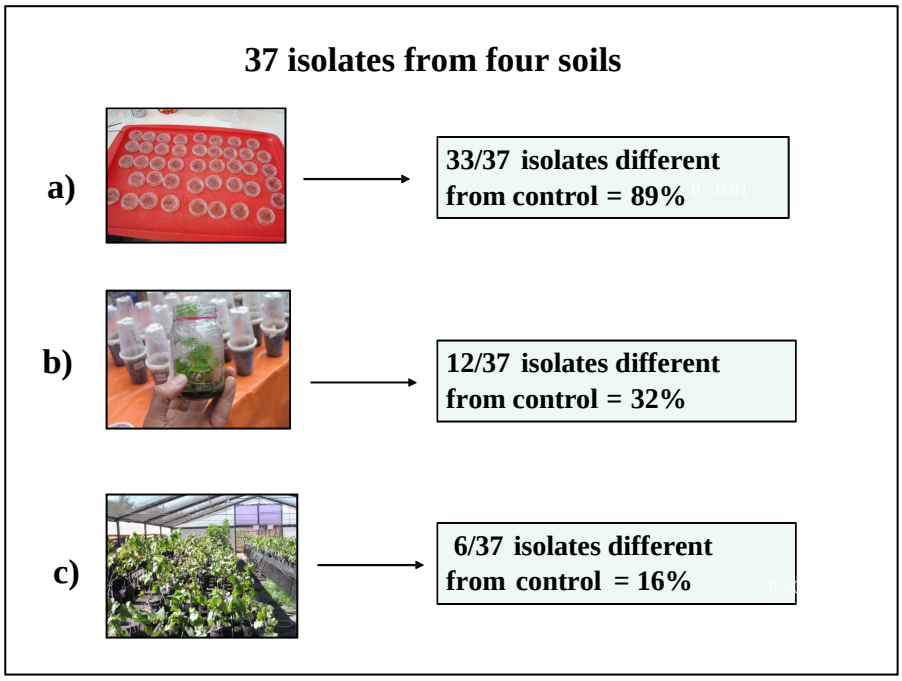

Figure 2. Decrease in percentages of mortality of *X. index* tested under three different assessment systems. a) *in vitro*, b) sterilized substrate-*in vitro* plants c) agriculture soil-potted plants.

4.5 Effects of rhizobacteria on parasitism by *Meloidogyne ethiopica* on grapevines (Paper IV)

The 16 isolates tested previously against *X. index* were also evaluated for *M. ethiopica*, considering that in vineyards various nematode genera normally parasitise crop roots at the same time.

Meloidogyne ethiopica is an endoparasitic nematode that has most of its cycle life within the root of the host. The eggs are protected by a gelatinous matrix produced by the adult females and when they hatch, the second stage juveniles (J2) are released in the soil and start searching for new roots to enter and complete the life cycle. This is different from the life cycle of *X. index*, which has all its stages (eggs, four juvenile stages (J1, J2, J3, J4) and the adults) in the soil, while for *Meloidogyne* species, only J2 and some few adult males are in the soil (Sasser & Carter, 1985).

The effects of rhizobacteria on parasitism by *M. ethiopica* on grapevine roots and its reproduction in inoculated plants were evaluated after 6 months of incubation. For most of the parameters evaluated, plants treated with

rhizobacteria had a lower degree of damage or fewer eggs and juveniles compared with the controls.

In potted plants, all the bacterial isolates gave a lower number of galls than the control plants infected with nematodes and eight were significantly different ($p<0.05$) (Table 1 in Paper IV). However, this was not reflected in the number of eggs present in the roots or juveniles in soil, since of the eight more effective rhizobacteria isolates, only *Serratia marcescens* 6 also gave a significantly different number of eggs and juveniles compared with control plants.

Seven of the bacterial isolates tested had an effect on parasitism and on reproduction of the nematode in terms of either production of eggs or juveniles (Table 1 in Paper IV). Of these isolates, *S. marcescens* 6 was the most effective, showing good activity as regards number of galls, eggs and juveniles. The other effective isolates were *Comamonans acidovorans* 49, *Pantoea agglomerans* 54, *Sphingobacterium spiritivorum* 64, *Bacillus mycoides* 83, *Alcaligenes piechaudii* 97 and *Serratia plymuthica* 213.

The effect of the isolates in decreasing parasitism by the nematodes was not directly associated with positive effects on plant growth, since only two isolates, *P. agglomerans* 54 and *Pseudomonas savastanoi* 176, gave plants that had significantly greater root weight than plants inoculated with nematodes only (Table 2 in Paper IV).

Studies of culture filtrates on hatching showed that all rhizobacteria isolates were effective after 24 hours of immersion compared with TSB ($p<0.05$) (Table 3 in Paper IV). The lowest hatching rate was observed for eggs in culture filtrates of isolates of *Pseudomonas putida* 188 (14.3%), *Bacillus megaterium* 69 (16.9%), *Bacillus pumilus* 72 (20.6%) and *Pseudomonas fluorescens* 144 (25.4), all of which were as effective as the chemical control, fenamiphos ($p<0.05$).

Similar hatching results have been obtained in other studies, but with two or three days of immersion (Mendoza *et al.*, 2008; Ali *et al.*, 2002).

The inhibition of egg hatching observed in Paper IV may be caused by secondary metabolites produced by the rhizobacteria, which may result in egg lysis and affect egg viability (Abo-Elyousr *et al.*, 2010; Mendoza *et al.*, 2008; Neipp & Becker, 1999; Westcott & Kluepfel, 1993). Siddiqui *et al*. (2005) and Siddiqui & Shaukat (2003) reported that some metabolites such as 2,4 diacetylphloroglucinol (DAPG) and cyanhydric acid produced by *Pseudomonas spp.* inhibit hatching of *Meloidogyne javanica* and *M. incognita*.

The inhibitory effect of the rhizobacteria on hatching of nematode eggs was not related to inhibition of parasitism in plants, even with the two most effective strains, *P. putida* 188 and *B. megaterium* 69, or the least effective, *S.*

marcescens 6, which showed completely different results when inoculated onto roots of vines. This means that the effects observed *in vitro* do not necessarily reflect the effectiveness of a bioantagonist and that in a long-term assay (such as 6 months in this study), many other factors influence the biocontrol activity.

Evaluation of the strains in terms of vine growth showed that plants inoculated with *P. agglomerans* 54, *P. savastanoi* 176 and *B. megaterium* 69 had greater root weight (Table 2 in Paper IV), despite these strains not showing good antagonistic activity in potted plants. Some mechanisms such as fixation of atmospheric nitrogen, synthesis of hormones (IAA) and antibiotic production have been suggested to explain this (Rokhzadi *et al.*, 2008; Rives *et al.*, 2007; Asghar *et al.*, 2002; Glick *et al.*, 1998).

The species *B. megaterium* has been reported to produce metabolites and potentially to be a good candidate for biological control of nematodes (Oliveira *et al.*, 2007; Neipp & Becker, 1999). Huang *et al*. (2010) reported several nematicidal volatiles and an antagonistic effect on *M. incognita* infection, especially with inoculum concentrations between $1x10^7$ and $1x\ 10^9$ CFU/mL, much higher than in Paper IV, which could explain the difference in effect on potted plants. It has also been reported that strains of this bacteria can effectively promote plant growth by phosphate solubilisation or alter the root system through induction of auxin and ethylene formation (López-Bucio *et al.*, 2007).

The seven rhizobacterial isolates which gave a significant effect in controlling *M. ethiopica* in Paper IV gave different results in the study on the ectoparasitic nematode *X. index* (Paper II). There, most of the isolates showed nematicidal activity when culture filtrates were evaluated in Petri dishes, but only *S. plymuthica* 213 showed a good suppressive effect when *in vitro* plants were inoculated with *X. index* and bacteria.

In most previous studies on eggs or juveniles of *Meloidogyne* spp., the number of rhizobacteria strains able to kill nematodes is much larger *in vitro* than the number of strains with similar activity when inoculated into pots or in field assays (Huang *et al.*, 2010; Oliveira *et al.*, 2007; Becker *et al.*, 1988). In Paper IV, the 16 bacterial isolates tested were all significantly different from the control in the *in vitro* test on hatching rates, and seven showed a significant effect in decreasing the number of galls or nematode reproduction rate. This means that about 44% of the strains maintained their nematicidal activity when applied to potted plants, a high proportion by previous standards (Mekete *et al.*, 2009). The reason for this higher proportion may be that the strains used in this study were isolated from grapevine roots and selected from previous studies.

The effect of rhizobacteria on populations of nematodes from the genus *Meloidogyne* and their impact in limiting nematode damage and improving

growth of different crops has been studied previously. However, such studies have mainly focused on interactions with *M. incognita* (Huang *et al.*, 2010; Kokalis-Burelle *et al.*, 2002; Becker *et al.*, 1988), *M. javanica* (Siddiqui *et al.*, 2007; Ali *et al.*, 2002) and a few others, such as *M. exigua* (Oliveira *et al.*, 2007). To the best of my knowledge, this is the first assessment of the effects of rhizobacteria on *M. ethiopica*. The results obtained in Paper IV indicate that the effects of rhizobacteria treatment on *M. ethiopica* are similar to, or better than, those on other species.

Considering the different parasitism habits of *M. ethiopica* and *X. index*, it must be considered that with the former the most exposed stage to the presence of toxic elements is the J2 stage, while all the stages of the latter are exposed to elements exuded by microorganisms. Thus, once J2 of *M. ethiopica* enters the root, it is more protected by the root tissues (Sasser & Carter, 1985), unless some endophytic rhizobacteria have an effect on the other parasitic stages (J3, J4 and adults), which has not been reported to date.

It has been reported that different genera of PPN show different sensitivity to a same chemical active nematicide (Bunt, 1975), which may mean that they also react in a different way to other elements in the soil.

The different parasitism performance of PPN may also be associated with differences in their cuticle structure or composition, *e*.g. type of collagens or soluble cuticle proteins associated with the outer coat (Blaxter & Robertson, 1998). *Meloidogyne incognita* has collagen-like proteins distributed through the entire cuticle (Spiegel *et al.*, 1995), but in *X. index* the structure of the collagens is not known (Blaxter & Robertson, 1998). There are also differences between the species in terms of other epicuticle components, such as carbohydrate recognition domains, which means that they may contain components with a different collagen sequence, no collagenous domains, or collagens masked by other components (Spiegel *et al.*, 1995). Other studies have compared the nematicidal activity of exudates from a particular rhizobacterial isolate on two different nematodes species, *Panagrellus redivivus* and *Bursaphelenchus xylophilus*, and completely different answers have been obtained (Gu *et al.*, 2007). This selectivity must be considered in strategies for effective use of rhizobacterial isolates in control programmes.

5 Main findings, conclusions and future perspectives

According to the results obtained in this thesis, it may be concluded that in fields cultivated with vines under low nematode pressures, it is possible to find microorganisms able to play a role in limiting nematode growth and damage to roots caused by PPN.

Some of the rhizobacteria evaluated suppressed nematode population densities and stimulated plant growth and might naturally suppress *X. index* populations or damage caused to the root system.

Some of the isolates assessed for the control of the ectoparasitic nematode *X. index* in grapevines proved also to be effective against the endoparasitic nematode *M. ethiopica* in assays with potted plants.

The rhizobacteria able to antagonise the ectoparasitic *X. index* were not the same species as those showing activity against *M. ethiopica*. Only the bacterial strain *Serratia plymuthica* 213 had an effect on *X. index* in *in vitro* tests and on *M. ethiopica* in potted plants. Under natural cultural conditions, soils are infested with several genera and species of PPN, so if a biological control programme is proposed to be implemented, a mixture of isolates should be used.

Some of the rhizobacteria studied in this thesis are promising candidates for incorporation into prospective vineyard soils or for inoculation of the roots of new plants in order to control mixtures of PPN affecting vineyards. Further experimentation is needed to determine their ability to control parasitism by other very aggressive parasitic nematodes, such *Mesocriconema xenoplax* and *Tylenchulus semipenetrans*, under field conditions.

The different tests described in this thesis to evaluate the direct effect of rhizobacteria on PPN mortality showed different efficacy. For *X. index*, the percentage control was 89, 32 and 16 % for tests using culture filtrates, plants developed *in vitro* and potted plants grown in agricultural soil, respectively.

This means that the more complex the environment in which the roots are growing, the lower the nematode control effect of the different bacterial isolates.

The mortality or control effect obtained in assays with potted plants was not 100% and rhizobacteria-treated plants had nematode populations which may be harmful if the initial populations are too high, particularly with sensitive cultivars. To improve the efficacy of treatment, it may be necessary to modify it in some respects, *e.g*. by increasing the number of CFU/mL of carrier and/or the number of applications over the treatment period. In the longer term, if bacteria have successfully colonised the roots their action may be superior to that of chemical nematicides, since the latter are degraded or leached into deeper layers of the soil.

Isolates do not prevent grapevines from the risk of transmission of *grapevine fan leaf virus* by *X. index*, since infection may occur even at low vector densities, but can help to protect roots from direct damage.

Further studies are needed to identify the mechanisms of action involved in nematode suppression and the chemical nature of the bioactive compounds. More studies, particularly under commercial field conditions, are also necessary to evaluate their performance in different management practices and with a natural soil community.

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