UNIVERSIDAD DE LA FRONTERA
Facultad de Ingeniería, Ciencias y Administración
Programa Doctorado y Magíster en Ciencias de Recursos Naturales

POTENTIAL USE OF SELENIUM ACCUMULATING RHIZOBACTERIA FOR BIOFORTIFICATION OF WHEAT

DOCTORAL THESIS IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF SCIENCES IN NATURAL RESOURCES

JACQUELINNE JOVANKA ACUÑA SOBARZO

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Esta tesis fue realizada bajo la supervisión del Director de Tesis Dra. María de la Luz Mora Gil, perteneciente al Departamento de Ciencias Químicas y Recursos Naturales de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

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Abstract

Selenium (Se) is an essential micronutrient for human people which are related with around twenty five selenoproteins biologically active. Selenium deficiency in the human diet affected around 15% of world population increasing the diseasesincidence such as cancer, thyroid dysfunction, VIH, and the reduction of immune functions, among others). In Chile, cereal crops production (wheat, oat and barley) is one of the most important food sources of human nutrition. Cereal crops frequently have suboptimal Se-levels due to diverse factors associated with plant species and Se-availability in soils. In this context, the rhizosphere harbors a wide variety of bacterial species (rhizobacteria) which play an important role in the biogeochemical cycle of nutrients and micronutrients such as Se. Microbiological Se transformations (i.e. methylation, oxidation, and reduction) have been suggested as biotechnological tools for bioremediation of Se-contaminated soils. However, few studies are focused in the potential application of Se-utilizing bacteria in order to increase the Se-available in the rhizosphere and consequently in plants. According this, the general aim of this Doctoral thesis was to evaluate the Se-bioaccumulation by native bacteria present in the cereal rhizosphere grown in Chilean acid soils (Andisols), in order to develop a biotechnological tool for Se-biofortification of wheat plants. Firstly, we present a general vision of the Se problematic in human health and plant crops by a critical review of worldwide research in Chapter II. From a biofortification perspective, we described that Se-accumulating rhizobacteria (selenobacteria) can be used for Se enrich plants. In this context, the Chapter III showed the selenobacteria occurrence in the rhizosphere and its contributions to enhance Se content in plants. The results described that selenobacteria selected; belong to the genera: *Stenotrophomonas*, *Bacillus*, *Enterobacter* and *Pseudomonas*, similar to the genera previously reported in seleniferous soils. The selenobacteria have a great ability to tolerate and accumulate Se intra and extracellular in micro and nanospherical elemental Se deposits. Furthermore, effectively the inoculation of wheat plantlets with selenobacteria inocula showed increased Se contentin plant tissues. In addition, the results suggest that selenobacteria

inocula can be used as a biotechnological tool for Se biofortification in plant. In fact, the effectiveness to enhance Se content in grain by the co-inoculation of selenobacteria strains and mycorrhizal arbuscular fungus (*Glomus claroideum*) demonstrated a great potential of these rhizosphere microorganisms for biofortification of wheat and derivates foods (i.e. Se enriched flour). This microbial association enhanced the Se content inwheat grains around to 23.5% compared with non-mycorrhizal plants, associated with a higher microbial biodiversity on the rhizosphere (Chapter IV). Thus, our results showed that Se biosynthesized by selenobacteria can be translocated inside to the plants toward the grains. These results support the hypothesis that selenobacteria have a great potential for Se-biofortification of cereals (Chapter V).

In summary, Se-biofertilizer based on selenobacteria isolated from volcanic soils is more effective than Se inorganic source according to Se-uptake and grain translocation in wheat plants. We think that the selenobacteria are a promising strategy for Se biofortification for intensive cereal crops production.

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Chapter I- Introduction and objectives

1. Introduction and objectives

1.1 Introduction

Selenium (Se) is an essential micronutrient with antioxidant properties for animal and human (Birringer et al., 2002). Selenium is found as selenocysteine (SeCys) in the active site of many selenoenzymes, including glutathione peroxidase, iodothyroninedeiodinase, and thioredixinreductase (Brigelius-Flohe, 1999). Essential Se-based roles in enzymes, antioxidants, and protective pathways have been discovered and have recently related with as cancer suppression, HIV treatment, free radical induced diseases, and protection from toxic heavy metals (Bordoni et al., 2008). However, Se was considered as a toxic element until 1973 (Dubois and Belleville, 1988) and studies have demonstrated that exist a narrow gap between toxic and essential levels (Suhajda et al., 2000). Selenium deficiency on human health is becoming an interest topic in public health systems around the world (Fairweather-Tait et al., 2010). According to recommendations of National Institutes of Health (NIH-USA) the optimal doses vary from 30 to 85 $\mu g day^{-1}$ for human and 100 and 300 $\mu g\ kg^{-1}$ of dry weight (DW) for animals.

The Se content in soils is the main route of Se-intake in human dietary. The principal strategy against Se-deficiency used is agronomic fortification applying Se-supplemented fertilizers, particularly in some areas like New Zealand, Australia, Denmark, Siberia, China, Bangladesh, and Finland (Combs et al., 2001). In Chile, cereal crops production (wheat, oat and barley) is one of the most important sources of human nutrition. Cereals are estimated that contribute over 50% of the total dietary fiber intake consumed by the Chilean population (INIA, 2007). Chilean acid soils, derived from volcanic ash (Andisol), support cereal production in southern Chile. More than 44% of cereal production in the period 2012-2013 was grown in acid soils present in the La Araucanía region (ODEPA, 2012). Andisol are characterized by high amounts of interchangeable aluminum (Al), organic matter, and low pH; properties that limit the Se-bioavailability in the soil solution (Mora et al., 1999; Mora and Demanet, 1999). In

this context, several studies have been described that the Se-content in soil-plant system in Chilean pasture is very low (Ceballos et al., 1998; Wittwer et al., 2002; Cartes et al., 2005). In particular, the agronomic fortification using Se-fertilizer increased the Se acquisition by plants, this leads to ameliorate Al-stress across the strengthening antioxidant defense system in plants (Mora et al., 2008; Cartes et al., 2010). However, the application of Se-fertilizer in Andisol conditions entails to low fertilizer use efficiency, associated with high selenite adsorption, displayed in the low translocations rates to plants (Cartes et al., 2011)..

Bacteria play an essential role in the Se cycling in the environment. The relative proportions of Se oxidation states and selenium compounds in environment also depend of the bioprocesses involved in the bacteria metabolism (Simonoff et al., 2007). Thus, reduction-oxidation and methylation reactions are involved in bacteria detoxification and also can be contribute as energy source for bacterial metabolism. Several researcher have proposed the potential use of bacteria with capacity to reducing inorganic Se to elemental forms (Se-nanosphere) for bioremediation of contaminated soils, sediments, industrial effluents, and agricultural drainage waters (Sarret et al., 2005). However, according our perspective Se-compounds from Se- bacteria could be to provide a available Se-source for plant uptake that decrease the leaching and adsorption of inorganic Se forms (selenate and selenite) in soil system. In despite that Se-utilizing bacteria have been isolated from diverse terrestrial habitats, including seeds (Lindblow-Kull et al., 1982); there few studies are focused to the potential application of these bacteria to improve the Se availabilityto plants. Thus, the Chilean positioning as agri-food potency has enormous challenge in nutritional cereal crops area to ensure food quality and human health through the biotechnological tools.

1.2 Hypotheses

It has been shown that some bacteria groups isolated from seleniferous soils can metabolize selenium through oxidation, reduction and methylationmechanisms. Whereas the metabolic processes of oxidation-reduction are involved in the energy generation for the bacteria, it is expected that under conditions of Se-enriched bacteria present some of these mechanisms.

In this study we propose the following hypothesis:

1- Bacteria present in the rhizosphere of cereals growing in acid soils of southern Chile have the ability to bioaccumulate Se.

2- Inoculation of selobacteria in the rhizosphere of wheat plants increase the uptake and content of Se in shoots and grains.

3- Selenobacteria can improve the antioxidant system in wheat plants.

1.3 General Objective

To evaluate the bioaccumulation of selenium by native bacteria present in the cereal rhizosphere for improving seleniumbio fortification of wheat plants grown in volcanic soils of southern Chile.

1.4 Specific objectives

- To evaluate the effect of selenite additions on bacterial communities structure present in the rhizosphere of wheat grown in volcanic soils.
- To isolate bacteria with ability to chemical reduce of selenium from rhizosphere of cereals (wheat, oat and barley) grown in volcanic soils.
- To assess the effect of the selenobacteria selected on biofortification of wheat plants grown in volcanic soils.
- To develop and evaluate different inoculation methods for Se biofortification using Se-biofertilizer in wheat plants.

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Chapter II- Theoretical background

Selenium Biofortification: Potential use of soil microorganisms

Abstract

Selenium is an essential micronutrient for human health due its antioxidant capabilities. During the last decade, many studies have been carried out on accumulation, speciation, and biological functions of Se in relation to human health. Selenium persists in the environment in four oxidation states with different concentrations influenced by natural and anthropogenic processes. Both toxicity and deficiency of Se occurs in the agroecosystems and the abiotic (i.e. adsorption, precipitation, redox reactions) and biotic (microorganisms) factors govern its chemical speciation regulating the transport pathways and fate of Se between different environmental compartments (i.e. soil and plant systems). Selenium deficiency affect around the one billion people worldwide. Thus, studies related with strategies for Se biofortification of plants for human nutrition has significantly increased due to this metalloid is incorporated into human metabolism mainly as a constituent of food plant.

Microorganisms play an important role in the transformations and availability of Se. During bacterial metabolisms, Se can be transformed from one chemical form to another, through mechanisms of oxidation, reduction and methylation. In this context, considerable research has demonstrated the potentiality of application of plant growth promoting rhizobacteria (PGPR) for improvement of plant nutrition associated with enhanced the availability of essential nutrients.

This review is focalized in describing the use of PGPR for plant biofortification, with special emphases in selenium crop biofortification as a biotechnological tool for increasing this metalloid in the food chain.

2. Introduction

Selenium (Se) has been considered during long time as a metalloid toxic and dangerous for human health. Only in the last decades its physiological importance as a micronutrient fundamental to human health has been assessed (Fernández-Martínez and Charlet, 2009), because Se is incorporated into selenoproteins as the twenty-first amino acid selenocysteine (SeCys) (Tormay et al., 1996; Tapiero et al., 2003). Selenium deficiency affects their critical role in iodothyron inedeiodinases, thioredoxin reductases and glutathione peroxidases, which prevents oxidative damage in tissues (Rotruck et al., 1973). Thus, optimal Se levels decrease the incidence of important diseases such as cancer, HIV (Human immudeficiency virus) and heavy metal toxicity (Bordoni et al., 2008, Fernandez-Martinez and Charlet, 2009; Méplan and Hesketh, 2012).

In the last decades the study of strategies for increasing Se content in human nutrition has a primordial attention for scientist due to Se is incorporate into human metabolism mainly through vegetables and cereals (Govasmark and Salbu, 2011). The nutritional importance of food Se-enriched plants is the aminoacid incorporation mainly as Selenomethionine (SeMet). Thus, SeMet can account for >50% of the total Se content of the plant whereas other selenium forms (Tapiero et al., 2003). SeMet is the Se-chemical specie more efficiently absorbed and retained in plant, animal and human tissues (Fairweather-Tait et al., 2010).

Selenium content in plants is highly dependent on soil Se concentration and their availability. Nevertheless, several soils around the world are Se-deficient, which means that food crops do not supply enough amount of Se in terms of the human requirements. In this sense, the enrichment of agricultural crops through the Se fertilizers application (Agronomic Biofortification) is used in different countries as a China, UK, Europe, Australia and New Zealand (Bañuelos et al., 2012). However, inorganic Se is toxic at high concentrations. Where sodium selenite is feasibly bound to soil constituents,

selenate may be leached under wet fall conditions (Govasmark and Salbu, 2011; Hawkesford and Zhao, 2007).

Microorganisms play an important role in the speciation, mobility and availability of Se in the agroecosystem. Plant growth promoting rhizobacteria (PGPR) and Arbuscular mycorrhizal fungi (AMF) has shown promise as biofertilizer for Se-biofortification. Studies realized by Acuña et al., (2012) and Duran et al., (2013) reported that the inoculation of selenobacteria (PGPR with abilities to bioaccumulate Se) in synergism with AMF can increase Se-content in wheat grain significantly respect to inorganic Se-supplementation without causing damage to the environment. This review summarized current information about the environmental Se implications and biotechnological tools for Se- biofortification emphasizing in soil microorganisms.

2.1 Selenium in agroecosystems

Selenium occurrence in agroecosystems is related to natural and anthropogenic process and the global Se-distribution in soils varies greatly from 0.005 mg kg^{-1} in Finland to 8,000 mg kg^{-1} in Tuva-Russia (Chasteen and Bentley, 2002). Although Se concentrations are in normal range between 0.01–2.0 mg Se kg^{-1}; mean of world is about 0.4 mg Se kg^{-1} and concentrations >1200 mg Se kg^{-1} can occur in seleniferous soils (Fordyce, 2005).

In relation with natural process, Se is associated with volcanic eruptions, weathering and evaporation in the process of soil formation and alluvial fan deposition in soil (Haygarth, 1994). In fact, the persistence of Se in soil is associated with parent material, principal source of Se soil formation (Fernandez and Charlot, 2009). Igneous and volcanic rocks generally contain around 0.035 mg kg^{-1}; whereas sedimentary rocks present concentration between 0.05-0.06 mg kg^{-1} (He et al., 2005). Sedimentary rocks represent the principal compounds of earth surface and its Se concentration is much higher than in igneous rocks (Fernandez and Charlot, 2009). This can be attributed to Se

transfer to the atmosphere and hydrosphere during volcanic processes (Haug et al., 2007). On the other hand, early studies published by Lag and Steinnes, (1974) have reported that Se-supply from the sea via rain and snow and sulphuric acid-rich polluted rain was as important Se source.

Anthropogenic processes also generated inputs of Se in agroecosystems (He et al., 2005) as a extraction and processing of various minerals, pharmaceutical manufactures, veterinary medicine, the glassware manufacturing industry, electronics devices industry, lubricants manufacturers, etc. (Frankerberger and Benson, 1994; Wen and Carignan, 2007). It has been estimated that between 30% and 40% of total Se emissions to the atmosphere are due to human activities (Wen and Carignan, 2007), such as: extraction and processing of different elements (copper, zinc, uranium and phosphorus), the use of pesticides and the combustion of oil and coal. In the atmosphere, Se is transported associate to particulate matter (Bosco et al., 2005) and then is deposited in the in agroecosystems. In addition, the extensive uses of Se-containing fly ash as soil amendments (Dhillon and Dhillon, 2003) and the irrigation of cultivated soils with Se-contaminated waters (Lemly, 1998), have a major impact upon the selenium cycle.

The selenium cycle in the agroecosystems (Figure 2.1) is complex because Se has a broad range of oxidation states, from Se^{2-} (completely reduced) to Se^{6+} (completely oxidized), and can be transformed both chemical and biological processes (Zhang et al., 2000). Microorganisms play an important role in the transformations, mobility and availability of Se in the agroecosystems. The relative proportions of selenium oxidation states also depend of bioprocesses involved in the metabolism of microorganisms (Simonoff et al., 2007), especially those mechanisms of reduction and oxidation and methylation reactions by bacteria, determining the Se concentrations in soils, the mobility and uptake of selenium into plants and animals.

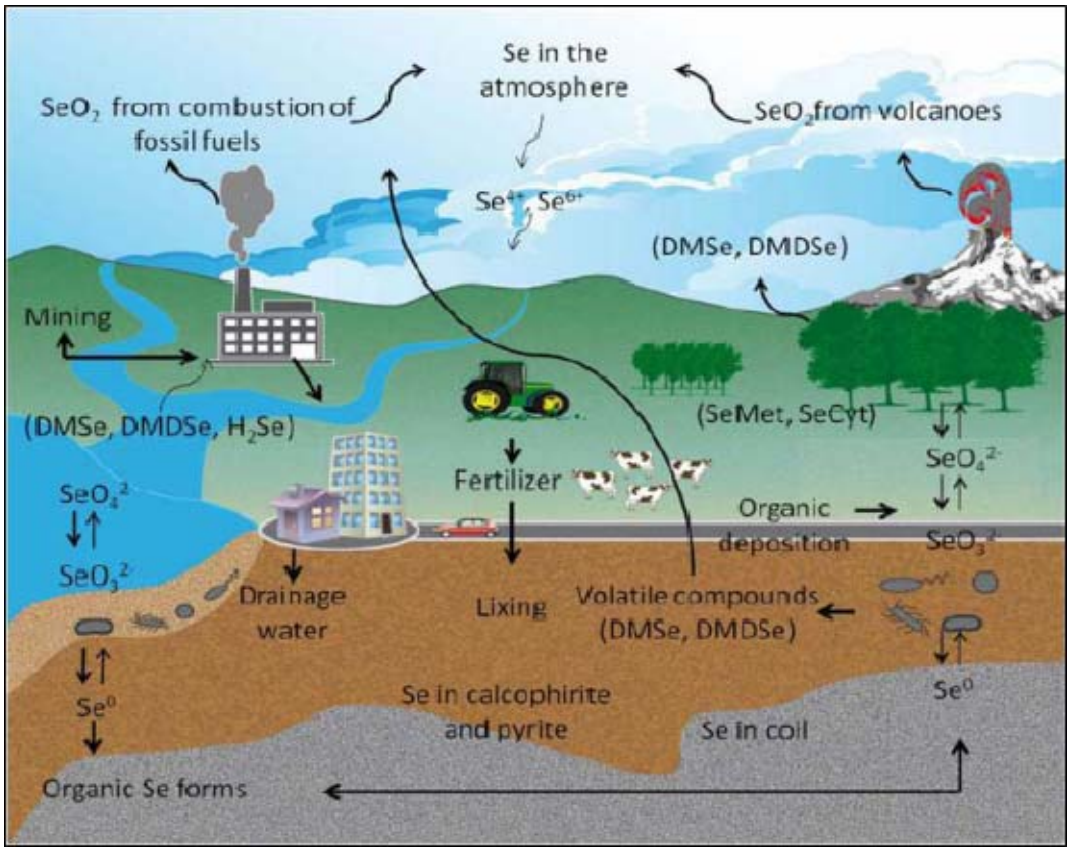

Figure 2.1 Selenium cycling in agroecosystems. The largest Se reservoirs on the Earth are iron sulphides (pyrite and calcophyrita) in sediments, rocks and coil, and selenate in seawater. Se, which is a necessary element for life, is taken up as selenate (Se^{+6}) by microorganisms and plants, and subsequently by animals. The combustion of fossil fuels and emission of volcanic fumes releases selenide dioxide (Se^{2-}) into the atmosphere. Microorganisms play an important part in the recycling of these selenium compounds.

2.1.1 *Agroecosystems Se-contaminated*

Soils containing more than 0.5 mg kg^{-1} are considered "seleniferous", an example is Kesterson Reservoir in California (USA) with a Se-content >100 mg Se kg^{-1} (Dhillon and Dhillon, 2005; Fordyce, 2007). Parts of the Great Plains of the USA and Canada, Enshi County in China, and parts of Ireland, Colombia and Venezuela are seleniferous (Combs, 2001). Dhillon and Dhillon (2003) have performed an extensive review about seleniferous soils. Lange and Berg, (2000) have showed some wildlife problems,

particularly at Kesterson Wildlife Refuge in California, attributed to the irrigation of cultivated soils with Se-contaminated waters. Similar issues and concerns are associated with application of activated sewage sludge as amendment on agricultural lands in areas located near urban centers in California (Fisher, 2000). In farm animals, selenosis have been described in different countries such as; Australia, China and USA (Peterson and Butler, 1971; Yang et al. 1983) by consume to Se accumulating plant species (capable to accumulate over 4,000 mg Se kg^{-1}; Terry et al., 2000), derivates from Se contaminated areas.

Selenium contamination in aquatic system in California has been found in the range of 140 to 1400 μg L^{-1}, mainly results from agricultural drainage water (Amweg et al., 2003). The bioaccumulation of Se in aquatic systems produce serious hazard to fish and waterfowl (Lemly, 1994). A review conducted by Fordyce et al., (2003) indicates that the natural source of Se is very important factor to considered in terms of risk health the animal and human exposure and requires careful consideration in Se-related health.

2.1.2 *Agroecosystems Se-deficient*

Selenium deficient soils are characterized by low Se-content and limited availability. In most of soils Se content is found between 0.01 and 2.0 mg Se kg^{-1} (Neal, 1995; Mayland 1994; Fordyce, 2007). Dhillon and Dhillon, (2003) proposed that soils with concentrations below 0.1 mg Se kg-1 of soil are described as Se deficient. Soils from New Zealand, Denmark, Finland, central Siberia, and a belt from north-east to south-central China are notably Se-deficient (Combs, 2001).

The low Se concentration in soil depend on geological source (parental rock) from which it derives and on biogeochemical processes. In volcanic soils, little is known about the specific interactions of Se oxyanions (predominant species of

inorganic selenium in volcanic soils) and the mineralogical components specific to these soils (imogolite and allophane), which could be responsible of the low Se bioavailability (Fernandez-Martinez and Charlot, 2009). In addition, the organic matter has an important role in Se content in soil due to propensity for Se to be adsorbed to organic materials (Ander et al., 2010; Fordyce et al., 2010). In this context, studies carried out by Cartes et al., (2005), have been described that Andisol from Southern of Chile, exhibit Se concentrations between 0.02 y 0.18 mg Se kg^{-1}.

2.2 Bioavailability of selenium and role of bacteria in the agroecosystems

Selenium in the agroecosystems is found in both inorganic and organic forms. The inorganic Se forms is present four oxidation states, which are denoted as selenide (Se^{2-}), elemental Se (Se^{0}), selenite (Se^{4+}) and selenate (Se^{6+}) (Fernandez-Martinez and Charlot, 2009).

The concentration, transformation, mobility and accumulation of these oxidation states depends on abiotic and biotic processes involved (Table 2.1), as well as on the local physico-chemical properties, including redox potential (Eh) and potential hydrogen (pH). Moreover various mechanisms such as precipitation, surface adsorption or absorption of minerals or organic matter also regulate the incorporation and therefore the availability of Se in the soil (Fernandez-Martinez and Charlot, 2009). Selenate is the predominant Se species in alkaline and oxidizing soils. This anion is highly soluble in water and readily available for plant uptake because is weakly adsorbed on soil surfaces. On the other hand, Se^{4+} occurs in acidic soils with high content of organic matter, such as humic and fulvic acids (Jayaweera and Biggar, 1996). Compared with Se^{6+}, Se^{4+} is less available to organisms because is absorbed by clays, calcites, organic matter or hydroxides, particularly ferric oxyhydroxides (Su and Suarez, 2000).

In the anaerobic and acid environments, Se^{4+} can mainly be reduced to Se^{0} by sulfur dioxide. However, Se^{0} has reducing rate, especially under acidic condition where requires a strong oxidizing action. Finally, Se^{2-} is strong reduced and therefore can easily be oxidized to volatile forms. In this oxidation state, Se^{2-} can be part of seleno-amino acids which are Se organic forms present in the metabolism of organisms.

It is widely accepted that bacteria present in soil systems have capability to reduce, oxidized and methylated Se oxyanions to some differents Se-compounds. The reduction processes have been described as the ability of some bacteria to convert the Se^{6+} and Se^{4+}, to Se^{0} and finally generating Se methylated compounds (Losi and Frankerberger, 1996), decreasing the availability and transport of Se in the environments. In addition, the oxidation, reverse process, was found in a few bacteria (i.e. *Bacillus megaterium*, *Thiobacillus* ASN-1 and *Leptothrix* MnB1), due to the oxidation of Se occurs in rates four orders of magnitude less than the reductive part of the Se cycle (Dowdle and Oremland, 1998). These redox transformations of Se by bacterial metabolisms determining the bioavalability of Se for plant uptake.

Table 2.1 Abiotic and biotic factors acting in the Se availability in the agroecosystems

Factors	Speciation toward	Mechanisms involved	Environment where occur	References
Abiotic				
pH and potencial redox (Eh)	Inorganic species SeO_4^{2-} SeO_3^{2-}, Se^0, and SeO_2	Oxidation and reduction	Oceans, rivers and soils, volcanic eruption, Industrial combustion processes	Dhillon and Dhillon, 2003
Biotic				
Bacteria	SeO_4^{2-} SeO_3^{2-}, Se^0	Oxidation and reduction	Oceans, rivers and soils, volcanic eruption, Industrial combustion processes	Frankerberger and Losi, 1997
	H_2Se	Methylation	Oceans	Amouroux et al., 2000
	Organic species DMSe, DMDSe	Methylation	Oceans and soils	Chasteen et al., 2003
	DMSeD, DMSeDS, GSSeSG, GSS-SeH, SeCys, SeMet, $SeCN^-$ and Selenoproteins (i.e Selenophosphate sintetase)	Methylation and metabolisms	Within microbial cells as part of its metabolism	Chasteen et al., 2003 Turner et al., 1998
Plants	DMSe, DMDSe, and DESe	Methylation	Within cells and tissues as part of its metabolism	Zhang and Frankerberger, 2000
Animals	Selenoproteins (i.e GPX, Selenoprotein P and W)	Metabolisms	Within cells (blood plasma, muscle and spleen) and tissues as part of its metabolism	Burk et al., 2003

2.3 Selenium in the human health

Selenium is an essential trace element with fundamental importance to human health. This recognized importance is due that this metalloid is a constituent of selenoenzymes such as glutathione peroxidase (GSH-Px), thioredoxin reductases (TR), and proteins with unknown functions that are involved in maintaining the cell redox potential (Rayman, 2000). Nowadays, 25 selenoproteins have been described in human metabolism, corresponding to selenoproteins in which Se is an enzymatic cofactor (Korlhe et al., 2000; Dodig et al., 2004; Rayman, 2000; Schomburg et al., 2004).

Selenium content in plants are derived food is the principal route of Se intake in the human diet, due to the capabilities for plants to metabolized organic Se compounds such as SeMet and SeCyt (Terry et al., 2000). In this context, several report have demonstrated the benefits of Se-compounds in the human diet like SeMet on the risk of breast, prostate, lung, bladder, liver and colorectal cancers (Wei et al., 2004; Duffield-Lillico et al., 2002; Zhuo et al., 2004; Ip et al., 2000). Other studies have reported that Se in humans have a antiviral effects in male and female reproduction and participates in the thyroid functioning, reducing AIDS virus growth(Campa et al., 1999; Rayman, 2000; Stranges et al., 2006, Hawkes, 2000). Recently, detailed and extensive reviews about the beneficial of selenium in the human diet have been published by Rayman, (2012).

In contrast, low Se content in human diet has been associated with different disease such cardiovascular disease (Rayman, 2002), dysfunction in inmune systems (Bodoni et al., 2008) as well as an increased risk as Keshan and Keshin–Beck (Tan et al., 2002), etc. Keshan and Keshin–Beck are two endemic disease associated with a Se-deficience which are produced in parts of China and Siberia. Keschan disease is produced generally in children and woman of childbearing age and its symptoms are

related with impairment of cardiac function, cardiac enlargement and arrhythmia (Xu et al., 1997). The diseases occurrence is involving Se and vitamin E deficiencies, and the presence of the Coxsackie B virus (Moreno-Reynes et al., 1998). Kaschin-Beck disease is an osteoarthropy, which manifests as enlarged joints, shortened fingers and toes, and in severe cases dwarfism and is attributable to Se and vitamin E deficiency (Coppinger and Diamond, 2001) and Iodine deficiency (Contempré et al., 1991). In relation to optimal Se status in human dietary, the Recommended Dietary Allowances (RDAs) indicates that 55 μg Se day^{-1} is an adequate dose for adult men and women (RDAs, 2000).

2.4 Strategies for increase Se content in food chain

The use of several Se supplements with organic selenium forms and Se enrichment samples has been used as the standard selenocompound in Se bioavailability studies (Rayman, 2002) and in most animal studies dealing with the relationships of antioxidant properties associated to carcinogenesis (Rojas et al., 1996). Selenium supplementation can be completed by multivitamin tablets, containing either sodium selenite, SeMet, from selenium enriched yeast (Barceloux, 1999).

In foods, the main source of organic Se is seafood and fish, whereas foods like cereals, meat, nuts, and eggs can also increase the dietary selenium intake. Recently, more researches showed that Se-enrichment yeast and some plant resources such as Se-enrichment onion, fungi and tea were considered as effective organic selenium supplement (Dumont et al., 2006; Whanger et al., 2000). In food plants, agronomic management for Se-biofortification (Agronomic Biofortification) and plant breeding (or genetic biofortification); they are supposed to be de most effective ways to increase selenium intake by population, whereby selenium reaches the different levels of food chain (Lyons et al., 2004, Hartikainen, 2005). Selenium enrichment of fertilizer

applications across pelletizing seeds directly with Se or foliar applications in crops has been described as an effective strategy in order to increase Se available to plants and concomitant enhanced content in leaves and cereals grain (Gissel-Nielsen et al., 1998; Lyons et al., 2005; Mora et al., 2008). Se application in pasture and forage is a common practice in Australia, New Zealand, China, Finland and part of North America for preventing some cattle diseases, such as muscular distrophy and white muscle disease both associated with low Se content soils, (Lee et al., 1999; Fordyce, 2005). While, Se in not recognized as essential micronutrients in plants, but the enhanced Se content in pastures also has been associated with the strengthening the antioxidant system against to the aluminum toxicity (Mora et al., 2008). Inverse relationship was observed between lipid peroxidation and the GSH-Px activity in ryegrass plant whose seeds were previously pelleted sodium selenite (Cartes et al., 2011). Thus evidence the important role of Se in the quality of higher plants and the beneficial for nutritive value in the food chain.

Cereals, primarily wheat (*Triticum aestivum* L.) have been described as a good source for bioavailable Se and the SeMet is the principal organic forms in grain (Lyons et al., 2004). Different countries such as: Australia, New Zealand, UK and Finland have been demonstrated the stronger linked between the Se concentration of wheat grain and the optimal Se status in human diet (Broadley et al., 2006). In this context, government of Finland is the pioneer in the application of inorganic Se fertilizer as a biofortification programs at a national scale; which was effective in enhanced Se concentrations in foods and dietary Se intake (Broadley et al., 2006). However, there are limitations to the effectiveness of agronomic biofortification in other regions due to Se is toxic at high concentrations and inorganic selenite is bound to soil constituents, thus it is unavailable to plants, whereas selenate may be leached under wet fall conditions (Govasmark and Salbu, 2011; Hawkesford and Zhao, 2007).

2.5 Biofortification by microorganisms

In the recent decades, the new strategies for the development of the crops biofortification as alternatives to the chemical fertilization and the agronomic practices have gained great interest for researchers. The use of traditional agronomic biofortification appears to have great potential for fight malnutrition; however, these practices have increased the environmental pollution, have a high-cost, and depend to political issues (Haug et al., 2008). Nowadays differents studies have proposed the use of plant growth-promoting rhizobacteria (PGPR) as alternatives for enhanced the uptake of micronutrients for plants (Tariq et al., 2007).

It is commonly accepted that the rhizosphere has a wide variety of bacterial species which carry out functions that are essential to plant growth, nutrition and disease control in agricultural systems (Nannipieri et al., 2003; Hawkes et al., 2007). Among them, members from different phylogenetic groups have attracted considerable interest due to their great biotechnological potential for improving crop yields. The ability to improve the availability of macronutrients such as, phosphorus (P) and nitrogen (N), are the most studied features in the rhizobacteria. The beneficial effects of the inoculation with phosphate-solubilizing bacteria in many crops have been commonly reported and they have been suggested as promising biofertilizer to increase the P supply from sources otherwise poorly available (Igual et al., 2001; Rodriguez et al., 2006). In addition, studies have been demonstrated that N-fixing bacteria have an important role in the N-recycling thus promoting plant nutrition (Bhattacharjee et al. 2008; Bashan and de-Bashan, 2010). Nevertheless, the role of PGPR in the micronutrient availability to plants and their effects in the growth promotion has been recently considered as an important mechanism as a part of multiple action mechanisms promoting plant growth in the microorganisms.

In this sense, recently studies showing that the application of PGPR with abilities to mobilizing of diverse micronutrients such as: Zinc (Zn), Iron (Fe), Manganese (Mn), and Copper (Cu) have a great potential for used in the biofortification programss and emerge as attractive alternative for replacing chemical fertilizer in cereal (de Santiago et al., 2011; Rana et al., 2012). Indeed, Rana et al., (2012) studied the effect of coinoculation with PGPR (*Bacillus* sp. AW1, *Providencia* sp. AW5, and *Brevundimonas* sp. AW7) in the Fe uptake in plants. These results indicated that inoculation with PGPR strains (isolates from the wheat rhizosphere) can enhance P, K and Fe content and plant yield. The authors suggested that the multiple benefits of plant growth promoting rhizobacteria (PGPR) can be used as a biofortification strategies and represent a promising candidate for inclusion integrated nutrient management in the wheat crop.

The practical use of PGPR to enhance plant nutrition in micronutrient-limited soils has been associated with the abilities to produce and release the siderophores for enhanced iron acquisition by plants (Mercado-Blanco and Bakker, 2007; Rroço et al., 2003). Meanwhile, the production of diverse exudates and the excretion of these compounds by microbes can also supply additional Mn, Zn and Cu to plants (Altomare et al., 1999; Howell, 2003; Tao et al., 2003). However, the competition for nutrients between microorganisms and plants sometimes could be limited the availability for plants. Examples of diversity of PGPR strains used as a biotechnological tool for biofortification with micronutrients in plants are summarized in Table 2.2.

Table 2.2 Diversity of PGPR used as a strategy of biofortification in plants.

Bacteria	**Micronutrient**	**Plants**	**References**
Pseudomonas sp	Iron	*Vigna radiata*	Sharma et al., 2003
Trichoderma asperellum T34	Iron	*Lupinus albus*	de Santiago et al., 2009
Penicillium chrysogenum	Iron		Hördt et al., 2000
*Pseudomon*as sp. GRP3A	Iron	*Zea mays*	Sharma and Johri 2003
Trichoderma asperellum T34	Iron	*Triticum aestivum*	de Santiago et al., 2010
	Copper		
	Manganese		
	Zinc		
Bacillus sp. AW1	Copper	*Triticum aestivum*	Rana et al., 2012
Providencia sp .AW5	Zinc		
Brevundimonas sp. AW7	Iron		
	Manganese		
Achromobacter xylosoxidans strain Ax10	Copper	*Brassica juncea*	Ma et al., 2009
Rhizobacteria N.D	Manganese	*Triticum aestivum*	Marschner et al., 1991
Arthrobacter sp.	Iron	*Glycine max*	Nogueira et al., 2007
Variovorax sp.	Manganese		
Ralstonia sp.			
Azotobacter chroococcum	Zinc	*Triticum aestivum*	Ebrahim and Aly 2004
Azospirillum brasilense			
Enterobacter sp. B16	Selenium	*Triticum aestivum*	Acuña et al., 2013
Stenotrophomonas sp. B19			
Enterobacter sp. B16	Selenium	*Triticum aestivum*	Duran et al., 2013
Stenotrophomonas sp. B19			
Pseudomonas sp. R12			
Bacillus sp. R8			

N.D; no indentified.

2.6 Biofortification by Selenobacteria

Selenium speciation, mobility and bioavailability in soils are highly affected by the presence of microorganisms in the environment (Dungan et al., 2003). Selenobacteria has been described as a Se-respiring and Se-tolerant bacteria, associated to the mechanisms of assimilation and metabolization of this metalloid inside the cells (Kessi et al., 1999; Losi and Frankerberger, 1997; Stolz et al., 1999). These mechanisms make to the selenobacteria an excellent candidate to be used in the biorremedation and phytoremediation of Se-contaminated sites. Diverse studies have described the positive effect associated with inoculation by rhizobacteria in plants to improved Se phytoextraction and Se volatilization in plant (Catafio et al., 1996; de Souza et al., 1999; Siddique et al., 2005; Bañuelos et al., 2005; Antonioli et al., 2007). Indeed, Wu et al., (2004) showed an extensive review about the bioremediation through soil microorganisms of Se-contaminated areas. Considering the duality (toxicity and deficiency) of Se in agroecosystems, the bioremediation and biofortification could be integrated closely and might be considered as a new biotechnological alternative for counteract the Se-deficiency in several agroecosystems (Figue 2.2).

From Se biofortofication point of view, recent studies have been demonstrated that the inoculation with Se-enriched rhizobacteria capable of metabolizing Se (selenobacteria) increased the Se concentration in plant tissue of wheat (Acuña et al., 2013). In addition, studies conducted by Duran et al., (2013) evaluated the synergism between selenobacteria and AMF in the Se uptake and translocation in wheat plants. This report showed that plants co-inoculated with a mixture of selenobacteria strains and *G. claroideum* increased the Se content in grain. These results demonstrated that Se associated with inoculant can be absorbed and it is an available source of Se by the plant. In this context, the use of Se-tolerant bacteria emerges as a viable alternative for

Se-biofortification of cereals grown on lands with low Se concentration or low Se available.

An important unresolved question concerns the transport mechanism of Se uptake into roots systems, the chemical forms of Se in biofortified wheat plants and their metabolic pathways within the plant.

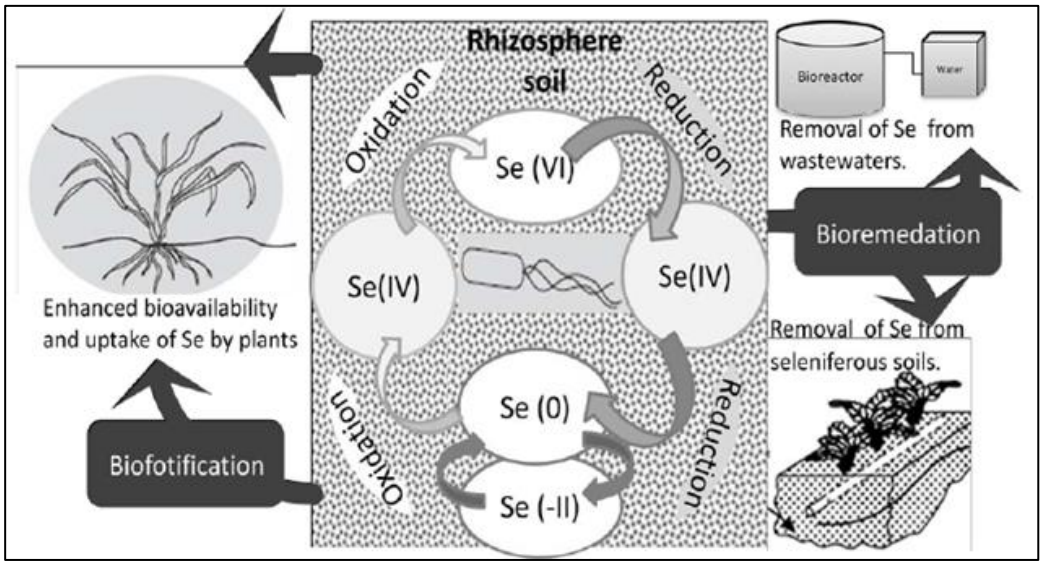

Figure 2.2 Schematic illustration of the potential biotechnological application of bacteria present in the rhizosphere with ability to metabolize selenium.

2.7 Ocurrence of selenobacteria

Selenium is toxic for microorganisms; however the continues exposure to high Se concentration provides natural selection of Se resistant strains (Burton et al., 1987). During bacterial metabolism, Se is transformed by diverse processes such as oxidation, reduction and methylation (Se^{6+}/ Se^{4+} to Se^{0}), under aerobic or anaerobic condition (Dhanjal and Cameotra, 2010; Dwivedi et al., 2013; Ghosh et al., 2008; Prakash et al., 2010). These reactions protect the bacteria from the toxic effects of Se (Se-tolerant bacteria) and in the most cases; Se provide part as energy source for bacterial metabolism (Se-respiring bacteria). In effect, bacteria capable to tolerate Se are related with aerobic systems by using diverse enzymes that confer Se resistance usually involve

glutathione reductase (Watts et al., 2003; Ridley et al., 2006). On the other hand, Se-respiring bacteria those are capable of growing anaerobically systems by using selenium oxyanions as a sole electron acceptor for respiration (Oremland et al., 2004).

Bacteria with ability to metabolized Se have been called as a selenobateria (Acuña et al., 2012), and these have a wide distribution in the ocean, acuatic sediments, wetlands, soil, and rhizosphere of plants (Hunter et al., 2007; Hunter et al., 2009; Baesman et al., 2009; Narasingarao and Haggblom, 2007; Dhanjal and Cameotra, 2010, de Souza et al., 1999; Acuña et al., 2013).

The rapid appearance of selenobacteria in enrichment cultures suggests that they are widespread and metabolically active in seleniferous and non-seleniferous environments.

The first report of a Se-respiring strain was in *Thauera selenatis* where acetate disappearance in the culture medium was observed to be proportional to the conversion of Se^{6+} to Se^{0} (Macy et al., 1993). After this report, the reports of Se-respiring microorganisms increased considerably. The isolation of Se-respiring bacteria have been described as belonging to different phylogenetic groups (Gram positive, β-, γ- and ε-proteobacteria), thus displayed that selenobacteria are widely spreaded in bacterial domain (Macy et al., 1993; Newman et al., 1997; Blum et al., 1998).

The majority of bacteria have been described that Se-respiring oxyanions isolate from anaerobic systems (Oremland et al., 1994; Blum et al., 1998, Takai et al., 2002 Oremland et al., 2004). However, studies of Se-tolerant aerobic microorganisms have gained advantages in terms of scale-up processes associated with strains grown under anaerobic conditions (Dhanjal and Cameotra, 2010). Currently, several reports have described diverse aerobic Se tolerant strains such as: *Pseudomonas aeruginosa*, *Bacillus* sp., *Enterobacter* sp., *Stenotrophomonas* sp., *Pantoea* sp. *Acinetobacter* sp. and *Klebsiella* sp. (Prakash et al., 2009; Yadav et al., 2008; Dhanjal and Cameotra, 2010;

Dwivedi et al., 2013; Acuña et al., 2013; Torres et al., 2013). In this context, differential mínimum inhibitory concentration (MIC) has been observed in the selenobacteria strains associated with to source from isolation. Recently, *Pseudomonas seleniipraecipitatus* isolated from soil, have been shown an unusual level of tolerance to selenite (>150 mM) (Hunter and Mater, 2011). In contrast, strains isolated from sediment showed a MIC of 1.56 mM (Burton et al., 1987).

2.8 Metabolisms of Se in Selenobacteria

2.8.1 Selenium uptake

Is generally accepted that selenate enter into the cell through the sulfate ABC transporter complex enconded by the *cysAWTP* operon, which comprises *cys*A, *cys*T and *cys*W genes (La Rossa, 1996). Studies have demonstrated that mutations in these genes give selenate resistance in bacteria (Turner et al., 1998). In *Escherichia coli*, selenate can enter into the cell via the sulfate transporter activated by ATP sulfurylase; while selenite uptake is associated with ABC transporter (Turner et al., 1998). The complex is composed of two CysA ATP-binding proteins, two transmembrane proteins, CysT and CysW, and a periplasmatic sulfate binding proteins (Turner et al., 1998). However, in *Rhodobacter sphaeroides*, a polyol transporter has been suggested as the transporting agent of selenite into the cell (Bebien et al., 2001). Thus, it has been suggested that specific membrane transport proteins are expressed in some microorganisms when exposed to selenite. These proteins could be responsible for selenite transport into the cytoplasm. However more studies are necessary for elucidate the specific mechanism of active transport of selenite ions into bacterial cells.

2.8.2 Intracellular metabolic pathways

Selenium inside the bacterial cell can be i) reduced to elemental selenium ii) metabolized to volatile compounds and iii) incorporated into selenoproteins as selenoaminoacids. Some bacteria can use selenate and selenite as an electron acceptor in the respiratory pathway. Several studies have been carried out to elucidate the molecular mechanisms of selenite reduction in bacteria. Studies by Schroder et al., (1997) showed the isolation of the most common Se-respiring bacteria *Thauera selenatis*. The reduction of selenate by *T. Selenatis* is mediated by selenate reductase enzymes (*ser*ABCD) (Krafft et al., 2000). The *ser*ABCD operon, encoding in a soluble periplasmic protein, with three subunits; a catalytic subunit containing a molybdenum cofactor (*ser*A), other subunits contains binding Fe–S cluster-containing (*ser* B), and a b-type cytochrome (serC). A fourth gene, *ser*D is a member of the *Tor*D family of peptide-binding proteins was also sequenced which is foretold to be an assembly chaperone for the selenite reductase (Lowe et al., 2010). McEwan et al., (2002) propose that *Ser*ABC is the only member of the DMSO reductase family. The reduction process is mediated by *Ser*ABC enzymes as a proton-motive force generation by accepting electrons from cytochrome c_4 (cytc4) wich is reduced by a quinol-cytochrome *c* oxidoreductase (QCR), mediated by quinol oxidation and generating two electrons, eventually resulting in the reduction of selenate to selenite (Lowe et al., 2010).

In the case of Gram-positive bacteria, studies carried out by Kuroda et al., (2011) showed that *Srd*BCA operon encoded membrane enzymes belong to the DMSO reductase family responsible to selenate reduction (Rothery et al., 2008). The *Srd*BCA is membrane bound and molybdopterin-containig oxidoreductases. Unlike the reduction in Gram-negative bacteria, the reduction of selenate to selenite occurs by quinol oxidation mediating by *Srd*C, providing two electrons to *Srd*B. The reductions continue toward delivering electrons to selenate via molybdenum subunit cofactor (Kuroda et al.,

2011). However, the carrier proteins that catalize the selenite or selenate uptake and reduction have not been identified in Se-tolerant bacteria.

2.8.2.1 Byosinthesis of Se-nanoparticle

The capability of bacteria to reduce both Se^{6+} and Se^{4+} to insoluble and non-toxic elemental selenium has been widely described in some bacteria. Reduction of Se oxyanios to elemental Se, which is visible as a brick red precipitated associated with intracellular deposites (cytoplasma and periplasmic space), other report have described the presence of extracellular spherical and oval-shaped nano- shaped nano-structures (Kessi et al., 1999; Roux et al., 2001; Bebien et al., 2001, Dungan et al., 2003; Van Fleet-Stadler et al., 2000). Oremland et al., (2004) indicated that the structural differences of Se nanospheres were attributed to the diversity of enzymes that catalyze the reduction of Se oxyanions. Several approach have been described to the the mechanisms by which selenite is reduced to selenium in bacterial cells (Yee et al., 2007; Ranjard et al., 2002; Oremland et al., 2004; Losi and Frankenberger, 1997).

Various studies attribute that the gene *fnr* (fumarate nitrate reduction regulator) confers selenate reductase activity, with selenite as an intermediary, and the ability to precipitate elemental selenium (Ma et al., 2007). In this sense, studies by Yee et al., (2007) showed that *E. cloacae* mutants with *fnr* knockout mutations lost the ability to reduce Se^{6+} and were unable to precipitate elemental Se. The authors suggest that the precipitation of Se^{0} particles by facultative anaerobes is regulated by oxygen-sensing proteins and occurs in periplasm. However, studies carried out by Debieux et al., (2011) showed that selenite reduction occurs in the cytoplasm and it is stabilized by the presence of the gen *Sef*A. The sequential reactions to reduce selenite to elemental Se (i.e *E coli*) are mediated by sulphate transporter which allows selenite entry into the cytoplasm (Turner et al., 1998). Once in the cytoplasm, selenite reacts readily with

glutathione (GSH) forming selenodiglutathione (GS–Se–SG) and subsequently in presence of GSH reductase the GS–Se–SG is reduced to selenopersulfide (GS–Se-), and then GS–Se- is unstable and dismutates into elemental Se (Se^0) and reduced GSH (Turner et al., 1998).

Different size, morphology and distribution of Se-nanoparticules have been described in some bacteria species. Se nanoparticules ranged from 100 to 550 nm, with an average size of ~300 nm (Fesharaki et al., 2009). In this sense, studies by Dobias et al., (2011) identified four proteins (AdhP, Idh, OmpC, AceA) that bound specifically to Se nanoparticules and observed that these proteins controlled the size and distribution of the elemental Se. However, the mechanism of how the Se nanospheres as a secreted across the inner and outer membranes has not yet been understood.

2.8.2.2 Biosynthesis of selenoproteins

In bacteria, the selenoproteins include formate dehydrogenases, hydrogenases, selenophosphate sintethase, or glycine reductase and proline reductase (Stock and Rother, 2009). As described above selenite once inside the cell is reduced to selenide and finally incorporated into amino acids cysteine and methionine as selenocysteine and selenomethionine (Turner et al., 1998).

Selenocysteine (SeCys), the 21st amino acid is cotranslationally incorporated into several prokaryotic and eukaryotic selenoproteins at inframe UGA stop codons (Böck, 2001) and provides an important active site residue in select redox proteins such as formate dehydrogenase H (*fdh*F gene) identified from *Echerichea coli* (Böck et al., 1991; Böck, 2001).

It is now known that UGA serves as a termination codon, but can be redirected by specific-specific mechanisms to code for insertion SeCys in the selenoproteins (Zhang et al., 2005; Böck, 2001). Four genes, SelA, SelB, SelC and SelD, are required

for Sec insertion and a SECIS (Selenocysteine insertion sequence) element in the mRNA coding to selenoproteins (Böck and Stadtman, 1998).

According to these processes, SECIS element as a part of mRNA structure is localized nearby to UGA codon leader the specific elongation factor SeIB (Rother et al., 2001). The SelB is the responsible to bind a selenocysteine specific- tRNA (tRNASec, SelC) only when it is charged with Sec residue begins at the insertion in UGA condon. Selenium is derived from an activated selenium donor, selenophosphate, from selenophosphate synthetase (SelD), which converts selenide and ATP to selenophosphate (Leinfelder et al., 1990).

Selenocysteine synthetase (SelA) converted selenocysteinyl to Seryl residue using in selenophosphate as a selenium donor. The Seryl residue is used for charging tRNASec thus generating selenocysteinyl-tRNASec (Forchhammer et al., 1991). The optimal stoichiometry between selenocysteinyl-tRNASec, SelB and mRNA (SECIS) have been described as a the most important factor to the normal Sec insertion in selenoproteins (Tormay et al., 1996). An extended review about the metabolic pathways of employed in selenoprotein synthesis in bacteria is summarized by Stock and Rother, (2009).

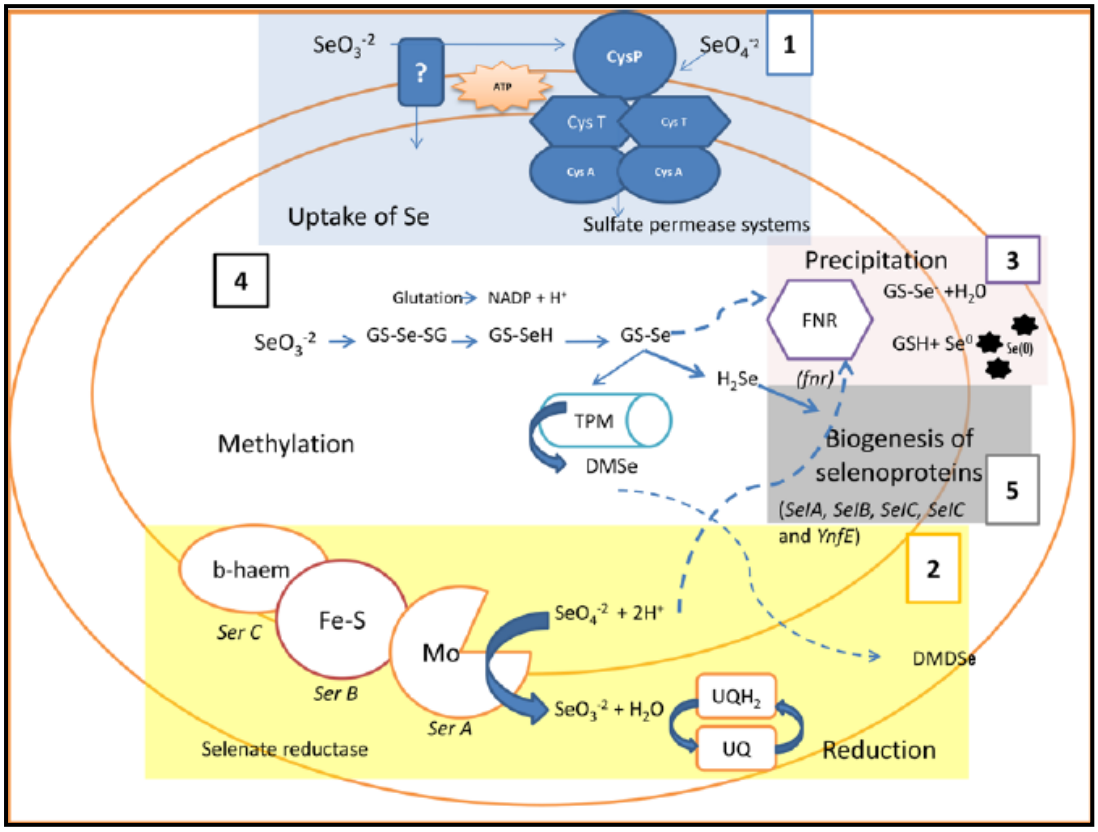

Figure 2.3 Diagram of the different microbial processes involved in selenium metabolism. (1) Selenium enters the cells through the sulphate transporters (Se^{6+} and Se^{4+}) or the undefined carrier (Se^{4+}). (2) Once inside the cells, Selenate is reduced to selenite by selenate reductase. (3) Selenite can also be detoxified by precipitation of Se^{0}. Inorganic selenium can also be transformed into organic species in a methylation (4) cascade and them be incorporated into the selenoproteins (5).

2.8.2.3Byosinthesis of methylate compounds

Bacteria have been identified as the principal organisms in the Se-methylated processes (Frankerberger et al., 1994). Thus, selenite or organic forms of Se (selenocystine, selenocysteine, selenomethionine) follows the route of methylacion to undertake training of dimethyl selenide (DMSe) or dimethyl diselenide (DMDSe) (Ranjard et al., 2002). The DMSe and DMDSe are the principal Se compounds associated with detoxification route of Se in some bacteria (Chasteen and Bentley, 2003).

The methylation process in bacteria is mediated by thiopurine methyltransferase (TPMTs) encoded by gene (*tmp*) indentified in *Pseudomans syringae* (Ranjard et al., 2002). Two TPMTs (TPMT-I and TPMT-E) from bacterial cell have been vinculated with the production of DMSe and DMDSe from selenate, selenite, and selenocysteine (Ranjard et al., 2002). In addition, MmtA enzyme is defining a new group of methyltransferases and has homologs in many species of bacteria (Ranjard et al., 2003). Report by Favre-Bonté et al., (2006) evaluated the diversity of bTPMT gene (*tpm*) sequences among the differents soils. Their analysis showed the main distribution of TPMTs among the γ-proteobacteria, presence among few β-proteobacterial species and observed, for the first time, TPMT sequences in a species of α-proteobacteria. The authors suggested that a widespread distribution of bacteria encoding *tpm* gene sequences. However, the specific biochemical pathways are not clear; thearefore more studies are requeried.

2.9 Concluding remarks and future trends

Selenium content in soil is regulated by environmental and anthropogenic factors affecting it speciation and bioavailability. In agroecosystems, bacteria play an important role in selenium cycle by reduction, oxidation and methylation processes. Selenium deficiency occurs in some areas on the world, thus affecting the protective role of Se as an antioxidant in the human health. It is becoming increasingly apparent that agronomic biofortification can increase selenium content in plants, but most studies currently focus in the enhanced Se amounts in plants and have not been able yet to sort out the bioavailability of the element and cycling through the environment. The potential biotechnological applications of PGPR with abilities to mobilizing micronutrients in plants have been addressed, particularly in areas such as plant biofortification where bacteria can take part as carrier for biofortification.

Several studies have been proposed the potential biotechnological applications of Se tolerant bacteria as an effective strategy for Se biofortification front to use of Se chemical fertilizer. However more work is required to understand the Se forms in grains and the biochemical and genetic pathways involved in Se biofortified plants, in order to generate innovative technologies for Se biofortification programs.

In addition, future studies could be directed towards evaluating the real feasibility and effectivenessof this biotechnological tool, in terms of to improve the Se status in human and enhanced the yields of crops.

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Chapter III

Selenobacteria selected from the rhizosphere as a potential tool for Se biofortification of wheat crops.

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Selenobacteria selected from the rhizosphere as a potential tool for Se biofortification of wheat crops

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Abstract

Cereal production in southern Chile is based on ash-derived volcanic Andisols, which present suboptimal levels of available selenium (Se). Strategies are needed to improve Se content in cereal crops and concomitantly improve the nutritional quality of grain. Here, we investigated the occurrence of Se-tolerant bacteria (selenobacteria) in Andisols and evaluated Se tolerance and accumulation in selenobacteria. The inoculation of wheat with selenobacteria and the contributions of these bacteria to Se content in plants were also evaluated under greenhouse conditions. The results showed that Se amendment of Andisols stimulated some bacterial groups (*Paenibacillaceae* and *Brucellaceae*) but inhibited others (*Clostridia*, *Burkholderiales*, *Chitinophagaceae* and *Oxalobacteraceae*), as revealed by denaturing gradient gel electrophoresis. Furthermore, we found four selenobacteria isolates that displayed 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase activity) and that carried the *acd*S gene as revealed by PCR. The selected selenobacteria were characterised as *Stenotrophomonas*, *Bacillus*, *Enterobacter* and *Pseudomonas* according to partial sequencing of the 16S rRNA gene. After 24 h of culture in nutrient broth, the selected selenobacteria showed the ability to grow in high Se concentrations (5 and 10 mM) and

to accumulate elemental Se in micro- and nanospherical deposits, transforming 50–80 % of the Se initially added. Greenhouse experiments with wheat showed that Se associated with selenobacteria (micro- and nanospheres of elemental Se and other intracellular forms) can be translocated into leaves of wheat plantlets.

3. Introduction

Selenium (Se) is a metalloid present in nature in many different forms (inorganic, organic, solid, liquid and gas). Se is associated with several natural processes (e.g. volcanic activity, rock and soil erosion) and anthropogenic processes (mining activity, lubricant manufacturing) (Fernandez-Martinez and Charlot, 2009; Lenz and Lensa, 2009). Studies have reported Se-induced toxicity at a cellular level, the effects of which include cellular apoptosis, DNA damage and inhibition of enzyme activity (Spallholz and Hoffman, 2002). However, bacteria play an important role in the biogeochemical cycle of Se in nature (Haudin et al., 2007; Ike et al., 2000). During bacterial metabolism, Se is transformed by diverse processes (oxidation, reduction and/or methylation), and Se-tolerant bacteria (selenobacteria) have shown a great potential for use in environmental sciences (bioremediation and phytoremediation) and technology (glassware manufacturing, electronic devices) (Fesharaki et al., 2010; Prakash et al., 2010; Narayanan and Sakthivel, 2010). Despite its potential toxicity, Se is also a recognised micronutrient with antioxidant properties, and dietary deficiencies of Se in humans can affect cancer suppression, HIV treatment, free radical-induced diseases and protection from toxic heavy metals (Fairweather-Tait et al., 2010; Combs, 2001). Thus, agronomic biofortification with Se-supplemented fertilisers is a common practice in cereal crops to increase the Se content and nutritional quality of grains (Rayman, 2002; Banuelos et al., 2005). However, the transformation of Se by bacteria and the effect of these bacteria on the Se availability to plants are poorly understood.

In the rhizosphere (the portion of the soil influenced by plant roots), a wide variety of bacteria known as plant growth-promoting rhizobacteria (PGPB, Bashan and Holguin, 1998) contribute to growth, disease suppression and stress tolerance of plants (Martinez-Viveros et al., 2010). Previous studies have shown that inoculation of plants with PGPB that possess traits such as indoleacetic acid production and 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) activity can enhance root growth and increase general tolerance of plants to a variety of environmental stresses, such as salinity, heat and drought (Yang et al., 2008; Barret et al., 2011). Recent work has also described higher growth rates and enhanced tolerance and bioaccumulation of heavy metals (nickel, lead, cadmium, chromium, copper, cobalt, zinc) in plants inoculated with PGPB that have ACC deaminase activity and in transgenic plants expressing bacterial ACC deaminase genes (Arshad et al., 2007; Zhuang et al., 2007; Farwell et al., 2006). However, Kuffner et al., (2010) indicated that PGPB with ACC deaminase activity is not involved in the metal mobilisation in plants and the enhanced extraction of cadmium and zinc by willow seedlings inoculated with PGPB was associated with a variety of mechanisms. Studies on phytoremediation have also noted that the presence of rhizosphere bacteria can enhance Se content in plants (de Souza et al., 1999). However, to our knowledge, there has not yet been any research aimed at evaluating the potential of PGPB with ACC deaminase activity for biofortification of Se.

In Chile, the Se content in soils varies widely. Soils from northern Chile have been described as one of the major reserves of Se in the world, containing 20,000 metric tons of Se (USGS, 2011). In contrast, ash-derived volcanic soils (Andisols) from southern Chile exhibit suboptimal levels of plant-available Se (Wittwer et al., 2002; Cartes et al., 2005; Mora et al., 2008). Chilean Andisols are also characterized by their acidity (produced by the use of urea and other ammonia fertilisers) that can increase the concentration of toxic metal cations (Al^{3+} and Mn^{2+}) in the soil solution, limiting crop yields (Mora et al., 2006). Recently, Cartes et al., (2010) reported that Se alleviated the Al-induced oxidative stress in ryegrass roots growing in an acidic Andisol.

Thus, strategies are needed to increase soil Se content to counteract the Se deficiency and mitigate the Al-induced oxidative stress in crops grown in acidic Andisols.

In this study, we hypothesise that the rhizosphere of cereal crops grown in Chilean Andisols is a reservoir of Se-tolerant bacteria with potential applications in biotechnology, such as phytoremediation, biofortification and other technological applications. The main objectives of this study were as follows: (1) to isolate Se-tolerant bacteria with ACC deaminase activity from the rhizosphere of cereals growing in acidic Andisols and (2) to evaluate the ability of bacteria to accumulate Se and the potential use of these bacteria as inoculants for Se biofortification in cereal wheat. The occurrence of selenobacteria populations in an Andisol from southern Chile was examined by culture-dependent approaches in soils and rhizosphere soils of cereal plants.

3.1 Materials and methods

3.1.1 Soil sample description

The soils used in this study were collected from Rio Bueno (39° S, 72° W), Región de Los Ríos and classified as an Andisol belonging to the Piedras Negras series with the following properties: 4 mg kg^{-1} of Olsen phosphorus, pH_{H20} 5.0, 16 % organic matter, 15 % aluminium saturation, 2.0 cmol $_{(+)}$ kg^{-1} cation exchange capacity and 0.94 mg kg^{-1} of total Se. The samples were aseptically collected in triplicate (20 cm topsoil), stored in coolers at 4°C and immediately transported to the laboratory for analysis.

3.1.2 Denaturing gradient gel electrophoresis of the bacterial communities from Se-treated soils

Soil samples were sieved (<2 mm), and from each sample, three 20 g subsamples were placed in sterile plastic tubes. These subsamples were supplemented with 0.2, 2 and 20 mM of sodium selenite ($Na_2SeO_3 \cdot 5H_2O$; SigmaAldrich, USA), prepared as a 1 M stock solution (sterilized by filtration), and were added with nutrient broth (diluted 10-fold, Oxoid Ltd.) to stimulate the growth of bacterial populations. The soil moisture levels

were adjusted to 50 % of the water-holding capacity, after which the samples were incubated for 1 week at 30°C.

Bacterial community structures were examined by PCR−denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes. Total DNA was extracted using a PowerSoil® DNA Isolation Kit (Mo Bio Inc., USA) according to the manufacturer instructions. PCR−DGGE was performed according to the method previously described by Jorquera et al. (2010) using the primer sets 358F-GC and 907R (Muyzer et al. 1998). In brief, the DGGE electrophoresis was run for 10 h at 100 V, and 20–70 % gradient (urea and formamide) gels were stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on a UV transilluminator. Representative bands in DGGE gels were carefully excised, re-amplified and sequenced by Macrogen, Inc. (Korea). The consensus nucleotide sequences were deposited under accession numbers ranging from JN644925 to JN644944 and were compared with those present in the GenBank database from the National Center for Biotechnology Information (NCBI, http://blast. ncbi.nlm.nih.gov) by using the BLAStn tool. UPGMA cluster analysis and principal components analysis (PCA) of DGGE banding profiles was also carried out by using Phoretix 1D analysis software (TotalLab Ltd.) and JMP statistical software (version 5.0; SAS Institute, Inc.), respectively. Samples were analysed in triplicate, and samples from soils that were not amended with Se were included as a control.

3.1.3 Culturable bacteria

Samples from the rhizospheres of wheat, barley and oat plants were aseptically collected (in triplicate), and rhizosphere soil was removed from the roots by shaking the roots for 5 min in 50 mL of sterile saline solution (0.85 % NaCl) (see appendices i). The occurrence of culturable selenobacteria was examined by plating serial dilutions of the root washings onto nutrient agar (Oxoid Ltd.) plates supplemented with 2 mM of sodium selenite, according to Vallini et al., (2005). Plates were incubated for 4 days at

30°C, after which colonies were counted. Diverse colonies were isolated based on their phenotypes (size, edge and colour) and used in further study.

3.1.4 ACC deaminase activity

Eight representative selenobacteria colonies showing red and whiteorange centres were chosen and screened for PGPB traits based on ACC deaminase activity. This activity was determined for cultures grown in Dworkin–Foster (DF) salts minimal medium containing ACC compounds as the sole N source (Glick 2003). Four strains showing the ability to grow in DF broth were screened by PCR to examine the presence of the genes encoding ACC deaminase activity. PCR reactions were performed as previously described by Blaha et al., (2006) using the primer set F1936/F1939 specific for the *acd*S gene.

Four representative isolates (denoted as B19, R12, B16 and R8) were selected, purified by streaking on agar and identified by sequencing of their 16S rRNA genes. The 16S rRNA genes were amplified using the universal primers 27f and 1492r (Peace et al., 1994), and the DNA fragments obtained were sequenced, deposited under accession numbers from JN644921 to JN644924 and compared with the GenBank database. These selected isolates were used for further assays.

3.1.5 Analysis of Se tolerance of selected selenobacteria

The selected selenobacteria strains were grown in nutrient broth supplemented with 2, 5 and 10 mM Se. The bacterial cultures were incubated with shaking (100 rpm) at 37°C, and bacterial growth was estimated by quantification of total protein content in the microbial biomass. After 8 and 24 h of incubation, bacterial cells were collected by centrifugation (3,000× *g* for 10 min) and were lysed by sonication (20 kHz for 2 min). Cell debris was discarded by centrifugation (10,000× *g* for 10 min), and total protein concentration in the supernatant was precipitated with ammonium sulphate (0–85 %)

and subjected to the Bradford method with bovine serum albumin as the protein standard (Bradford, 1976). The cultures were produced in triplicate, and controls without Se were also evaluated.

The minimum inhibitory concentration (MIC) of selected selenobacteria isolates was determined on Tris-buffered low-phosphate agar medium (TBLP, Mergeay et al., 1985) supplemented with sodium selenite at increasing concentrations (in millimolar): 2, 5, 10, 20, 30, 40, 50, 100, 150 and 200. Serial dilutions were spread on agar plates and incubated for 4 days at 30°C. The growth of the bacterial colonies was recorded and compared to the values for colonies produced on control plates without Se. The Se tolerance criterion used in this study was defined as the growth rate (cellular biomass) equal to or greater than that of the control without Se for each bacterium.

3.1.6 Se accumulation and microscopic analysis of selected selenobacteria

Based on growth rates, the selected bacteria grew at 2 mM Se concentration as described. Changes of Se content in the supernatant (6, 8 and 24 h) were measured by atomic absorption spectrophotometry (AAS) with a HG3000 Hydride generator (GBC Scientific Equipment Ltd.). An air−acetylene flame was used, and the readings were recorded at a wavelength of 196 nm as described by Kumpulainen et al., (1983).

In parallel, samples of fresh cultures of selected selenobacteria isolates were examined by epifluorescence (Olympus BX41, Japan) and by laser scanning confocal microscopy (Olympus FluoView FV1000, Japan). Selected isolates were further examined by scanning electron microscopy (Jeol JSM-6380LV, Japan) and analysed by an energy dispersive X-ray (EDX, Spectrometer Link Analytical Model AN 10/ 85S) to determine the elemental composition of spherical deposits that were observed on or in the cells of selenobacteria colonies from agar media containing Se.

3.1.7 Wheat rhizosphere inoculation assay

Two selected strains showing lower and higher Se accumulation rates and an ability to produce micro- and nanospherical deposits of Se (*Pseudomonas* sp. R8 and *Stenotrophomonas* sp. B19, respectively) were used as a Se carrier to increase the Se content in wheat tissue under greenhouse conditions. Wheat seeds were surface sterilised by treatment with 70 % ethanol for 30 s and 20 % hypochlorite for 20 min, followed by three washes with sterile distilled water. Then, the seeds were germinated for 4 days on a moistened filter paper and transferred to hydroponic systems containing standard Hoagland solution (Hoagland and Arnon, 1938). Bacterial cells from fresh cultures grown in nutrient broth supplemented with 2 mM of sodium selenite were repeatedly washed in the sterile saline solution and directly inoculated in the Hoagland solution. *Stenotrophomonas* sp. B19 grown in the nutrient broth supplemented with 5 and 10 mM of sodium selenite was also used in inoculation experiments.

After incubation, the cultures were centrifuged (3,000× *g* for 10 min), twice washed in 50 mL of sterile saline solution (0.85 % NaCl) and re-suspended in 30 mL of saline solution. Then, 10 mL of bacterial suspension (~10^6 cfu mL^{-1}) was applied to the rhizosphere solution and maintained for 7 days to enable bacterial colonisation of plant roots. After colonisation, the plants were transferred to pots containing 500 g of soil and incubated under greenhouse conditions (20°C, 70 % relative humidity and 16:8 h of light/dark). All treatments were performed in triplicate, and controls without bacterial inoculation were also evaluated. After 14 days, roots and leaves were harvested, washed with sterile distilled water and dried (65°C for 24 h) and Se content in tissues was analysed by AAS as described above.

3.1.8 Statistical analysis

The data were analysed by a one-way ANOVA, and comparisons were carried out for each pair with Tukey test using JMP statistical software (version 5.0; SAS Institute, Inc.). All experiments were carried out in triplicate, and the values are given as means ± standard errors. Differences were considered to be significant when the *P* value was less than or equal to 0.05.

3.2 Results

3.2.1 Selenobacteria in Chilean Andisols

The addition of Se to the soils produced changes in the soil bacterial communities that could be observed by PCR−DGGE. Many bands in the DGGE gel appeared or became more intense with selenite addition. These bands were sequenced and tentatively identified by comparison to reference sequences in the GenBank public database. The main effect of the addition of 0.2 and 2 mM Se on the bacterial community composition appeared to be the stimulation of the bands numbered 1, 2, 3 and 9 (Figure 3.1 a). The sequences from these bands aligned with various species of Firmicutes, primarily belonging to the Clostridia family. However, at the addition of 20 mM Se, other bands were stimulated corresponding to members of *Paenibacillaceae* (band no. 4) and *Brucellaceae* (band no. 8), while bands representing Clostridia (band nos. 1, 2, 3 and 7), *Burkholderiales* (band no. 6), *Chitinophagaceae* (band no. 13) and *Oxalobacteraceae* (band nos. 14 and 15) were inhibited (Fig. 1a, Table 1). Other bacterial taxa that were represented by predominant bands in the gels did not show a response to Se; these taxa included *Bacillaceae* (band no. 11), *Rhizobiaceae* (band no. 12) and *Gemmatimonadetes* (band no. 19). Moreover, differences between bacterial communities structure of

selenite addition treatments and the control were generally consistent as shown by hierarchical cluster analysis visualised in the dendrogram (Fig. 1b). The cluster analyses demonstrate that selenite treatments showed a higher similarity among each other than with control treatments; also, the doses of 0.2 and 2 Mm clustered together. In this context, PCA analysis revealed a strong effect of Se doses in the bacterial composition variability measured by 16S rRNA DGGE. The progressive increase of selenite doses represented an increment in data correlation with PC1 (73 %) and decrease in correlation with PC2 (9.5 %).

The range of counts of culturable STB was 2–8 × 10^4 cfu g^{-1} of rhizosphere soil (see appendices ii). The higher counts were observed in wheat and oat rhizospheres, and the lower counts were observed in barley rhizospheres. Culturable selenobacteria showed diverse phenotypes on Se-supplemented agar, mainly showing red and white-orange centres (Figure 3.2 a).

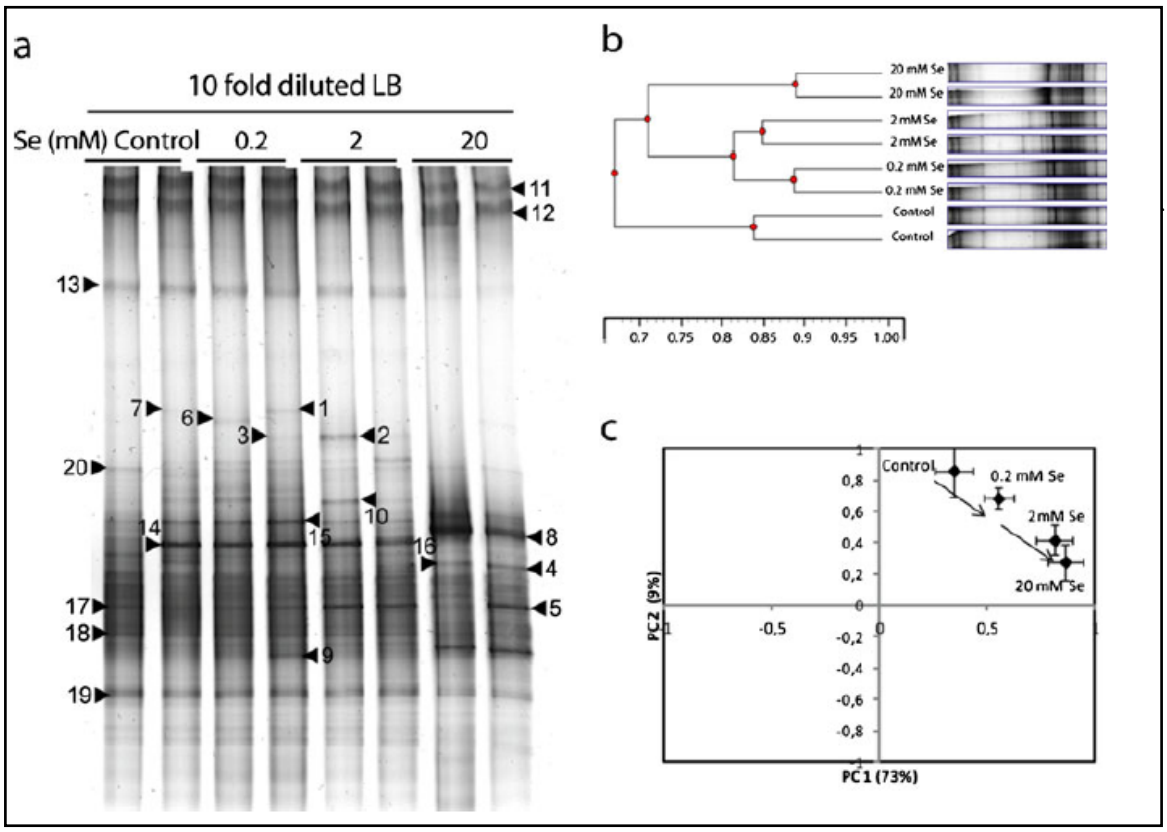

Figure 3.1 DGGE banding patterns (a), cluster analysis (b) and principal component analysis(c) of bacterial communities present in soil supplemented with Se (0, 0.2, 2.0 and 20 mM) and nutrients (LB broth diluted 10-fold) after 7 days of incubation at 30°C.

3.2.2 ACC deaminase activity and Se tolerance of selected selenobacteria

The isolated were selected on based with capabilities to metabolized Se and the presence of multiple mechanisms of PGPR (see appendices iii).The ACC deaminase activity of three out of four representative strains showing the ability to grow in DF using ACC as the sole N source was confirmed by PCR using the specific primer set for the *acd*S gene (Figure 3.3). Identification of these selected strains by analysis of their 16S rRNA genes revealed bacteria related to *Stenotrophomonas* (isolate B19), *Bacillus* (isolate R12), *Enterobacter* (isolate B16) and *Pseudomonas* (isolate R8).

At 10 mM Se concentration, *Stenotrophomonas* sp.B19 had the highest growth rate compared to the other strains (*Bacillus* sp. R12, *Enterobacter* sp. B16 and

Pseudomonas sp. R8). This result was particularly apparent at 8 h of incubation, when the relative growth rates were 51.3 and 1.7–8.6 %, respectively, compared to cultures grown in broth without Se. Significant differences ($P \leq 0.05$) between selenobacteria were observed in growth rates for cultures grown with 2 mM Se for 24 h (Table 3.2). These isolates showed also a high tolerance of Se in the TBLP medium, with an MIC of 10 mM Se. Growth of mutants of the isolate B19 was observed at concentrations of 20 mM Se. It should be noted here that colonies with white-orange centres (*Stenotrophomonas* sp. B19 and *Enterobacter* sp. B16) became redder in colour after exposure to high doses of selenite, which suggests adaptation to Se toxicity.

Table 3.1 Phylogenetic assignment of DGGE bands

Band[a]	Taxonomic group[b]	Closest relatives or cloned sequences (Accession no.)	Similarity (%)[c]	Accession no.
1	*Firmicutes; Clostridia*	Uncultured clostridium from anoxic rice field soil (AY607209)	96	JN644925
2	*Firmicutes; Clostridia*	Uncultured bacterium from rice paddy soil (AB517718)	99	JN644926
3	*Firmicutes; Clostridia*	Uncultured bacterium from rice paddy soil (AB486224)	78	JN644927
4	*Firmicutes; Bacillales; Paenibacillaceae*	*Brevibacillus* from biological soil crust (AJ871421)	90	JN644928
5	*Firmicutes; Bacillales; Paenibacillaceae*	*Aneurinibacillus aneurinilyticus* from soil (GU549488)	83	JN644929
6	*Proteobacteria; Betaproteobacteria; Burkholderiales*	Uncultured bacterium from rice paddy soil (AB487774)	87	JN644930
7	*Firmicutes; Clostridia*	Uncultured bacterium from sediment (JF326054)	79	JN644931
8	*Proteobacteria; Alphaproteobacteria; Brucellaceae*	*Ochrobactrum* sp. from non-rhizospheric soil	99	JN644932
9	*Firmicutes; Clostridia*	Uncultured bacterium from rice paddy soil (AB486724)	91	JN644933
10	*Proteobacteria;Alphaproteobacteria; Bradyrhizobiaceae*	*Bradyrhizobium* sp. from grassland soil (FN600560)	78	JN644934
11	*Firmicutes; Bacillaceae*	Uncultured *Bacillus* from bauxite soil (GU477359)	82	JN644944
12	*Proteobacteria; Alphaproteobacteria; Rhizobiaceae*	Uncultured bacterium from greenhouse soil (FR871436)	75	JN644935
13	*Bacteroidetes; Sphingobacteria; Chitinophagaceae*	Uncultured bacterium from soil (FJ380145)	94	JN644936
14	*Proteobacteria; Betaproteobacteria; Oxalobacteraceae*	Uncultured bacterium from rice paddy soil (AB487774)	90	JN644937
15	*Proteobacteria; Betaproteobacteria; Oxalobacteraceae*	Uncultured bacterium from rice paddy soil (AB608689)	96	JN644938
16	*Firmicutes; Bacillaceae*	Uncultured bacterium from rice paddy soil (AB488378)	88	JN644939
17	*Proteobacteria;Alphaproteobacteria; Bradyrhizobiaceae*	*Bradyrhizobium elkanii* from root nodule (AY904789)	85	JN644940
18	*Acidobacteria*	Soil bacterium (HM748732)	96	JN644941
19	*Gemmatimonadetes*	Uncultured Gemmatimonadales from rhizosphere (EF018673)	99	JN644942
20	*Proteobacteria; Betaproteobacteria*	Uncultured beta proteobacterium from cropland (EF662768)	95	JN644943

[a] Corresponding DGGE bands shown in Figure 3.1
[b] The phylogenetic assignment is based on sequence analysis by using the BLASTn tool from NCBI (http://www.ncbi.nlm.nih.gov) or the RDP classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp). The phylum is given along with the lowest predictable phylogenetic rank

3.2.3 Se accumulation and microscopic analysis of selected selenobacteria

Atomic absorption spectrophotometry analysis showed significant differences in the reduction of Se soluble concentrations in culture broth by *Stenotrophomonas* sp. B19 strain (33 % of reduction) after 8 h of incubation, with respect to *Enterobacter* sp. B16 strain (8 % of reduction), *Pseudomonas* sp. R12 strain (0 % of reduction) and *Bacillus* sp. R8 strain (0 % of reduction). After 24 h, the isolates showed growth rates similar to controls without Se (100 %; see in appendices iv), and the soluble Se concentrations in the broths had been reduced by 70–80 % by these three isolates. *Pseudomonas* sp. R8 showed lower Se transformation capacity, with Se removal of only 50 % after 24 h (Figure 3.4).

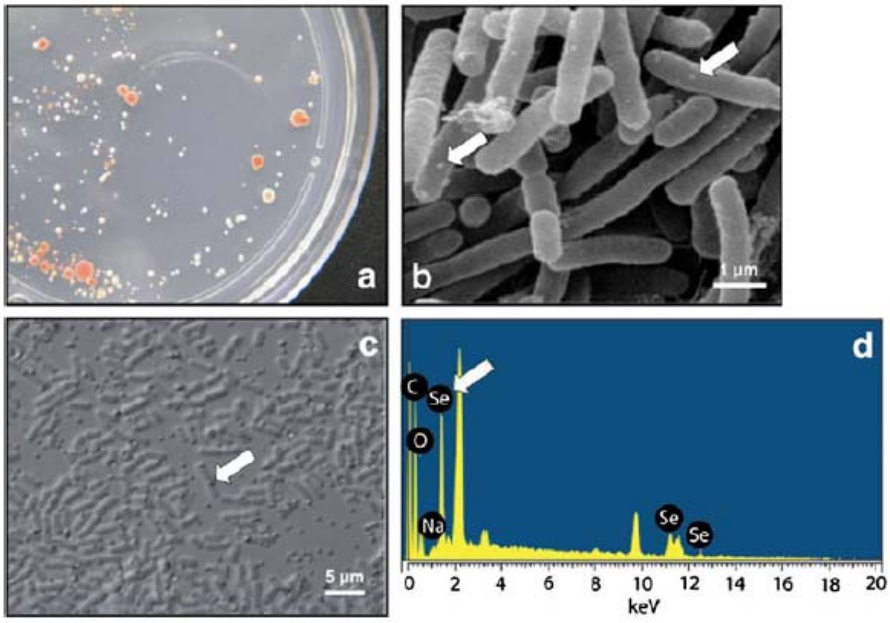

Figure 3.2 Selenium-tolerant bacteria (selenobacteria) cultured in nutrient media supplemented with 2 mM of sodium selenite. *White* and *red* colonies of STB grown on Se-supplemented agar. Visualisation by scanning electronic microscopy (b) and laser scanning confocal microscopy (c) of spherical deposits (*arrows*) produced by selenobacteria in Se-supplemented broth. (d) Analysis of elemental composition by energy dispersive X-ray (EDX) of spherical deposits produced by selenobacteria in Se-supplemented nutrient broth. *Arrow* indicates selenium microsphere.

Observations of the cells by scanning electronic microscopy and laser scanning confocal microscopy showed the formation of numerous micro- and nanostructures around and within the bacterial cells incubated in the presence of Se (Figure 3.2b, c). Observations under laser scanning confocal microscopy and EDX analysis also revealed spherical deposits of elemental Se (Se^0) within these structures (Figure 3.2c).

Table 3.2 Relative growth percentage (in percent) by selected rhizobacteria after incubation for 8 and 24 h at 30°C in nutrient broth (pH 7.0) supplemented with 2, 5 and 10 mM of Se.

Isolate	8 h			24 h		
	2 mM	5 mM	10 mM	2 mM	5 mM	10 mM
B19	35.7[a]±6.8[b] B, C, a	50.2±8.7 C, a	51.3±8.1 C, a	83.5±9.7 A, a	70.3±5.0 A, B, a	49.7±9.7 A, B, C, a
R12	24.5±2.6 C, D, a	8.5±1.8 E, b	8.6±0.6 E, b	90.0±1.2 A, a	38.7±1.2 B, c	30.2±3.5 C, a
B16	20.8±3.7 B, C, a, b	17.7±2.0 C, b	7.7±0.4 C, b	93.6±3.9 A, a	49.0±5.2 B, b, c	39.7±6.7 B, a
R8	15.7±0.8 D, b	9.4±0.7 D, b	1.7±0.4 D, E, b, c	102.2±6.7 A, a	65.5±8.5 B, a, b	35.6±3.9 C, a

Capital letters denote significant difference in the same row, and lower letters denote significant difference in the same column ($P \leq 0.05$)
[a] Measured as total cell proteins (in milligrams per millilitre) compared to control without Se addition (defined as 100 %)
[b] Mean ± standard error (n=3)

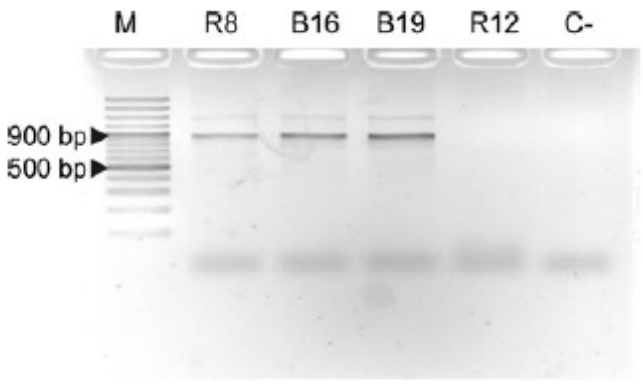

Figure 3.3 Detection of the *acd*S gene by PCR in selenium-tolerant bacteria: *Pseudomonas* sp. R8, *Enterobacter* sp. B16, *Stenotrophomonas* sp. B19 and *Bacillus* sp. R12. M molecular marker, C- negative control.

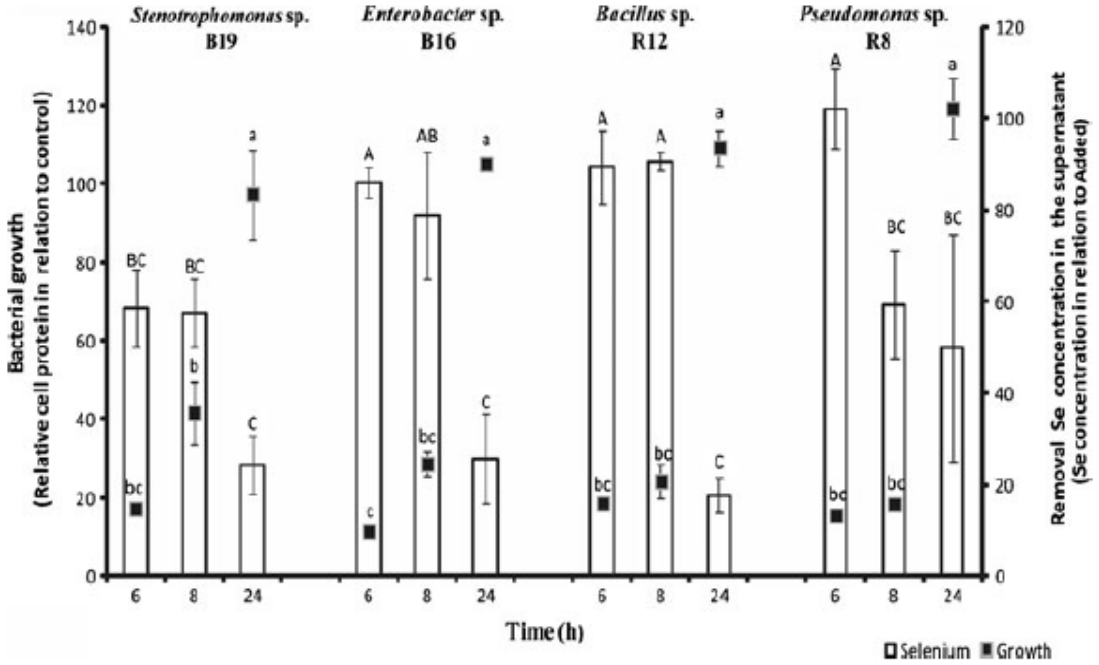

Figure 3.4 Bacterial growth and changes in selenium content in the supernatant of liquid cultures supplemented with 2 mM of sodium selenite. Different *letters* denote statistical difference ($P \leq 0.05$, Tukey test) ($n=3$). Capital *letters* denote significant difference in the selenium content, and lower letters denote significant difference in the growth between strains.

3.2.4 Wheat rhizosphere inoculation assay

Analyses showed significantly higher Se content in roots and leaves of plants inoculated with both STB strains, *Pseudomonas* sp. R8 and *Stenotrophomonas* sp. B19, compared with uninoculated controls (Figure 3.5a, b). A significantly higher Se content in both tissues was also observed when plants were inoculated with *Stenotrophomonas* sp. B19 grown at concentrations of 5 and 10 mM of sodium selenite, compared to those grown at 2 mM. It is noteworthy that in all plants, the Se content in roots was significantly higher than in leaves. Inoculated plants did not show significant differences in biomass with respect to uninoculated controls (Figure 3.5c, d).

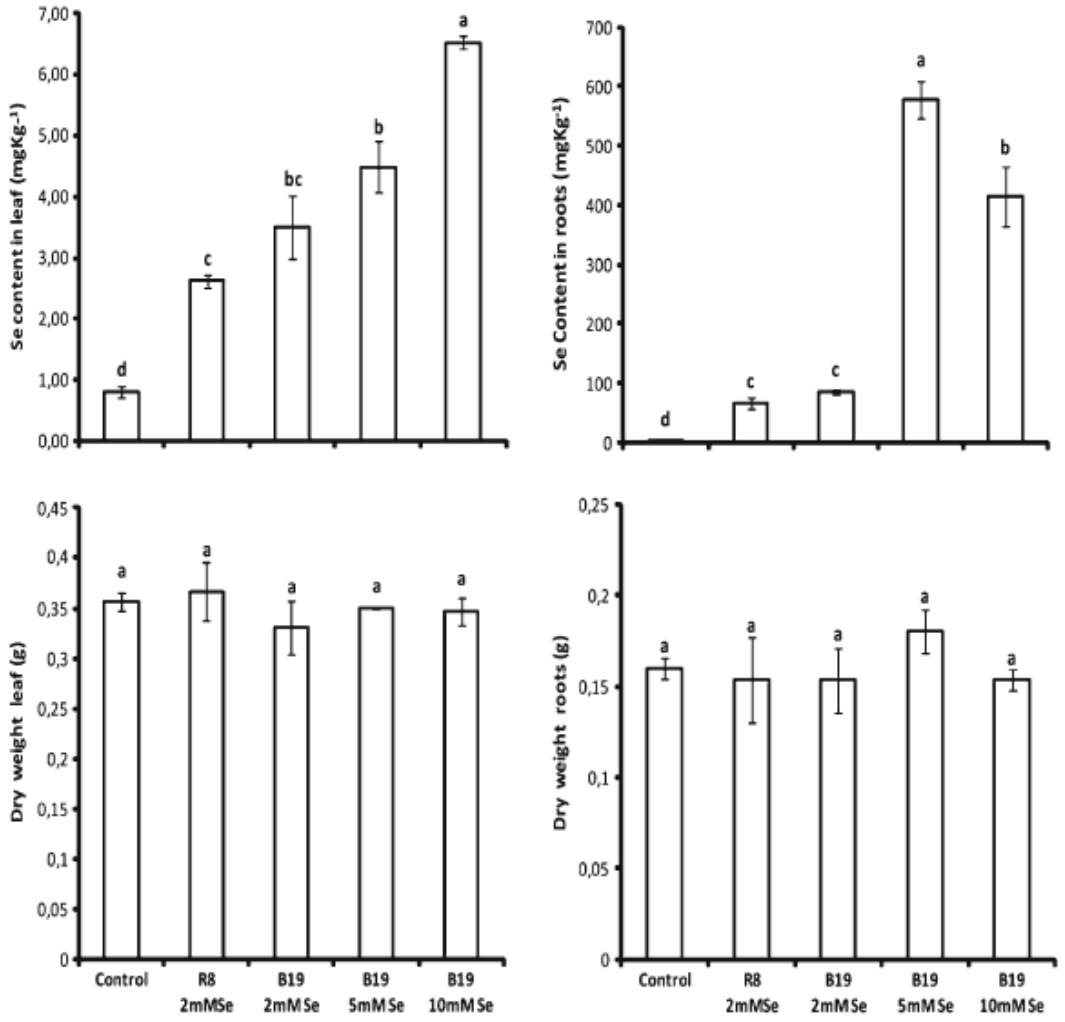

Figure 3.5 Selenium content (a, b) and plant biomass (c, d) of roots and leaves of wheat plantlets after 14 days of inoculation with *Pseudomonas* sp. R8 and *Stenotrophomonas* sp. B19. Different *letters* denote statistical difference ($P \leq 0.05$, Tukey test) ($n=3$).

3.3 Discussion

This study showed that the addition of Se to bacteria from a Chilean Andisol soil altered the bacterial community structure that developed following a nutrient pulse to initiate bacterial growth. Siddique et al., (2005) observed greater bacterial diversity during Se reduction when rice straw was added as an energy and C source to agricultural drainage water. Previous studies have shown that Firmicutes (*Bacillus* and *Paenibacillus*) and Proteobacteria (*Pseudomonas* and *Enterobacter)* stimulate Se transformation through oxidation, reduction and methylation (Losi and Frankenberger, 1997; Fordyce, 2007;

Fernandez-Martinez and Charlot, 2009). In this context, our study reported the stimulation of various Firmicutes and Proteobacteria.

Although the Chilean Andisol studied here is considered to be Se deficient, our investigation revealed the occurrence of abundant culturable bacteria with high Se tolerance. We also observed significantly lower loads of culturable selenobacteria in the rhizosphere of barley compared with the rhizosphere of wheat and oats. It is well established that root exudates and plant species strongly determine bacterial community composition in the rhizosphere (Marschner et al., 2001; Barret et al., 2011). However, De Ridder-Duine et al., (2005) showed that the bacterial community diversity of *Carex arenaria* rhizosphere appeared to be determined for bulk soil community composition. Therefore, the main factor that probably influences bacterial communities is the complex interaction between soil type, plant species and root zone location (Nannipiere et al. 2008a, b).

Thus, Se biofortification would likely alter rhizosphere community structures differently for different plant and soil combinations, but it would hypothetically still enrich the same types of bacteria that are able to use Se as an electron acceptor. On the other hand, cultures of selenobacteria on Se-spiked agar media produced cells having both white and red colouration. It is known that the reduction of Se (selenite and selenate) by bacterial activity precipitates Se^0 as a tolerance mechanism, which is revealed by a red colouration in culture medium (Vallini et al., 2005). The occurrence of colonies with white-orange centres suggests the presence of selenobacteria with different Se tolerance mechanisms.

Three of the selected selenobacteria showed ACC deaminase activity and presence of the *acd*S gene. The occurrence of bacteria with ACC deaminase activity is commonly reported in the rhizosphere of diverse plants (Martínez-Viveros et al., 2010), and it has been suggested as a key PGPB trait for plants under environmental stress,

including heavy metal toxicity (Dell'Amico et al., 2008; Rodriguez et al., 2008; Rajkumar et al., 2009; Zhang et al., 2011a, b). The partial sequencing of the 16S rRNA genes of selected STB revealed similarities with known bacteria belonging to the genera *Stenotrophomonas*, *Bacillus*, *Enterobacter* and *Pseudomonas*. Diverse *Stenotrophomonas* strains have already been reported to be involved in Se transformations in soil. Antonioli et al., (2007) and Dungan et al., (2003) isolated *Stenotrophomonas maltophilia* strains from Se-contaminated mining soils and seleniferous agricultural drainage pond sediments, respectively. Investigators have also described the biotransformation of Se by *Enterobacter cloacae* and suggested the potential use of this bacterium for bioremediation of seleniferous agricultural drainage water (Ghosh et al., 2008). Members of the genera *Pseudomonas* and *Bacillus* are well known to be resistant to various metals and metalloids (Stolz et al., 2006). Garbisu et al. (1999) reported the ability of *Bacillus subtilis* to tolerate high concentrations of Se, and Hunter and Mater, (2011) recently showed an unusual level of tolerance to selenite (>150 mM) by *Pseudomonas* sp. $CA5^{T}$; they proposed *Pseudomonas seleniipraecipitatus* as the scientific name for this new strain.

Our results showed a high Se tolerance (MIC of 10 mM) and a rapid reduction of Se (50–80 %) in culture broths of selected selenobacteria supplemented with 2 mM Se after 24 h. Reduction of selenite after 24 h of incubation by bacteria isolated from seleniferous habitats has been previously observed (Dungan et al., 2003; Dhanjal and Cameotra, 2010). However, the occurrence and role of selenobacteria in Chilean Andisols are poorly understood. It is known that Se shows a similar chemical behaviour to sulphur and tellurium, and ash-derived volcanic soils are a natural reservoir of sulphur compounds (sulphites and sulphates) and bacteria involved in sulphur cycling (sulphur-oxidising and sulphur-reducing bacteria) (Wend-Potthoffr and Koschorreck, 2002). We hypothesise that these high rates of Se tolerance and transformation by

bacteria could be related to sulphur cycling in Andisols. However, the reason for high Se tolerance and efficient Se transformation by bacteria isolated from Andisols is still unknown.

Microscopic analysis of the red-coloured colonies revealed the accumulation of Se0 in micro- and nanospherical deposits. The ability to accumulate Se^0 in spherical deposits in a wide variety of bacteria isolated from seleniferous soils (*Ralstonia*, *Rhodospirillum*, *Stenotrophomonas*, *Rhodobacter*, *Bacillus*, *Sulfurospirillum* and *Selenihalanaerobacter*) has been reported in many studies (Kessi et al., 1999; Bebien et al., 2001; Roux et al., 2001; Dungan et al., 2003). Studies have also indicated the potential of Se deposited in nanostructures for a wide variety of electronic, optical, catalytic and medical applications (Prakash et al., 2010). Se^0 is a nontoxic form of Se and could be an adequate candidate for biofortification of cereal crops using bacteria as bacterial inoculation. Despite the fact that Se was mainly deposited as unavailable Se^0 by selenobacteria, our study demonstrated that the inoculation with Se-enriched selenobacteria resulted in the translocation of Se into leaves of wheat. Studies carried out in sterile soils, inert media and hydroponic systems supplemented with Se have reported that plants show higher phytoextraction of Se when inoculated with rhizobacteria (de Souza et al., 1999; Azaizeh and Hemons, 2003; DiGregorio et al., 2005; Lampis et al., 2009). Investigations have demonstrated that Se^0 nanoparticles could show similar bioavailability to other bioavailable Se forms (e.g. selenite) and could facilitate absorption of Se in plants, animals, humans and microorganisms (Zhang et al., 2004). The selective uptake and translocation of metal nanoparticles (Au, Ag, Cu and Fe) in edible plants has recently been revised by Rico et al., (2011). Thus, there is evidence that the production of Se deposits as nanospheres by selenobacteria could enable better incorporation of Se by plants. However, how the Se accumulated in bacteria (including Se^0 in deposits and other intracellular forms, such as organic and

methylated Se forms) was translocated in wheat seedling remains unknown. This research suggests that the presence of PGPB with ACC deaminase activity could not only protect plants against the stress produced by phytotoxic cations (Al^{3+} and Mn^{2+}) (Mora et al., 2008) commonly found in Andisols but could also boost plant Se content and alleviate the stress caused by toxic forms of Se produced during transformation of Se^0.

3.4 Conclusions

Interestingly, Se-tolerant bacteria isolated from Se-deficient Andisols from southern Chile were from similar genera to the ones previously reported in seleniferous soils. Our study showed that these bacteria, belonging to the genera *Stenotrophomonas*, *Bacillus*, *Enterobacter* and *Pseudomonas*, have a great ability to tolerate and accumulate Se intra- and extracellularly in micro- and nanospherical Se^0 deposits.

The inoculation of wheat plantlets with Se-enriched bacteria inocula showed an increased tissue Se accumulation. Therefore, plants inoculated with Se-tolerant bacteria were capable of incorporating and translocating the Se associated to bacterial inocula into leaves. These Se-enriched bacterial inocula can be used as a biotechnological tool for plant Se biofortification.

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Chapter IV

Enhanced selenium content in wheat grain by co-inoculation of selenobacteria and arbuscular mycorrhizal fungi: A preliminary study as a potential Se biofortification strategy.

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Enhanced selenium content in wheat grain by co-inoculation of selenobacteria and arbuscular mycorrhizal fungi: A preliminary study as a potential Se biofortification strategy

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Abstract

Cereal crops grown in southern Chilean Andisol provide suboptimal levels of this metalloid for human diet. Certain rhizosphere microorganisms, such as rhizobacteria and arbuscular mycorrhizal fungi can increase the selenium uptake in plants. The purpose of this study was to evaluate selenium acquisition by wheat plants through the co-inoculation of native selenobacteria strains (*Stenotrophomonas* sp. B19, *Enterobacter* sp. B16, *Bacillus* sp. R12 and *Pseudomonas* sp. R8), both individually and in mixture, as selenonanosphere source with one arbuscular mycorrhizal fungus (*Glomus claroideum*). Total selenium content in plant tissues and substrate was analyzed. According to our results, significant higher selenium content was found in inoculated plants in comparison to uninoculated controls ($P < 0.05$). Independently of

fungal presence, selenium content in grain from plants inoculated with Enterobacter sp. B16 (236 mg kg^{-1}) was higher than the rest of the strains (116-164 mg kg^{-1}). However, when plants were coinoculated with a mixture of selenobacteria strains and *G. claroideum*, selenium content in grain was 23.5% higher (725 mg kg^{-1}) than non-mycorrhizal plants (587 mg kg^{-1}). The results suggest a synergistic effect between the selenobacteria mixture and *G. claroideum* associated to major biodiversity and demonstrate a great potential of these rhizosphere microorganisms for biofortification of cereals and its derivates.

4. 1 Introduction

In recent decades, it has been suggested that selenium (Se) has an important function in a wide range of physiological processes associated with antioxidant activity in organisms. Thus, Se dietary deficiency increases the risk of oxidative damage and different human pathologies, such as cancer, HIV and heavy metal toxicity (Méplan and Hesketh, 2012). Recommended Dietary Allowances (RDAs) indicates that 55 µg Se day^{-1} is an adequate dose for adult men and women. However, this value does not consider its different chemical forms (Thiry et al., 2012).

Selenium content in plants is highly dependent on soil Se concentration. Thus, Se dietary intake varies greatly across the different regions of the world (Méplan and Hesketh, 2012). In volcanic soils from southern Chile (Andisol), Se can form stable complexes with clays and/or can be strongly adsorbed, resulting in low Se bioavailability to plants (Cartes et al., 2005; Mora et al., 2008). This is especially significant because Se is incorporated into human metabolism mainly as a dietary constituent of vegetables and cereals (Govasmark and Salbu, 2011). Thus, biofortification of wheat is a good alternative for increasing Se content in human diet

because Chile is the second largest bread consumer in the world (100 kg of bread per person $year^{-1}$ with a yield of 940,000 Mg of cereals $year^{-1}$ in southern Chile) (ODEPA, 2012).

Agronomic biofortification, through the application of Sefertilizers, has been used to raise Se content in plants in different countries such as Australia, Finland, and New Zealand (Hartikainen et al., 1997). In Chile, studies have shown that selenite-pelleted seeds increase both Se content in forage and the antioxidant ability of white clover and ryegrass (Cartes et al., 2011; Mora et al., 2008). However, Se is toxic at high concentrations and inorganic selenite is bound to soil constituents, thus it is unavailable to plants, whereas selenate may be leached under wet fall conditions (Govasmark and Salbu, 2011; Hawkesford and Zhao, 2007).

Selenium speciation, mobility and bioavailability in soils are highly affected by the presence of microorganisms in the soil environment (Dungan et al., 2003). Acuña et al. (2012) recently reported that the inoculation of selenobacteria harboring Se in micro- and nanospheres of elemental Se (Se^0) and other intracellular forms (such as selenomethionine, selenocisteine and methylated forms) can be translocated toward leaves of wheat plants. On the other hand, arbuscular mycorrhizal fungi (AMF) favor the growth of the bacterial microflora adjacent to the fungus hyphae, accelerating their metabolic activity and nutrient cycling and can influence plant acquisition of some elements such as P, metalloids and heavy metals (Barea et al., 2005). To our knowledge, there are no reports on synergic effects between selenobacteria and AMF inoculation for enhancing Se content in food plants. Thus, the aim of this study was to evaluate the feasibility of co-inoculation of selenobacteria and AMF for enhancing Se content in wheat grain as an effective strategy for Se biofortification.

4.2 Experimental

4.2.1 Selenobacteria strains

Four native strains of selenium tolerant bacteria, called selenobacteria (*Stenotrophomonas* sp. B19, *Enterobacter* sp. B16, *Bacillus* sp. R12, and *Pseudomonas* sp. R8) previously isolated from the rhizosphere of cereal plants growing in an Andisol, were used (Acuña et al., 2012). The strains were grown in 200 mL of nutrient broth (Oxoid, Ltd., UK) supplemented with 5 mM of sodium selenite [Se(IV), Na_2SeO_3] and sodium selenate [Se(VI), Na_2SeO_4] (Merk, Inc.). After growth at 30°C for 24 h with continuous shaking (150 rpm), the bacterial cells were collected by centrifugation (1,500 × g) for 10 min, rinsed twofold with sterile saline solution (SSS) (0.85% NaCl) and resuspended in 30 mL of SSS (1-2 $\times 10^9$cfu mL^{-1}). This solution was used as selenobacteria inoculum for pot experiments.

4.2.2 Selenium content in selenobacteria biomass

The Se content accumulated in bacterial biomass was measured. Briefly, 1 mL of selenobacteria suspension in SSS was centrifuged (1,500 × g) for 10 min. The cell pellet was weighed and Se content was measured according to the methodology described by Kumpulainen et al. (1983). Cell pellet was digested in 10 mL of acid mixture (65% HNO_3, 70% $HClO_4$ and 95% H_2SO_4) and incubated overnight at room temperature. After incubation, the mixture was heated at 120 °C for 3 h, 220 °C for 5 h and then HCl (12%) was added up to 15 mL. Finally, the mixture was boiled at 120 °C for 20 min and Se content was measured by Atomic Absorption Spectrophotometry (AAS) with a HG 3000 Hydride generator (GBC Scientific Equipment Ltd.) using $NaBH_4$ solution as reducing agent. Two Se-enriched flour samples supplied by the Department of Applied Chemistry and Microbiology of Helsinki University (Finland) were used as reference.

4.2.3 Plant mycorrhization

Wheat (*Triticum aestivum* L. cv. Otto) seeds were surface disinfected by dipping in 0.8% (v/v) NaOCl for 15 min. Then, the seeds were rinsed, soaked with sterile distilled water and germinated in wet filter paper for 4d in a controlled temperature chamber (20 °C). After germination, the seedlings were placed in a box containing 25 g of sand:vermiculite:peat (SVP) (1:1:1) mixture with 25 g of *Glomus claroideum* inoculum. *G. claroideum* is a native arbuscular mycorrhizal fungi (AMF) isolated from the rhizosphere of *Sorghum bicolor* and *Trifolium repens* grown in agricultural volcanic soils of the Southern Chilean Region. The seedlings were maintained under greenhouse conditions for 7d and then plants were transferred to a pot containing 500 g of sterile SVP mixture for selenobacteria inoculation in the greenhouse experiment for twelve weeks.

4.2.4 Greenhouse experiment

A completely random experimental design was adopted. Two mL of 1-2 $\times 10^9$ cfu mL^{-1} grown in selenite and selenate were inoculated in each pot. The inoculum was directly injected in the rhizosphere of wheat plants. The selenobacteria inoculation included: (1) control (non-inoculated), (2) *Stenotrophomonas* sp. B19, (3) *Enterobacter* sp. B16, (4) *Bacillus* sp. R12, (5) *Pseudomonas* sp. R8 and (6) a mixture of the four strains. These treatments were applied to both mycorrhizal and non-mycorrhizal wheat plants. Pots with non-mycorrhizal plants were obtained as described above, except containers with 50 g of sterile SVP substrate. Pots were inoculated at 14, 24, 34, 44 and 54 days with 2 mL of selenobacteria inoculum as described above. The wheat plants were irrigated every 10 days with Taylor and Foy nutrient solution (Taylor and Foyd, 1985).

4.2.5 Selenium content in plants and SVP mixture

Plant samples (root, leaf + stem and spike) and SVP were collected and fresh and dry (65 °C for 48 h) weights were determined. Then, dried SVP samples were sieved (2 mm) and Se content was measured by AAS as described above (see 4.2.2)

4.2.6 Bacterial community composition analyses

The bacterial community composition in the rhizosphere (considered as the portion of SVP mixture influenced by plant roots) was examined by Denaturing Gradient Gel electrophoresis (DGGE) according to the method described by Acuña et al. (2013). For bacterial community analysis, fragments of 16S rRNA gene were amplified by touchdown PCR using EUBf933-GC/EUBr1387 primers set (Iwamoto et al., 2000).

The DGGE analysis was performed using a DCode system (BioRad Laboratories, Inc.). The PCR product (20 mL) was loaded onto a 6% (w/v) polyacrylamide gel with a 50−70% gradient (urea and formamide). The electrophoresis was run for 12 h at 100 V. The gel was then stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on a UV transilluminator. Clustering of DGGE banding profiles using a dendrogram was also carried out by using Phoretix 1D analysis software (Total Lab Ltd.). Analysis of microbial community diversity by Shannon-Weaver Index was also carried out according to the method described byYang et al. (2003).

4.2.7 Statistical analyses

The data were analyzed by a one-way analysis of variance (ANOVA), and comparisons were carried out for each pair with Tukey test using SPSS software (SPSS, Inc.). All experiments were carried out in triplicate, and the values were given as means ±

standard errors. Differences were considered to be significant when the P value was less than or equal to 0.05.

4.3 Results

4.3.1 Reduction capacity of selenobacteria strain

The *Bacillus* sp. R12 strain was the most effective in reducing selenite and *Pseudomonas* sp. R8 in reducing Se (VI). According to Se content in cell biomass, selenobacteria strains were able to accumulate high Se content with respect to control without selenium, i.e. 722−1224 and 52−357 mg kg^{-1} selenite and selenate, respectively (Table 4.1).

Table 4.1.Selenium in bacterial biomass and total Se added to pots in greenhouse experiment

Strains	Se biomass (mg kg^{-1})		
	Selenite	Selenate	Control
Stenotrophomonas sp. B19	908.7	115.3	0.54
Enterobacter sp. B16	948.4	209.9	0.21
Bacillus sp. R12	1224.4	52.8	0.54
Pseudomonas sp. R8	722.5	357.5	0.63

4.3.2 Root colonization and plant development

Mycorrhizal plants (27−35% colonization rate) showed significantly ($P \leq 0.05$) higher root length with respect to non mycorrhizal (i.e. 13−18 and 12−16 cm respectively); however, plants did not show significant differences in biomass production with respect to non mycorrhizal plants (Figure 4.1).

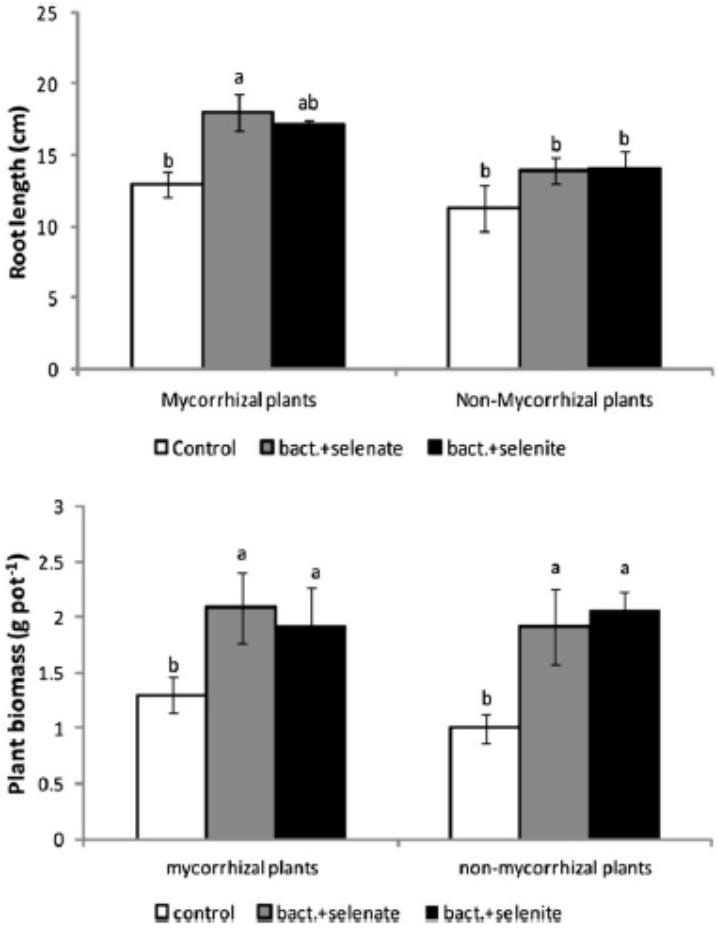

Figure 4.1 Root length (cm) and plant biomass (g pot^{-1}) in mycorrhizal and nonmycorrhizal plants. Tukey test to compare treatments means, values followed by the same letter do not differ at $P \leq 0.05$ ($n = 3$).

4.3.3 Se content in SVP substrate and plant tissues

Higher Se contents were recorded in plant tissues than SVP substrate in all treatments. The SVP mixture showed lower Se content (0.62−1.61 mg kg^{-1}) from pots with mycorrhizal plants compared to those with nonmycorrhizal plants (3.6−12.1 mg kg^{-1}) in both selenite and selenate (Table 4.2), while in SVP from plants inoculated with selenobacteria mixture, no significant differences were observed (Table 4.3).

Plants inoculated with selenobacteria grown in selenite showed higher Se content in tissues compared to those inoculated with selenobacteria grown in selenate

(Table 4.2 and 4.3). Selenium was found in all sampled tissues. In aerial tissues from mycorrhizal and non mycorrhizal plants inoculated with *Enterobacter* sp. B16 grown in selenite, significantly ($P \leq 0.05$) higher Se content were found, similar to mycorrhizal plants inoculated with this strain grown in selenate. In contrast, the lowest Se content was observed in plants inoculated with *Pseudomonas* sp. R8 and *Bacillus* sp. R12 (Table 4.2). In roots, the highest Se content was found in non mycorrhizal plants; whereas wheat plants inoculated with selenobacteria mixture showed no significant differences in roots between mycorrhizal and non mycorrhizal plants (Table 4.3).

Table 4.2

Se content (mg kg^{-1}) in SVP substrate and wheat plant tissues inoculated or non inoculated with selenobacteria strains and AMF (*Glomus clarideum*) grown in nutrient broth supplemented with selenite and selenate.

Strains	Mycorrhizal plants				Non mycorrhizal plants			
	Stenotrophomonas sp. B19	*Enterobacter* sp. B16	*Bacillus* sp. R12	*Pseudomonas* sp. R8	*Stenotrophomonas* sp. B19	*Enterobacter* sp. B16	*Bacillus* sp. R12	*Pseudomonas* sp. R8
Selenite								
SVP†	1.61‡e	0.7g	0.62g	0.87f	4.4c	6.2 b	3.64 d	12.1a
Root	133.8 (31.5)d	137.4 (19.7)d	133.7 (32.2)d	234.2 (46.7)c	23.7 (8.4)e	298.9 (33.1)b	342.9 (53.4)a	303.8 (46.1)b
Stem+leaf	129.8 (30.6)b	154.2 (22.1)a	85.2 (20.5)c	50.9 (10.2)e	70.8 (25.2)d	159.1 (17.6)a	71.5 (11.1)d	54.9 (8.3)e
Waste spike	47.9 (11.3)g	170 (24.4)b	76.2 (18.3)f	92.3 (18.4)e	70.7 (25.2)f	210.2 (23.3)a	113 (17.6)d	135.9 (20.6)c
Grain	112.6 (26.6)d	235.6 (33.8)a	120.6 (29.0)c	124 (24.7)c	115.6 (41.2)d	235.1 (26.0)a	114.9 (17.9)d	164.3 (24.9)b
Selenate								
SVP	2.0c	0.68e	0.52e	1.4d	7.0a	5.4b	7.3a	5.9 b
Root	99.3 (37.0)b	85.5 (23.8)c	39.2 (36.7)f	24.6 (27.8)g	234.1(51.5)a	97.2 (35.1)b	49.7 (22.1)e	68.9 (25.3)d
Stem+leaf	57.9 (21.6)d	67.3 (18.8)b	30.3 (28.3)e	20.5 (23.2)f	59.4 (13.1)d	62.1 (22.4)c	73.7 (32.8)a	74.5 (27.3)a
Waste spike	34.1 (12.7)e	88.4 (24.6)a	9.2 (8.6)f	11.9 (13.5)f	79.5 (17.5)b	50 (18.1)d	53.3 (23.7)d	61.4 (22.5)c
Grain	77.3 (28.8)b	117.6 (32.8)a	28.2 (26.4)e	31.4 (35.5)e	81.5 (17.9)b	67.7 (24.4)c	48.3 (21.5)d	67.7 (24.8)c

¥SVP= sand:vermiculite:peat (1:1:1) substrate. † values represent mean (% Se relative content in relation to total Se content in SVP substrate and plant tissues). Different letters in the same row denote significant difference ($P \leq 0.05$; $n = 3$).

4.3.5 Selenium content in grain

Wheat plants inoculated with *Enterobacter* sp. B16 strain showed greatest Se concentration in grain (Table 4.2). The relative Se content in grain from plants inoculated with selenite enriched selenobacteria in relation to total Se in plant tissues was 25−34% (113−236 mg kg^{-1}) and 18−41% (115−235 mg kg^{-1}) in mycorrhizal and nonmycorrhizal plants, respectively. A similar result was observed in selenobacteria grown in selenate where the relative content of Se was 26−35% (28-118 mg kg^{-1} grain) and 18-25% (68−81 mg kg^{-1}) in mycorrhizal and non mycorrhizal plants, respectively. Wheat plants inoculated with selenobacteria mixture grown in selenite also showed higher Se contents in tissues compared to those inoculated with selenobacteria grown in selenate (Table 4.3). Significant differences ($P \leq 0.05$) between mycorrhizal and non mycorrhizal plants were also observed in Se content in grain from plants inoculated with selenobacteria mixture grown in selenite, but not in plants inoculated with selenobacterial mixture grown in selenate (Figure 4.2).

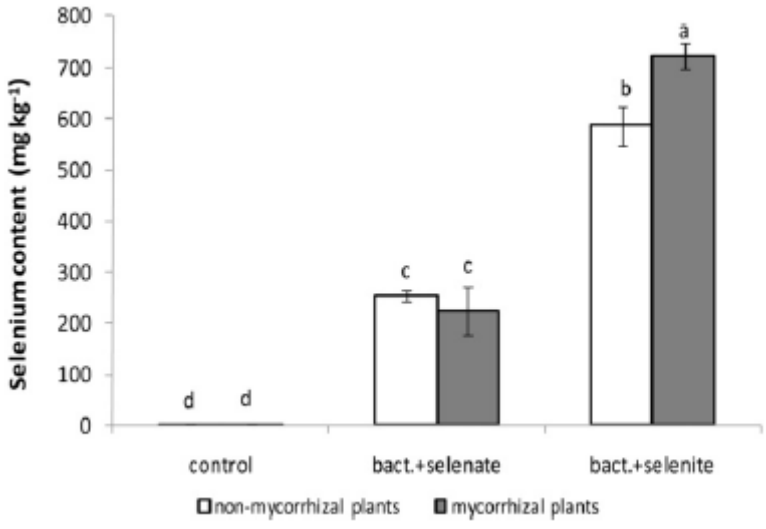

Figure 4.2 Total Selenium content in grain of non-mycorrhizal and mycorrhizal plants inoculated with selenobacteria mixture grown in nutrient broth supplemented with5 mM of selenite (Se^{4+}) and selenate (Se^{6+}). Tukey test to compare treatments means,values followed by the same letter do not differ at $P \leq 0.05$ (n= 3).

Table 4.3 Se content (mg kg^{-1}) in SVP substrate and wheat plant tissues inoculated with selenobacteria mixture and AMF (*Glomus claroideum*) grown in nutrient broth supplemented with selenite and selenate.

	Mycorrhizal plants	Non mycorrhizal plants
Selenite		
SVP†	25‡a	17 a
Root	541 (25)a	568.6 (28.3)a
Stem+leaf	563.3 (26)a	532.8 (26.6)a
Waste spike	339.4 (15.7)a	313.8 (15.7)a
Grain	724.6 (33.4)a	587.4 (29.3)b
Selenate		
SVP	1.65 a	1.61 a
Root	183.8 (22.5)a	206.9 (22.7)a
Stem+leaf	278.8 (34.2)a	324.7 (35.6)a
Waste spike	127.7 (15.7)a	124.9 (13.7)a
Grain	225 (27.6)a	255 (28)a

¥SVP: sand:vermiculite:peat (1:1:1) substrate. †values represent mean (% Se relative content in relation to total Se content in SVP substrate and plant tissues). Different letters in the same row denote significant difference ($P \leq 0.05$; $n = 3$).

4.3.5 PCR-DGGE

Analysis of DGGE profiles is shown in Figure 4.3. In mycorrhizal and non mycorrhizal plants, the DGGE profiles showed predominant ribotypes similar to inoculated selenobacteria mixture (Figure 4.3A). Moreover, differences between bacterial communities of mycorrhizal and non mycorrhizal plants were generally consistent as shown by hierarchical cluster analysis (Figure 4.3 B). In this context, ShannoneWiener Index analysis revealed a higher diversity (0.8−1.1) in mycorrhizal plants compared with non mycorrhizal plants (0.7−0.8). Principal Component Analysis (PCA) revealed a strong effect of AMF inoculation in the bacterial composition variability. Two distinct clusters were observed in the bacterial communities composition measured by 16S rRNA DGGE affected by mycorrhizal inoculation in wheat plants (Figure 4.3C).

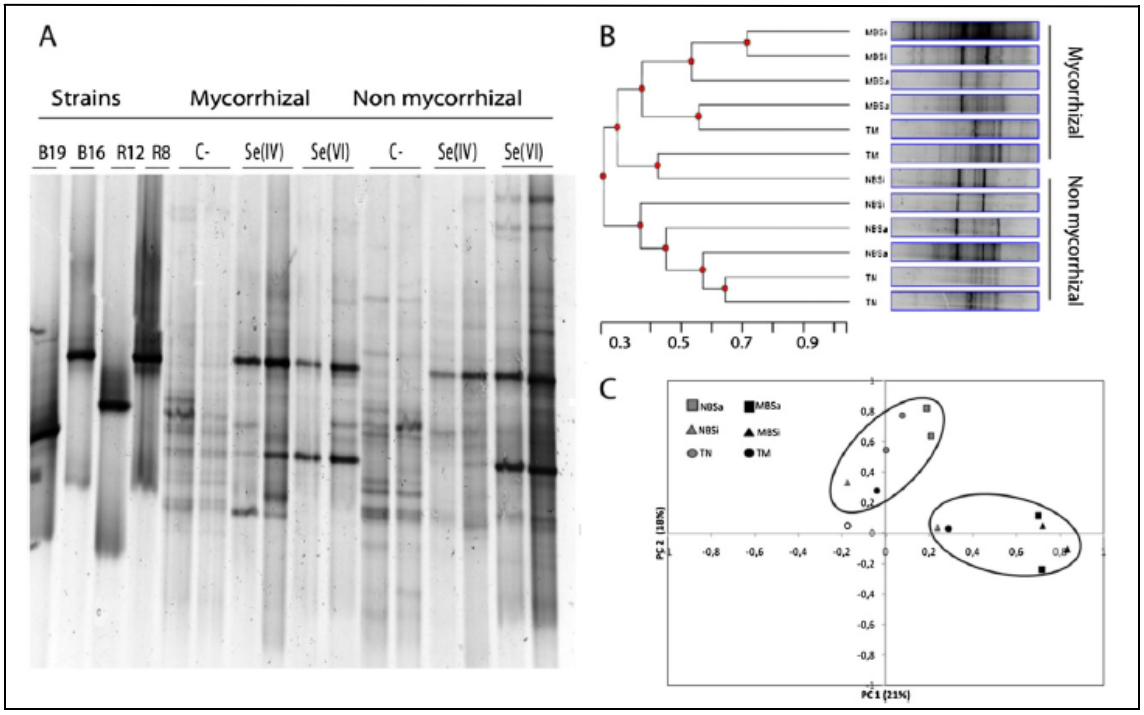

Figure 4.3 A) Denaturing gradient gel electrophoresis (DGGE) analysis, B) dendrogram of DGGE profiles and C) principal component analysis (PCA) of bacterial communities in non mycorrhizal and mycorrhizal plants inoculated with selenobacteria mixture grown in nutrient broth supplemented with 5 mM of selenite (Se^{4+}) and selenate (Se^{6+}). Selenobacteria strains (*Stenotrophomonas* sp. B19; *Enterobacter* sp. B16; *Pseudomonas* sp. R12; *Bacillus* sp. R8) were used as band control in DGGE gel. C: uninoculated controls.

4.4 Discussion

It is widely known that bacteria participate in the Se cycle in nature and selenobacteria have been studied for bioremediation of Se contaminated soils (Ghosh et al., 2008; Lampis et al., 2009). However, the application of selenobacteria as a tool for improving Se content in cereal crops has not been explored. Our research group recently described the isolation and characterization of selenobacteria from the cereal crop rhizosphere grown in Andisol and their potential for biofortification of wheat plants (Acuña et al., 2013). Using the same selected selenobacteria supplemented with selenite and selenate,

our results showed that selenite treatments were better translocated to shoots than selenate. This result is in opposition to biofortification with inorganic Se-containing fertilizers, where selenate is more available due to the fact that it is only weakly adsorbed in soils and therefore more easily incorporated to roots, while selenite shows stronger adsorption to soil matrix, diminishing its root accumulation with a concomitant lower translocation to shoots (Govasmark and Salbu, 2011; Hawkesford and Zhao, 2007). Similarly, in Chilean Andisols, selenite is more strongly adsorbed than selenate in soil surfaces. Also, plants treated with inorganic selenate showed higher shoot Se-concentration (Cartes et al., 2005).

In relation to bacterial biomass, selenobacteria grown in selenite supplemented medium showed higher Se content than selenobacteria grown in selenate. This result can be attributed to the fact that selenite is more easily reduced to elemental Se (Se0) by bacteria in comparison to selenate, as described by Dungan et al. (2003). Plants inoculated with *Enterobacter* sp. B16 grown in selenite accumulated higher Se content in stem þ leaf (i.e. 155 mg kg^{-1}) and grain (i.e. 235 mg kg^{-1}), in both mycorrhizal and non mycorrhizal plants, and in relation with the rest of strains. In contrast, plants inoculated with *Bacillus* sp. R12 and *Pseudomonas* sp. R8 showed the lowest Se content. According to Losi and Frankerberg (1997), *Enterobacter* strains are useful for removing Se oxyanions from agriculture drainage water.

The inoculation of selenobacteria resulted effectively in the Se translocation to shoots with the concomitant accumulation of Se in wheat grain. We observed that Se nanoparticles produced by selenobacteria are apparently related with Se plant uptake as reported previously by other authors. Thus, Rico et al. (2011) reported that the nanoparticles can enter plants by binding to carrier proteins, through aquaporins, ion channels, or endocytosis, by creating new pores, or by binding to organic chemicals.

Also, Kurepa et al. (2010) showed that nanoparticles may form complexes with membrane transporters or root exudates and subsequently be transported into the plant. This can be produced through the vascular systems as reported for some nanoparticles, such as zinc and carbon (Corredor et al., 2009; Kurepa et al., 2010), and could be accumulated in vacuoles or citoplasmic strands (Parsons et al., 2010).

Plants co-inoculated with selenobacteria mixture plus AMF showed the highest Se content. Larsen et al. (2006) and Yu et al. (2011) reported that AMF inoculation increased Se content in garlic, alfalfa and maize. Mycorrhiza promote the root length with a concomitant major exploration capacity (Munier-Lamy et al., 2007), exudation (higher nutrient availability), and absorption (higher absorption area) by roots (Rico et al., 2011). The highest Se content in plants can also be attributable to rhizobacteria, as it can stimulate root hair production and benefit the establishment of mycorrhizal symbioses (de Souza et al., 1999). Our results also showed a higher bacterial diversity in mycorrhizal plants inoculated with selenobacteria mixture (Fig. 3A). Higher bacterial diversity can be due to a high number of bacteria associated with AMF structures and the establishment of complex bacteria-mycorrhiza interactions (Budi et al., 1999). It has been speculated that AMF inoculation would not only influence Se accumulation but also speciation transformation of Se in plants due to its effects on microbial activity and community (Yu et al., 2011). Barea et al. (2005) also reported that AMF release organic compounds, increasing bacterial density and accelerating microbial metabolic activity and nutrient cycling in the rhizosphere. Thus, as revealed by our results, the use of this consortium for bioaccumulation strategy is more effective for improving Se content in wheat plants than single selenobacteria inoculum.

It is important to mention that values found in grain are significantly higher than those obtained by agronomic biofortification with Se-containing fertilizers (Galinha et

al., 2012) and significantly exceed the recommended doses for human consumption (55 mg day^{-1}), although Se-rich grain produced by this technology can be used as a supplement to increase Se content in flours, as well as "semolina", which has the highest retention capacity (Cubadda et al., 2009). Our study provides an intriguing first view of coinoculation and synergism between selenobacteria and AMF as a strategy to enhance Se content in wheat grain. However, deeper studies are clearly required to evaluate this technology. Finally, we recommend future studies focused on researching: i) molecular mechanisms involved in uptake and translocation of supplemented Se in plants, ii) analysis of Se forms present in grain, and iii) determining selenobacteria doses which can be used to produce flours for human nutrition.

4.5 Conclusions

Selenium content in wheat grain was increased by almost 23% with the co-inoculation of selenobacteria mixture (*Stenotrophomona* sp. B19, *Enterobacter* sp. B16, *Bacillus* sp R12. and *Pseudomonas* sp. R8) and arbuscular mycorrhizal fungi (*Glomus claroideum*) compared with non mycorrhizal plants. This directed microbial association represents a promising strategy for biofortification ofwheat plants in order to produce Se enriched flour for supplementing foods for human consumption.

Further studies are needed to elucidate the chemical forms and transport mechanisms of selenium in wheat plants inoculated with selected selenobacteria and arbuscular mycorrhizal fungi.

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Chapter V

Development and evaluation of different strategies of Se biofortification using Se-biofertilizer in wheat plants.

This manuscript will be present as a patent

Abstract

The nutritional importance of selenium (Se) to human health is universally recognized and their essential role in selenoproteins has been widely described. The low Se availability in soil for cereal crops is a principal factor associated to Se deficiency in the human diet. We have reported that exist a beneficial interaction between plants and soil microorganisms (rhizobacteria and arbuscular mycorrhizal fungi) for increasing Se content in plants. In order to evaluate different Se-biofortification strategies in wheat plants, three biofertilizers based on selenobacteria were formulated (lyophilized, microencapsulated and liquid culture) and compared with the inorganic selenite application as Se-inorganic fertilizer. For the first assay *in vitro* conditions we analyzed seed germination and plant growth. The second experiment was conducted for Se acquisition evaluation and antioxidant performance in wheat plants in response to Se-biofertilizer under greenhouse conditions using an acidic Andisol (Gorbea Series) limed or no limed. The first results indicated that the Se biosynthesized supplied at doses of 300 g Se ha^{-1}not produce toxic effect on germination rates in contrast with selenite. Interestingly, in the second assay, we found that Se-biofertilizer application in all formulations enhanced Se concentration in wheat plants. However, the highest Se concentration was found in plants growing in no limed soils. On the other hand, plants showed differential patterns of antioxidant responses to Se-biofertilizer applications. Thus, plants growing in no limed soil decreased the oxidative membrane damage which was associated to MDA reduction and SOD activity decline. In addition, according to transmission electron microscopy (TEM) analyses different Se nanoparticles sizes were found in roots (~70-200nm). According toresults, Se-biofertilizer huge great potential because is readily available to plants and can alleviate plant stress. Further studies are needed to elucidate the chemical forms and transport mechanisms of Se biosynthesized in biofortified wheat plants.

5.1 Introduction

Seleniumdeficiency in human diet affects around to 15% of world population causing a negative impact on the human health (Tan et al., 2002). Selenium deficiency is associated with the immune function, antioxidant defense system, and thyroid hormone metabolism (Rayman et al., 2008). In fact, Se has been related with 25 genes expressing selenoproteins and it is associated with the optimal iodothyronine deiodinases, thioredoxin reductases and glutathione peroxidasesfunction. In addition, optimal Se levels decrease the incidence of cancer, immune systems, heart disease, and reproduction (Kryukov et al., 2003).Selenium intake by humans is determined by Se content in foods. Therefore, low Se availability in soils is the principal limiting factor to provide an optimum Se content in plant-derived foods (Combs et al., 2001). Thus, Se levels decline in crops is directly related with a low Se intake, because is the main route of Se incorporation in the human diet (Govasmark and Salbu, 2008). In Chile, cereal crops including wheat, oat and barley are the principal nutrition sources for the population. Indeed, particularly wheat contributes over 50% of the total dietary fiber intake (INIA, 2007). Acidic soils derived from volcanic ashes, like Andisol of Southern Chile; support about 40% of the national cereal production (ODEPA, 2012). These soils are characterized by high exchangeable aluminum (Al) (Al saturation > 20%), inducing selenite adsorption through Al polyvalent bridging in aluminosilicates and Al-hydrous oxides (Mora et al., Mora et al., 2002; Mora et al., 2005; 2006; Barrow et al., 2005). Thus, Se deficiency in soil (ranging between 21 -180 μg Se kg^{-1}) and Al toxicity (over 5,000 mg Al kg^{-1} plant dry weight) coexist in Andisol, limiting the crops production (Soil and Plant Analysis Services Laboratory, Universidad de La Frontera). Nowadays, the main strategy to amend Se deficiency is through agronomic Se fertilization (Whelan and Barrow, 1994; Oldfield, 1998; Hartikainen, 2005). In Chile, previous studies developed in Andisols have demonstrated an increase of Se acquisition by plants whose seeds were previously pelleted with sodium selenite (Mora et al., 2008; Cartes et al., 2011). The increase of Se content improves the forage quality, because this metalloid

increases the antioxidant ability that mitigates the Al-induced oxidative stress (Cartes et al., 2010). Recent studies carried out using wheat plants growing in field conditions (fertilized with 150 g Se ha^{-1}), showed that different cultivars increased slightly Se shoot concentration (8 μg kg^{-1}) in response to Se supply (unpublished data). These results can evidence the low Se translocation from soil to shoot as well as the low efficiency of inorganic Se fertilizers. Therefore, we need to implement strategies to increase efficiently Se content in cereal crops, and thereby improve the nutritional quality and antioxidant activity. We are interested in crops biofortification by selenobacteria able to accumulate intracellular Se and to provide an available Se source to plant uptake that decrease the risk of leaching and adsorption of inorganic Se forms (selenate and selenite) in soil system. Previously, we reported the occurrence of selenobacteria isolated from cereal rhizosphere with ability to metabolize and biosynthesize Se nanospheres and other intracellular Se forms. In addition, we showed that the inoculation with selected selenobacteria enhanced Se content in the wheat shoots (Acuña et al., 2013) and grain by co-inoculation of selenobacteria and arbuscular mycorrhizal fungi (*Glomus claroideum*) associated with a higher biodiversity of rhizospheric microorganisms (Duran et al., 2013). Thus, our results evidenced that Se biosynthesized by selenobacteria can be translocated inside the plants. However, their antioxidant role in plants and the effectiveness at higher doses in comparison with inorganic Se-fertilization has not been studied. Furthermore, we need more technological development to implement biofertilizer strategies in a large-scale forintensive crops production. The main goal of this study was to develop and evaluate different inoculation methods for Se biofortification using Se-biofertilizer in wheat plants.

5.2 Material and Methods

In order to evaluate different Se-biofortification strategies in wheat plants (*Triticum aestivum* L.) cv. Fritz Baer®, two experiments were conducted. The first assay was carried out under *in vitro* conditions in order to evaluate the seed germination and plant growth. Subsequently, the second experiment was conducted on Andisol (limed and no limed) to evaluate Se content and antioxidant performance in plants under greenhouse conditions.

5.2.1 Se-biofertilizer development

Selenobacteria were previously isolated from the cereal rhizosphere (wheat, oats and barley) growing on Andisol (Acuña et al., 2012). Based on their effectiveness to translocate Se in wheat plants, *Enterobacter* sp. strain B16 was selected for this study (Duran et al., 2013). We developed three Se-biofertilizer formulations from *Enterobacter* sp. B16: lyophilized, microencapsulated and liquid culture (see appendices vi). Methodologies related to the Se biofertilizer formulation such as inoculum obtain, lyophilization, microcomposite preparation and microencapsulation are not incorporated in this report due to patenting process. With the purpose to provide the same Se concentration in treatments, total Se content in Se-biofertilizer was determined according to Kumpulainen et al., (1983). Thus, Se concentration in each sample was measured by Atomic Absorption Spectrophotometry-Hydride Generation coupled (AAS-HG) using sodium borohydride ($NaBH_4$) solution as the reducing agent and measured at wavelength 196.1 nm.

5.2.2 Seed germination and plant growth under Se- biofertilizer treatments

Wheat seeds (*Triticum aestivum L.*) cv. Fritz Baer®, were sterilized with 70% ethanol (v/v) for 5 minutes, washed three times with sterile deionized water, treated with commercial sodium hypochlorite at 5% and successively washed with sterile deionized

water (Özgen et al., 1998). For the pelletization process, Se concentration was determinated according previous studies. Thus, doses above to 300g ha^{-1} of sodium selenite equivalent to field applications affect the seed germination (data unpublished). In order to evaluate the Se-biofertilizer effects on germination and plants growth, seeds were pelletized with lyophilized and microencapsulated formulation with or without Se (control). The seed pelletization process was made according to methodology described by Cartes et al., (2011). Table 5.1 summarizes the different treatments evaluated.

Table 5.1 Se-biofertilizer treatments

Nomenclature	Treatments	Inoculation method
C	Control	Pelletized
Seb (L)	Se-biofertilizer lyophilized	Pelletized
Seb (M)	Se-biofertilizer microencapsulated-lyophilized	Pelletized
B (L)	Biofertilizer lyophilized (without Se)	Pelletized
B (M)	Biofertilizer microencapsulated-lyophilized (without Se)	Pelletized
Se	Sodium selenite	Pelletized

Seeds were sown in flask containing 1L of purified agar (10%) containing Murashige & Skoog medium (Phyto Technology Laboratories, LLCTM) described by Murashige and Skoog, (1962). The flasks were maintained in a growth chamber under experimental conditions (25°C, 80% of relative humidity and 16:8 h of light /dark). All treatments were performed in triplicate, and control without bacterial inoculation were also evaluated. Germination was monitored daily for two weeks. After 14 days, roots and shoots were harvested and dried (65°C for 48 h) to determine dry weight.

5.2.3 Greenhouse experiment: Se content in wheat plants

We evaluated different strategies for biofortification using Se-biofertilizer in three forms: lyophilized, microencapsulated and liquid culture. For lyophilized and microencapsulated experiments, pretreated pelleted seeds were used (Table 5.1), whereas for liquid form, fresh culture of Se-biofertilizer was employed. Wheat seeds were cultivated in greenhouse conditions. The assays were conducted in acidic Andisol

(Gorbea Series) limed and no limed from Southern Chile, never amended with Se-fertilizers. Chemical soil parameters are presented in Table 5.2, and they were determined according to the methodology described by Sadzawka et al. (2004). For the experiments, fifteen pretreated seeds for each treatment (C; Seb (L); Seb (M); B (L); B (M); and Se) were established in pots with 1 kg soil, and plants were harvested after 30 days. The effect of liquid Se-biofertilizer application was tested by direct injection of Seb (I) and B (I) treatments. After 15 days, the plants were injected with micropipettes in the rhizospheric zone using 2 mL Seb(I) or B(I) described above.

Table 5.2 Chemical properties of the Andisol of Gorbea Series from Southern of Chile

	Limed	No-limed
Parameter	Concentration¥	
N (mg kg^{-1})	30.5	24
P (mg kg^{-1})	17	20
K (mg kg^{-1})	139	91.5
pH (water)	5.52	4.99
K (cmol+ kg^{-1})	0.355	0.235
Na (cmol+ kg^{-1})	0.105	0.055
Ca (cmol+ kg^{-1})	2.435	0.58
Mg(cmol+ kg^{-1})	0.44	0.23
Al(cmol+ kg^{-1})	0.275	0.435
Al Saturation (%)	7.635	28.33
CICE (cmol+ kg^{-1})	3.61	1.31
S. Bases (cmol+ kg^{-1})	3.335	1.1

¥Data are means of three replicates ±SD

Plants were grown at 25 ºC in a daily cycle of 16:8 h light /dark respectively and 80% relative humidity. Three pots were used as replicates for each treatment, and control were also evaluated. During the growth period, plants were watered with distilled water. During plants harvest, fresh subsamples were taken to perform biochemical analyses. Later, shoots and roots samples were dried at 65°C for 48 h to determine dry weight and Se concentration by AAS-GF at wavelength 196.1 nm as a described above (see stage 5.2.1).

5.2.4 Plant biochemical analyses

Plant subsamples of fresh shoot (0.1 g) were frozen in liquid nitrogen and 50mM potassium phosphate buffer (pH 7.2) were used for extraction in order to determine catalase, ascorbate peroxidase, superoxide dismutase enzyme activities. Catalase activity (CAT, EC 1.11.1.6) was determined according to Hossain et al., (2010). The hydrogen peroxide (H_2O_2) decomposition was measured at 240 nm (extinction coefficient of 0.036 mM^{-1} cm^{-1}). Peroxidase activity (POD, EC 1.11.1.7) assay was based on the guaiacol oxidation determination (extinction coefficient 26.6 mM^{-1} cm^{-1}) at 470 nm by H_2O_2. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 20.1 mM guaiacol, 12.3 mM H_2O_2, and enzyme extract in a 3 mL volume. The activity of SOD was estimated according to the method described by Donahue et al., (1997). The absorbance was measured at 560 nm, once the reaction tubes were illuminated for 15 minutes. Non illuminated and illuminated reactions without supernatant were used as controls. All enzymatic activities were expressed based on protein content. The protein concentration in the enzyme extracts was determined by Bradford method. As oxidative stress indicator, lipid peroxidation in shoot tissues was assayed by measuring the thiobarbituric acid reactive substances (TBARS) according to the modified method described by Du and Bramlage, (1992). The reaction was measured at 532, 600 and 440 nm in order to correct the interferences produced by TBARS-sugar complexes.

5.2.5 Bacterial community composition analyses

The rhizospheric bacterial community composition was examined by denaturing gradient gel electrophoresis (DGGE) according to Jorquera et al., (2010). Briefly, total DNA from rhizosphere soil was extracted using a Power Soil® DNA Isolation Kit (Mo-Bio Inc., USA). For bacterial community analysis, fragments of 16S rRNA gene were amplified by touchdown PCR using EUBf933-GC/EUBr1387 primers set (Iwamoto et al., 2000).

The DGGE analysis was performed using a DCode system (Bio-Rad Laboratories, Inc.). The PCR product (20 μL) was loaded onto a 6% (w/v) polyacrilamide gel with a 50-70% gradient (urea and formamide). The electrophoresis was run for 12 h at 100 V. The gel was then stained with SYBR Gold (Molecular Probes, Invitrogen Co.) for 30 min and photographed on an UV transilluminator. Clustering of DGGE banding profiles using a dendrogram was also carried out by using Phoretix 1D analysis software (TotalLab Ltd.). Analysis of microbial community diversity by Shannon-Weaver Index it was also carried out according to described by Yang et al., (2003).

5.2.6 Transmission electron microscopy (TEM)

Se-biofertilizer powered samples were analysed by transmission electron micrographs and were recorded using a JEOL JSM 1200EX-II transmission electron microscope equipped with electron diffraction pattern. The mean diameter of Se-nanospheres and their detection inside root was measured from the images obtained by TEM in plants with and without Se-biofertilizers (Tam et al., 2010).

5.2.7 Statistical analyses

The data were analyzed by a one-way analysis of variance (ANOVA), and comparisons between means were carried out with the Tukey test (see appendix viii) using SPSS software (SPSS, Inc.). All experiments were conducted in triplicate, and the values are given as means ± standard error. Differences were considered to be significant when the *P* value was less than or equal to 0.05. Relationships between Se concentration and enzymatic activities were investigated with correlation analysis (Pearson correlation).

5.3 Results

5.3.1 Germination rates and plant growth of wheat under *in vitro* conditions

Selenite treatment (Se) exhibited lower germination in comparison to control and biofertilizers treatments. Similar results were obtained by Lyons et al., (2005), which showed that elevated doses of inorganic Se (70 mg L^{-1}) can inhibit wheat seed germination (Figure 5.1, A). Thus, Se biofertilizers based on lyophilized or microencapsulated formulations not caused seed toxicity neither physical damage in wheat seeds. According these results, we can conclude that Se-biosynthesized (Se nanospheres and other intracellular forms; see appendices vii) from Se biofertilizer are less toxic than selenite in terms of chemical stability, such has been reported by Zhang et al., (2004) for Se nanoparticles. Similarly, Song et al., (2013) showed that other nanoparticles (nano-TiO2 and Ag nanospheres) no produce a severe toxicity in tomatoes germination. Plant biomass production not varies significantly from lyophilized or microencapsulated formulations respect to control (Figure 5.1, B). Interestingly, the highest biomass production was observed in lyophilized bacteria without Se [B (L)] treatment. In this sense, we presume that bacteria inoculated directly to the seed coat have a higher interaction on the rhizosphere zone than microencapsulated bacteria (Bashan et al., 1988).

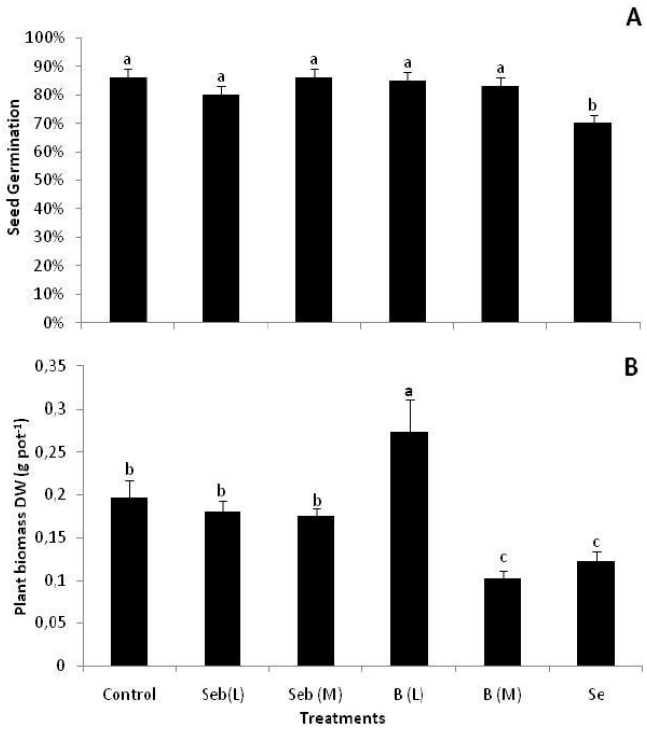

Figure 5.1 Effect of Se treatments on wheat seeds germination (*Triticum aestivum* L.) cv. Fritz. C: control; Seb (L): Se-biofertilizer lyophilized; Seb (M): Se-biofertilizer microencapsulated; B (L): biofertilizer lyophilized; B (M): biofertilizer microencapsulated; and Se: selenite. Means with the same lower case letters are not significantly different among the different treatments. ($P \leq 0.05$; $n = 3$).

5.3.2 Effect of Se-biofertilizer: Plant growth and Se uptake under greenhouse conditions

5.3.2.1 Plant growth

Biomass production of wheat plants was higher in all treatments respect to control in either liming or no liming soils (29% aluminum saturation). Plants grown under Seb (L) treatments showed an increase of plant biomass around 40,6 % and 75% respect to control in no limed and limed soil. This fact indicated that Se biosynthesized from selenobacteria affected positively the plant growth. Plants exposed to Se biofertilizer showed no significant differences in shoot elongation in response to treatments (Table

5.3). As expected, higher root growth was found in plants grown in limed soil. Thus, the higher Al saturation from no limed Andisol can affect the optimal root development (Lugany et al., 1995). Respect to selenite treatment (Se) there was no differences in plant growth in comparison to control treatments in both soil conditions; similar results have been reported by Chu et al., (2010). Unlike, the results showed by Cartes et al., (2011), Hawrylak-Nowak, (2009) and Hasanuzzanman et al., (2012) have demonstrated the positive effect of selenite on plant growth parameters in ryegrass, cucumber and brassica. Moreover, reports showed that both the Se content increase and growthimprovement as a consequence of Se fertilization in many plants species such as: ryegrass, lettuce, kenaf, rapeseed, potato, arabidopsis, and wheat (Cartes et al., 2011; Rios et al., 2009; Lavu et al., 2013; Hasanuzzaman et al., 2011; Turakainen et al., 2004; White et al., 2004; Broadley et al., 2010). Such differences could be related with the plant species and selenite concentration in the growth media. In addition, significant differences in root elongation were observed in plants cultivated on no limed soil as a response to bacteria (without Se) inoculations in all formulations [B(L), B(M), B (I)]. Thus, these treatments increased the root length by about 35% − 43 % with respect to control.

Table 5.3 The effect of Se biofortification treatments on growth parameters

Treatments	Length (cm)		Biomass dry weight (g pot^{-1})		
	Shoot	Root	Shoot	Root	Total
No-limed soil					
Control	25.5 ± 1.1^{aA}	7.9 ± 1.5^{bB}	0.22 ± 0.02^{bA}	0.10 ± 0.02^{bA}	0.32 ± 0.04^{bA}
Seb (L)	27.7 ± 1.1^{aA}	8.9 ± 0.9^{bB}	0.34 ± 0.05^{aA}	0.11 ± 0.01^{bB}	0.45 ± 0.06^{bA}
Seb(M)	26.1 ± 1.9^{aA}	9.3 ± 0.3^{bB}	0.35 ± 0.04^{aA}	0.11 ± 0.02^{bB}	0.41 ± 0.07^{bA}
Seb(I)	27.2 ± 1.5^{aA}	8.6 ± 1.5^{bB}	0.31 ± 0.01^{aA}	0.07 ± 0.01^{bB}	0.38 ± 0.01^{bB}
B (L)	28.7 ± 1.3^{aA}	10.9 ± 0.2^{aB}	0.33 ± 0.03^{aA}	0.08 ± 0.02^{bA}	0.42 ± 0.06^{bA}
B (M)	26.1 ± 1.1^{aA}	10.7 ± 0.5^{aB}	0.28 ± 0.05^{aA}	0.08 ± 0.01^{bA}	0.38 ± 0.04^{bA}
B (I)	29.8 ± 1.2^{aA}	11.3 ± 0.6^{aB}	0.38 ± 0.05^{aA}	0.21 ± 0.02^{aA}	0.59 ± 0.09^{aA}
Se	26.2 ± 1.3^{aA}	9 ± 0.4^{bB}	0.27 ± 0.02^{aA}	0.10 ± 0.01^{bA}	0.38 ± 0.03^{bA}
Limed soil					
Control	24.1 ± 1.7^{bA}	15.8 ± 0.5^{bA}	0.25 ± 0.06^{bA}	0.07 ± 0.01^{bA}	0.32 ± 0.07^{bA}
Seb (L)	27.2 ± 1.0^{aA}	14.3 ± 0.6^{bA}	0.40 ± 0.05^{aA}	0.16 ± 0.01^{aA}	0.56 ± 0.03^{aA}
Seb (M)	26.1 ± 1.3^{aA}	17.1 ± 1.0^{aA}	0.32 ± 0.01^{bA}	0.17 ± 0.02^{aA}	0.41 ± 0.04^{bA}
Seb (I)	28.3 ± 0.9^{aA}	17.7 ± 1.3^{aA}	0.33 ± 0.02^{bA}	0.17 ± 0.04^{aA}	0.50 ± 0.05^{bA}
B (L)	26.2 ± 0.9^{aA}	12.2 ± 0.4^{bA}	0.26 ± 0.04^{bA}	0.08 ± 0.02^{bA}	0.34 ± 0.05^{bA}
B (M)	26.7 ± 0.8^{aA}	14.6 ± 0.8^{bA}	0.29 ± 0.05^{bA}	0.09 ± 0.01^{bA}	0.37 ± 0.01^{bA}
B (I)	29.1 ± 0.5^{aA}	16.0 ± 1.1^{aA}	0.36 ± 0.04^{aA}	0.13 ± 0.01^{aB}	0.48 ± 0.07^{bA}
Se	27.2 ± 1.2^{aA}	13.5 ± 0.8^{bA}	0.30 ± 0.01^{bA}	0.09 ± 0.02^{bA}	0.39 ± 0.02^{bA}

C: control; Seb (L): Se-biofertilizer lyophilized; Seb (M): Se-biofertilizer microencapsulated; Seb (I): Se-biofertilizer inyected; B (L): Biofertilizer lyophilized; B M): biofertilizer microencapsulated; B(I): biofertilizer inyected, and Se: inorganic selenite. Means with the same lower case letters are not significantly different among the different treatments in a same soil; means with different uppercase letters are significantly different in a same treatment for different soil. ($P \leq 0.05$; $n = 3$).

5.3.2.2 Selenium uptake

Plants under Se-biofertilizer treatments showed higher Se concentrationrespect to selenite in all treatment ($P \leq 0.05$), (Figure 5.2). Selenite is strongly sorbed by soil colloids limiting their availability (Fernandez-Martinez and Charlot, 2009). Several studies haveindicated that Se-nanospheres (5~200 nm) has similar bioavailability than inorganic forms. In this context, our previous reports have been demonstrated the ability of Se biosynthetized by selenobacteria for enhanced Se content in plant tissues (Acuña et al., 2013). Indeed, was observed an increase by almost 23% in the selenium content in wheat grain when subjected to a co-inoculation with selenobacteria mixture (*Stenotrophomona* sp. B19, *Enterobacter* sp. B16, *Bacillus* sp R12. and

*Pseudomonas*sp. R8) and arbuscular mycorrhizal fungi (*Glomus claroideum*) comparedwith non mycorrhizal plants (Duran et al., 2013).

Selenium concentration in shoots was significantly higher in plants under Seb(L) treatment than Seb(M) and Seb(I) treatments. In comparison, Se concentration in shoots was 75% and 30% higher under Seb(L) treatments than selenite fertilization in no limed and limed Andisol, respectively (Figure 5.2, A ; $P \leq 0.05$). Interestingly, these results indicated that Se biofertilizer under lyophilized form [Seb(L)] are more readily taken up by plants than other formulations. The effectiveness of Seb(L) treatment could be attributed to the Se biosynthesized that remains in direct contact with seeds, unlike the microencapsulation and liquid culture formulations. Significantly higher Se concentration was found in roots than shoots; similar findings were reported by Mora et al., (2008), Cartes et al., (2010) and Cartes et al., (2005). The highest Se concentration in root was observed in plants under Seb(L) treatment in no limed soil, whereas wheat plants grown under selenite treatment showed the lower Se concentration (Figure 5.2, B). Selenium uptake by roots under Se-biofertilizer treatments no showed differences between liming and no liming soils. However, Se-uptake by roots under Seb(M) treatment was higher in no limed soil (266 mg Se kg^{-1} DW) than in the limed soil (158 mg Se kg^{-1}DW).

Comparatively, wheat grown in no liming soil showed higher Se content in shoots than those growing in liming soil, independently to the Se biofertilization strategy used. Thus, selenite is the predominant form in the acidic soils and their mobility is limited due to its high adsorption degree on organic matter and mineral surfaces (Fernandez and Charlot, 2009). Barrow et al., (2005) showed in Chilean volcanic soils that selenite is strongly absorbed. Our results, showed the highest Se concentration in wheat roots supplied with selenite in no limed soil, which exhibited 29% of aluminum saturation (Figure 5.2, B). These results indicated that selenite could be more available to plants in no limed soil possibly due to calcium-selenite complexes decreased Se availability in limed soil. According these results, we can confirm that Se

biofertilizer is effective to enhance Se content in wheat plants (Acuña et al., 2013 and Durán et al., 2013). Moreover, Se-biofertilizers induce a greater Se uptake for the plants in comparison to agronomic Se fertilization with selenite.

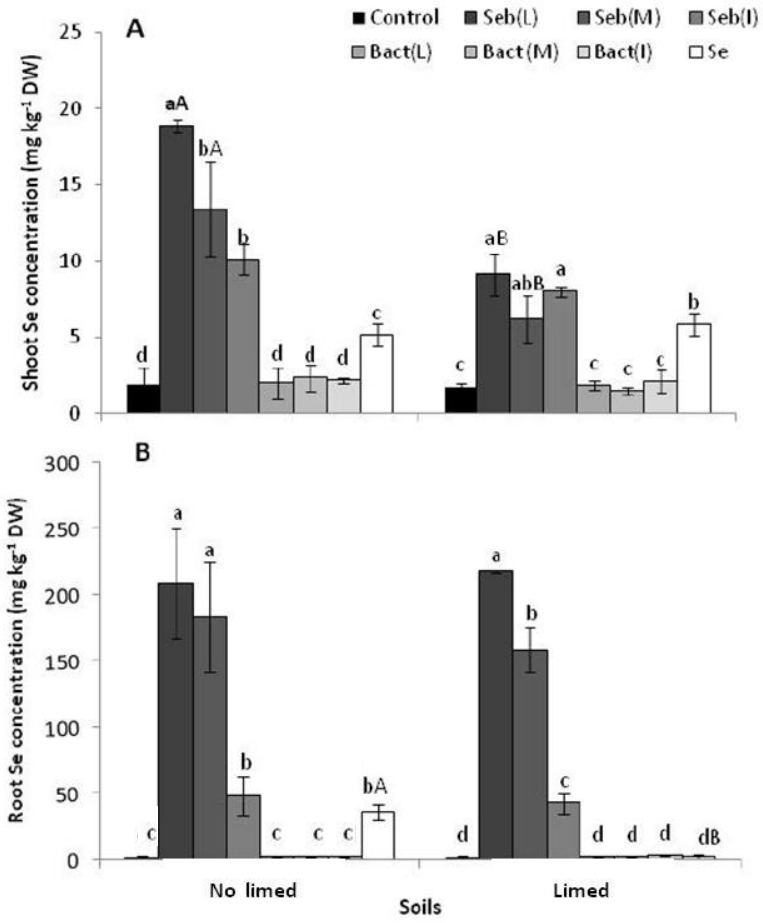

Figure 5.2 Selenium concentration in shoot (A) and root (B) of wheat (*Triticum aestivum* L.) cv. Fritz subjected to Se biofortification growing on no liming and liming Andisol. C: control; Seb(L): Se-biofertilizer lyophilized; Seb(M): Se-biofertilizer microencapsulated-; Seb(I): Se-biofertilizer inyected; B(L): biofertilizer lyophilized; B (M): biofertilizer microencapsulated; B(I): Biofertilizer injected, and Se: inorganic selenite. Different capital and lower case letters show significant differences between treatments and the soils evaluated ($P \leq 0.05$; $n = 3$).

5.3.3 DGGE profiles

The bacterial community structure was evaluated by DGGE after 30 days of plants growth under Se biofortification treatments. Clustering of the different samples was performed using an UPGMA (Unweighted Pair Group Method with Arithmetic) algorithm (Figure 5.3). The result shows an equally intense set of DGGE bands in all the analysed samples, indicating the large number and equally abundant of ribotypes presence in no limed and limed soil. Despite these similarities, UPGMA clustering analysis revealed the existence of three major groups with a similarity of 90% and 80% for no liming and liming soil, respectively. No significant differences were observed between bacterial communities under Se treatments respect to control samples. This result suggests that the Se biofortification experiments did no produce changes in the indigenous bacterial communities in soil.

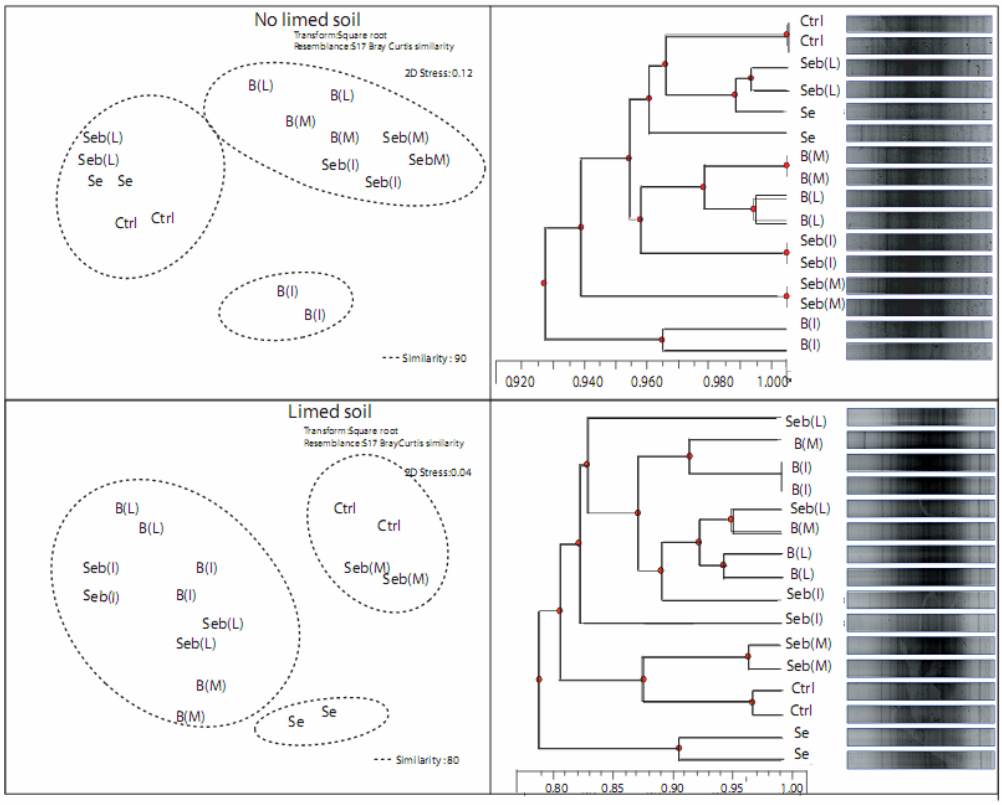

Figure 5.3 DGGE profiles of no limed and amended soil under C: control; Seb(L): Se-biofertilizer lyophilized; Seb(M): Se-biofertilizer microencapsulated; Seb(I): Se-biofertilizer inyected; B(L): biofertilizer lyophilized; B(M): biofertilizer microencapsulated; B(I): Biofertilizer injected, and Se: inorganic selenite.

5.3.4 Lipid peroxidation and antioxidant response to Se-biofertilizer treatments

Inoculations with Se-biofertilizer in Seb(L) induced a noticeable MDA reduction in shoots compared with control plants. Even though the evidence that Se decreases the MDA contents in plants has been described only for plants fertilized with inorganic Se, Our results suggests that Se biofertilizers also could be have a positive effect in protecting cell membranes against oxidative damage. Whereas lyophilized formulation [Seb(L)] diminished the lipid peroxidation level around 40%in comparison to control in no limed, respectively (Figure 5.4 A). Lipid peroxidation index is widely used for the Al- stress in plants determination (Mora et al., 2008). Thus, Se induced lower level of lipid peroxidation in rapeseed seedlings grown under Cd stress (Hasanuzzaman et al., 2012). Studies carried out by Wang et al., (2007) indicated that Se biosynthesized as Se-nanospheres act as a very effective antioxidant in animal tissue, without cause toxicity which is typical for other Se forms. It is widely accepted that the pro-oxidant attributes of Se play an important roles in its potential toxicity (Shen et al., 2001). However, Zhang et al., (2001) showed that the pro-oxidative effect of Se-nanospheres was significantly lower than those detected for selenitein Se-deficient rats, due to Se-nanospheres had lower reaction with GSH as compared to selenite. Whereas, Huang et al., (2003) reported that Se-nanosphere size react differently with free radicals due that small size had a high efficacy for scavenging free radicals.

The Se benefitson the plant antioxidant response have been often related with the SOD enzyme activity reduction (Hartikainen et al., 2000; Cartes et al., 2010; Cartes et al., 2011). In this context, we found that SOD activity decreased significantly in plants subjected to Se-biofertilizer applications in no limed soil. In fact, it was observed about 50% less SOD activity in plants under Seb(L) and Seb(I) and 30% diminution in Seb(M) treatments (Figure 5.4 B, $P \leq 0.05$). In this sense, studies conducted by Spallholz and Hoffman (2002) showed that Se-nanospheres have lower inhibitory effect

on SOD and CAT compared with selenite supply in rats. In contrast, Zhang et al., (2005) demonstrated that the SOD and CAT activities were inhibited, rather than promoted when Se-nanospheres were administrated orally to mice during 12 days. The disparity of these results can be attributed to Se dose, administration duration, tissue, and animal species (Zhang et al., 2003). Our results showed that in limed soil, SOD activity exhibited a similar behavior in response to Se biofertilization and not observed significant differences with control treatments. These observations are supported by Song et al., (2013), who indicated that nano-TiO2 treated plants only showed higher SOD values at the highest application (5000 mg kg^{-1}).

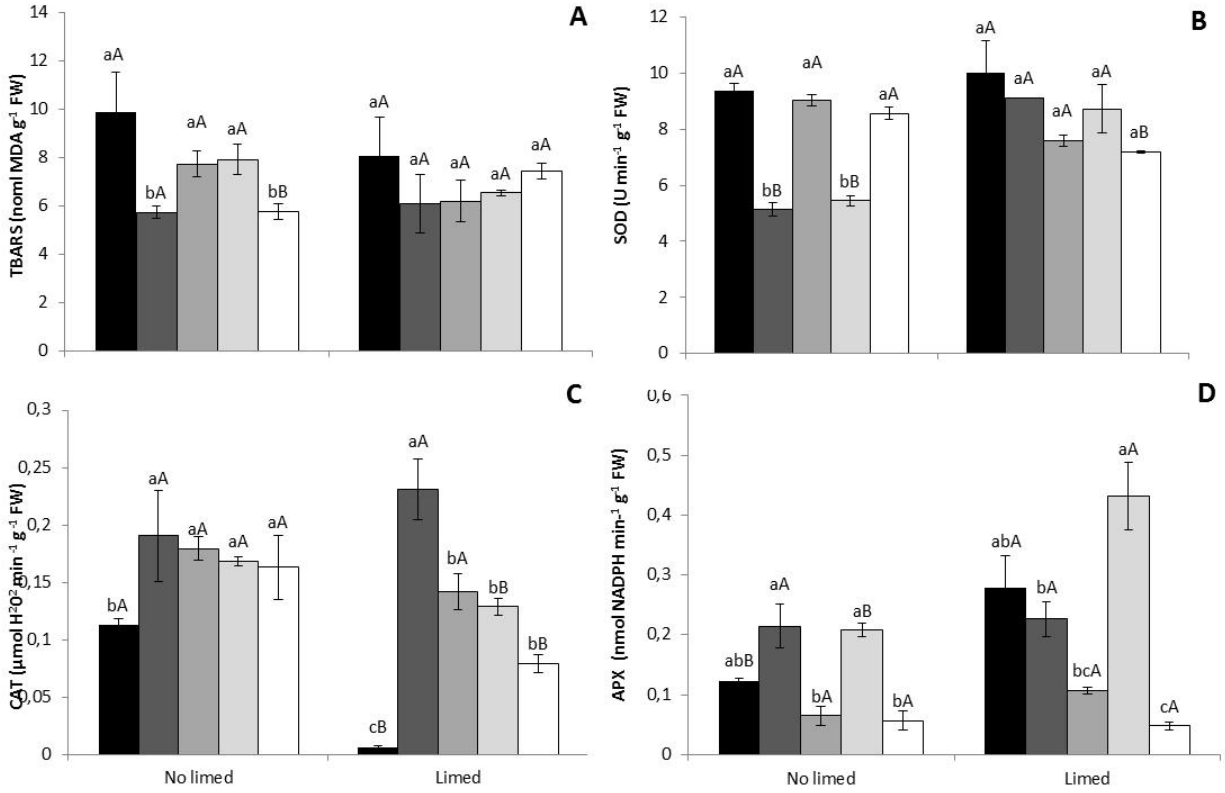

Figure 5.4 Activities of TBARS (A), SOD (B), CAT (C), and APX (D) in response of Se biofortification treatments at doses of 300 g Se ha^{-1} in wheat plants (*Triticum aestivum* L.) cv. Fritz established in no liming and liming Andisol. C: control; Seb (L): Se-biofertilizer lyophilized; Seb (M): Se-biofertilizer microencapsulated-lyophilized; Seb (I): Se-biofertilizer injected and Se: inorganic selenite. Different capital and lower case letters show significant differences between treatments and the soils evaluated ($P \leq 0.05$; $n = 3$).

Cartes et al., (2011) have been describing the Se role in the SOD reduction in ryegrass plants. According this, we showed significant correlations between Se content and SOD activity in plants grown under selenite treatments. Interestingly, our study shown a strong correlation among Se-biosynthesized and SOD activity (r=-0.5691; $P \leq 0.01$) in wheat plants for both soil conditions (Figure 5.5). These results support that Se-biosynthesized is capable of being metabolized by plant and have a key role in plant antioxidant system.

On the other hand, the activity of CAT significantly increased in response to Se biofortification and the higher activity was observed in plants under Seb (L) treatments in no liming soil. The activity of CAT increased between 19−52 % when we applied Se-biosynthesized compared with selenite treatment in no limed and limed soil, respectively. Moreover, significant correlations were found between shoot Se concentration and CAT activities (r=0.6429; $P \leq 0.01$). The results indicate that in presence of Se-biosynthesized, CAT activity could detoxify H_2O_2 to water and molecular oxygen. Our results are similar to those reported by Borowska and Koper (2011), which observed a strong correlation between Se content in red clover and catalase activity (r= 0.7716; $P \leq 0.05$) with selenite fertilization.

In relation to APX activity, significant increase ($P \leq 0.05$) was observed in plants grown in soil with high Al saturation (no limed soil) in contrast with the activity in plants cultivated on limed soil (Figure 5.4, D). Comparatively, the treatments with Seb caused significant higher APX levels in shoots with respect to plants subjected to selenite treatments in no limed and limed soil. Studies conducted by Mora et al., (2008) showed an increase in APX activities activated by Se increased concentration in shoot of white clover plants.

In synthesis, the benefits of Se-biosynthesized were associated to an enhanced of the plant antioxidant systems, demonstrated by SOD activity reduction and CAT enzyme activation.

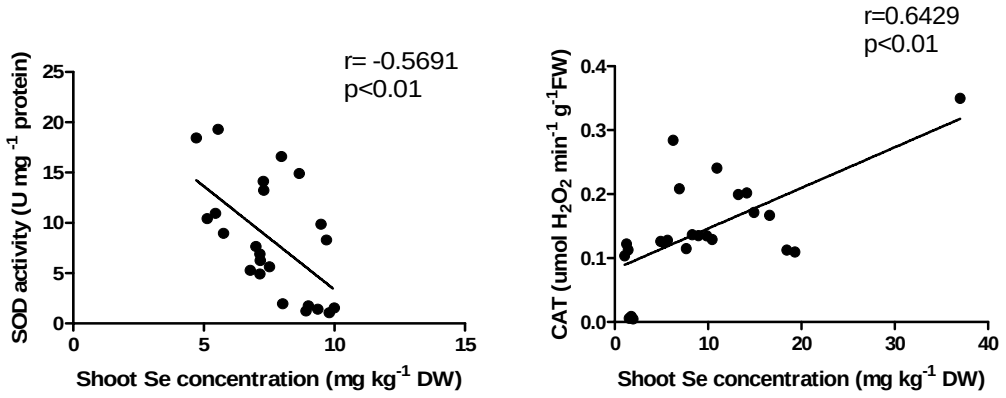

Figure 5.5 Pearson correlation between shoot Se concentration and antioxidant activities (SOD and CAT) in wheat plants subjected to Se-biofertilizer treatments (Seb (L); Seb (M) and Seb (I)).

5.4.4 Transmission electronic microscopic analysis

Se-biofertilizer in lyophilized form demonstrated different nanospheres sizes (~70-200nm) (Figure 5.6A), similar range were reported by Tam et al., (2010) and Torres et al., (2012). In addition, nanoparticles presence was not detected in control plants (data no shown).

The presence of Se-nanospheres inside and scattered around the cells as free deposits and also present as aggregates attached to bacterial cell mass, make evident the capacity of *Enterobacter* sp. B16 strain to tolerate and metabolize higher Se concentration. We previously published the presence of bacillary bacteria and nanoparticles by Scanning electron microscopy and EDS (Acuña et al., 2013). In addition, the electron diffraction patterns of the single Se nanospheres confirmed the occurrence of amorphous Se in lyophilized Se-biofertilizer (Figure 5.6A).

The ability of Seb to penetrate cell wall were visualized by high magnification TEM imaging of roots from plants with and without Seb (Figure 5.6 B,C). From TEM image, we observed amorphous Se nanospheres structure inside the root cells, which not observed in control samples. These results demonstrated that the Se nanospheres are able to penetrate the seed coat and cell root. According to the report by Khodakovskaya et al., (2012) carbon nanotubes can be penetrate in tomato seeds and affect their

biological activity and germination rates. In addition, specific studies on wheat plants have been described the presence of magnetic carbon-coat nanoparticules in the vascular tissues and their tolerance to the high nanoparticules concentration (Cifuentes et al., 2010). It is remarkable that Se nanoparticles were visualized accumulated in vesicules inside the cells. In fact, studies conducted by Corredor et al., (2009) indicate that gold nanoparticles entering to the protoplasts across of the endocytosis and were found accumulated in cluster inside the cells.

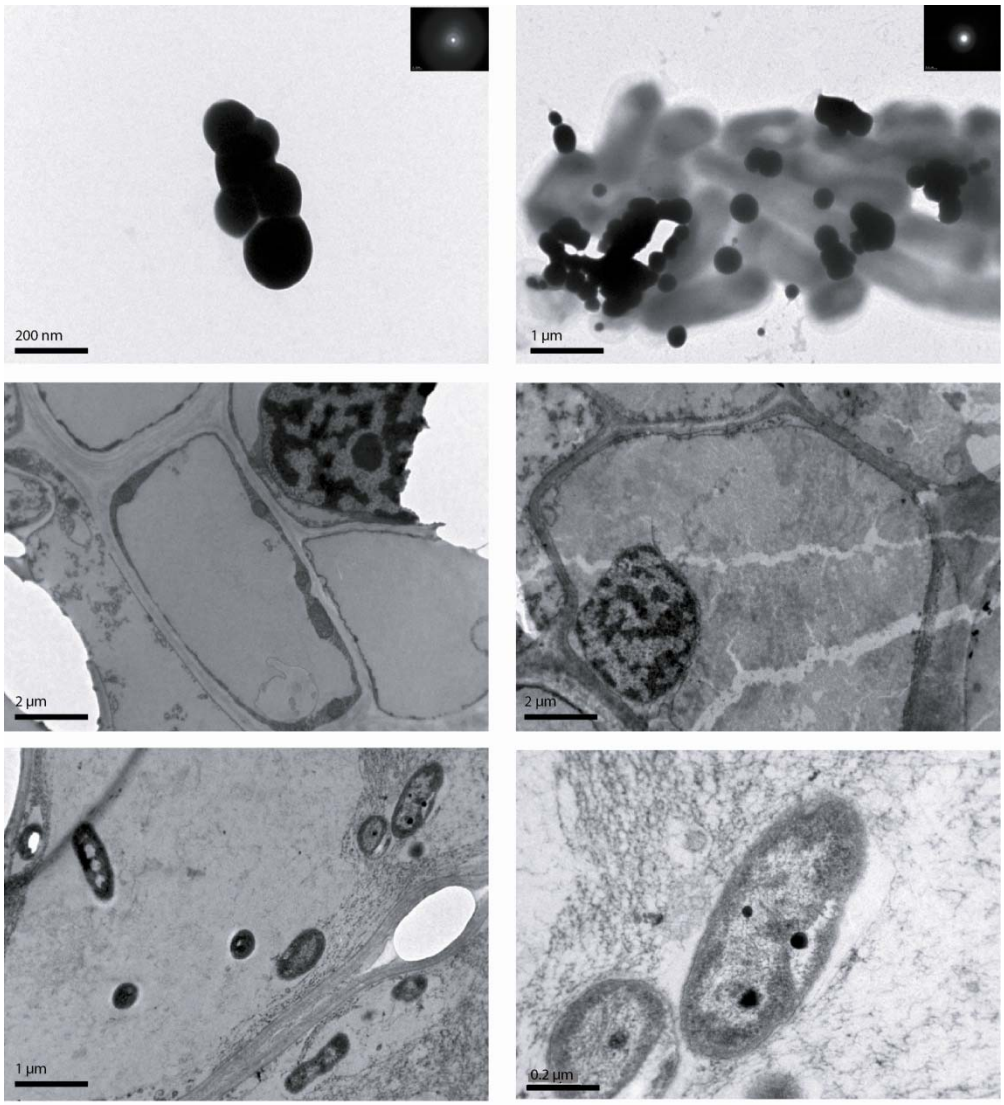

Figure 5.6 TEM images of the root of Se-biosynthesized as nanospheres of elemental Se (A), root cells of wheat seedling without Se biofortification treatments (B) and with Se biofortification treatments.

5.5 Conclusions

According to results we can conclude that Se-biofertilizer produced from selected selenobacteria isolated from volcanic soils is an interesting and environmental friendly biotechnological tool for cereal biofortification and crop production

Also, Se-biofertlizer does not affect seed germination and biomass production in contrasting with Se inorganic fertilizer (over 300 g ha^{-1} equivalents to field conditions)

Interestingly, Se-biosynthesized is more effective than selenite respect to selenium uptake and translocation in wheat plants. Also, Se-biofertilizer has huge potential because is readily available to plants and can alleviate plant stress associated to an enhancement of the plant antioxidant system and not affect native bacterial community. Se-biofertilizer in lyophilized form represents a promising strategy for Se-biofortification to be used in large-scale for intensive crops production. Further studies are needed to elucidate the chemical forms and transport mechanisms of selenium biosynthesized in biofortified wheat plants.

5.6 References

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Chapter VI

General discussion, conclusions and future challenges

6.1 General discussion

Selenium (Se) is a microelement present in agroecosystems with a differential distribution associated with natural and anthropogenic processes determining the occurrence of seleniferous or Se-deficient soils. Several studies have been described the important role of Se as a micronutrient with antioxidant properties for human, animals and plant health. It is estimated that at least 0.5 to 1 billion people worldwide consume food-derived from Se-deficient soil. Suboptimal Se levels in human diet increase the incidence of diseases such as cancer, HIV and heavy metal toxicity.

The food crops are the most important source of Se intake in the world population. Thus, several countries with Se-deficient soils, have implemented the use of Se-fertilizer as a strategy for agronomic fortification. Selenium is added under selenite and selenate inorganic forms, but under Andisol conditions (low pH, high content of organic matter, and high amounts of exchangeable Al), only a small proportion is taken up by plants and much of the remainder is lost by adsorption and leaching processes. Considering that bacteria play an important role in the geochemistry cycle of Se in the agroecosystems we think that emerge the possibility to use bacteria capable to Se-metabolize for enhancing Se content in plants growing in Andisol.

In this research we firstly isolated and selected PGPR strain from native rhizobacteria from cereal crops (wheat, barley and oat), able to tolerate and metabolize Se, called selenobacteria. After, from selenobacteria we selected strains with the presence of multiple action mechanisms such as: 1) phosphorus mineralization/solubilization, 2) indole acetic acid production, 3) siderophore production, and 4) AAC-deaminase activity.

Selenobacteria strains selected were identified belonging to the genera *Stenotrophomonas* sp. B19, *Bacillus* sp. R12, *Enterobacter* sp. B16 and *Pseudomonas* sp. R8. These genera previously have been reported as Se-tolerant bacteria isolated from seleniferous soils. Our results suggested that the Se-tolerance is widely distributed in bacteria and is associated with the Se redox capability to produce oxidation-reduction processes. However, the Se-tolerance in our selected selenobacteria (10mM) were lesser

than Se-resistant bacteria isolated from seleniferous environments (>150mM). The highest rates of growth of some selenobacteria were related to Se chemical reduction capacity detected by red precipitates occurrence in the bacterial biomass. These observations allow us to propose that the reduction of Se (IV) to Se (0) by selenobacteria (*Enterobacter* sp. B16) occurs at the intracellular level. Thus confirming the occurrence of selenobacteria in the rhizosphere with PGPR traits, of cereals crops growing on Andisol and their ability to metabolize and accumulate Se nanospheres.

In terms of biofortification, the inoculations of selenobacteria under a differential concentration of Se in the culture media showed an increased Se content in plants.These results support the hypothesis that the inoculation with selenobacteria is a viable source of Se for increasing Se uptake in plants. Additionally, the highest Se content in grains was observed in wheat plants inoculated with a mixture of four selenobacteria strains with *G. claroideum*, arbuscular mycorrhizal fungi, compared with non-mycorrhizal plants. The results suggest a synergistic effect between selenobacteria mixture and *G. claroideum*. We think that at rhizosphere level the michorrizal colonization can be an important factor to enhance the Se uptake to plants, mediated by root elongation representing an indirect mechanism of Se-uptake observed in mycorrhizal plants. The responses of indigenous soil bacterial community front to the inoculation processes have played an important role in the selective process and the viability of inocula in the rhizosphere. The inoculation of selenobacteria in the rhizospheric zone did not affect the native bacterial communities determined by DGGE analysis.

The inoculation of selenobacteria directly to rhizosphere of wheat plants was the main strategy for evaluating the effectiveness of selenobacteria as a source of Se to the plants. Although this application was effective for enhanced Se uptake in roots and their translocation in leaf and grain in green house conditions, we think is not viable strategy under field conditions.

This study allow us to propose the formulation of a Se-biofertilizer from *Enterobacter* sp. B16 and their inoculation on the seed of wheat by pelletization methodology as a new biotechnological alternative to the traditional fertilization with

inorganic selenium . Interestingly, Se-biofertilizers are more effective than selenite in the Se uptake and translocation in wheat plant under the same doses of Se. These results provide empirical evidence of the great potential of the Se-biofertilizer from selenobacteria for crops biofortification in intensive production system. In addition, wheat seed can tolerate high Se concentration applied as a Se-biofertilizer without affecting its germination and plant biomass production. Comparatively, these results showed the toxicity of selenite and their low availability for plants is not an effective strategy for Se-fortification in plants growing on Andisol.

It is widely accepted the beneficial role of selenite supplementation against to the oxidative damage in plants. In this sense, our study shown that plants growing in high Al saturation (no limed soil), Se supply as Se-biofertilizer decreased the oxidative damage in plants which was associated with reduction of MDA content, decline SOD activity, and increased of CAT activity. In this context, Se-biofertilizers has huge potential because is readily available to plants and can alleviate plant stress associated to high aluminum content and low Se available to plants in Andisols from southern of Chile.

From plant biofortification perspective, the use of Se-biofertilizer should be considered as an important biotechnological strategy to improve nutritional cereal crop status and to obtain a better plant quality. In turn, improved plant nutrition through the biotechnology processes, could increase the competitiveness of our Se biofortified crops in the international market, which generates added value to our food export product.

6.2 General conclusions

Considering that the principal objective of this research was evaluated the Se bioaccumulation by native cereal rhizobacteria bacteria for improving Se biofortification of wheat grown in volcanic soils of southern Chile.

We can conclude that selenobacteria isolated from rhizosphere of crops growing on Andisol, is an interesting biotechnological tool for Se-biofortification. The selenobacteria strains *Stenotrophomonas* sp. B19, *Bacillus* sp. R12, *Enterobacter* sp. B16 and *Pseudomonas* sp. R8. showed the abilities to metabolize Se compounds and their effectiveness to use these compounds as a source of Se available to plants. Furthermore, the co-inoculation with selenobacteria and mycorrhizal fungus, not only was effective in increasing the selenium content in the leaf, but it also was translocated within the plant to the grain demonstrated the great potential of this biotechnology for Se-biofortification programs.

The formulations of Se-biofertilizers in conjunction with strategies to seed inoculation were effective in the mechanisms of absorption, translocation and metabolization of selenium by wheat plants. Moreover, these formulations were not toxic to seeds although high doses used and diminished the oxidative stress in wheat plants because to strengthen the antioxidant system in wheat plants In addition, the Se-biofertilizer application in field conditions could be potentially effective because: 1) the application of selenobacteria is efficient in terms of Se available to plants and 2) the inoculation not affects the diversity of native bacterial communities. In fact, Se-biofertilizer using selenobacteria offers functionally beneficial of PGPR activities and an attractive approach to substitute chemical Se fertilizer, which can improve sustainable agronomic production.

This biotechnology can be use as a strategy for maximize of Se concentrations in grain in order to produce a new functional foods Se-enriched. . However, further studies are needed to elucidate the chemical forms and transport mechanisms of selenium biosynthesized in biofortified wheat plant

6.3 Future challenges

Ongoing and future research on Se-biofortification with Se-biofertilizer are necessaries for elucidating the speciation of selenium in grain and the occurrence of specific organic forms such as selenomethionine and selenocysteine, which are described as essential to counteract immune diseases, cancer and HIV.

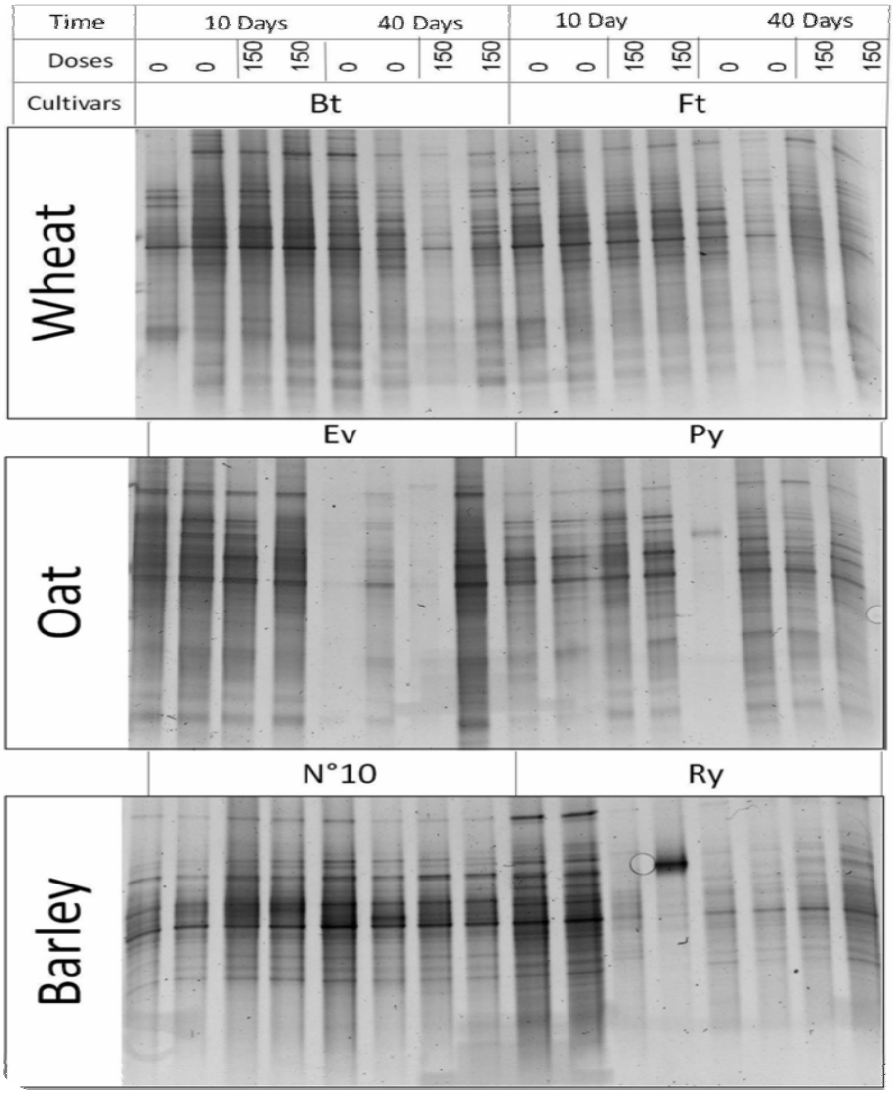
The use of biotechnological tools in field conditions could give an opportunity to understand the real impact of this biotechnology in terms of the nutritional quality of grains. In this sense, the study of Se organic forms into the grain would be an interesting way to know the metabolism induced by selenobacteria application in plants.

Considering the benefits associated with synergism between selenobacteria and arbuscular mycorrhizal fungi in the improvement of Se in plants, the use of Se-biofertilizer associated with early AM native colonization could be considered of biotechnological importance to maximize Se absorption and translocation in crops under field conditions. Futures studies related to the role of AM fungi in the speciation, translocation and uptake of Se under Se-biofertilizer application are needed. In addition, we have developed of pelletization techniques a large scale required for transfer this biotechnology to field conditions. Nowadays, the Se-biofertilizer applications are used to evaluate the response of different cereals species and cultivars

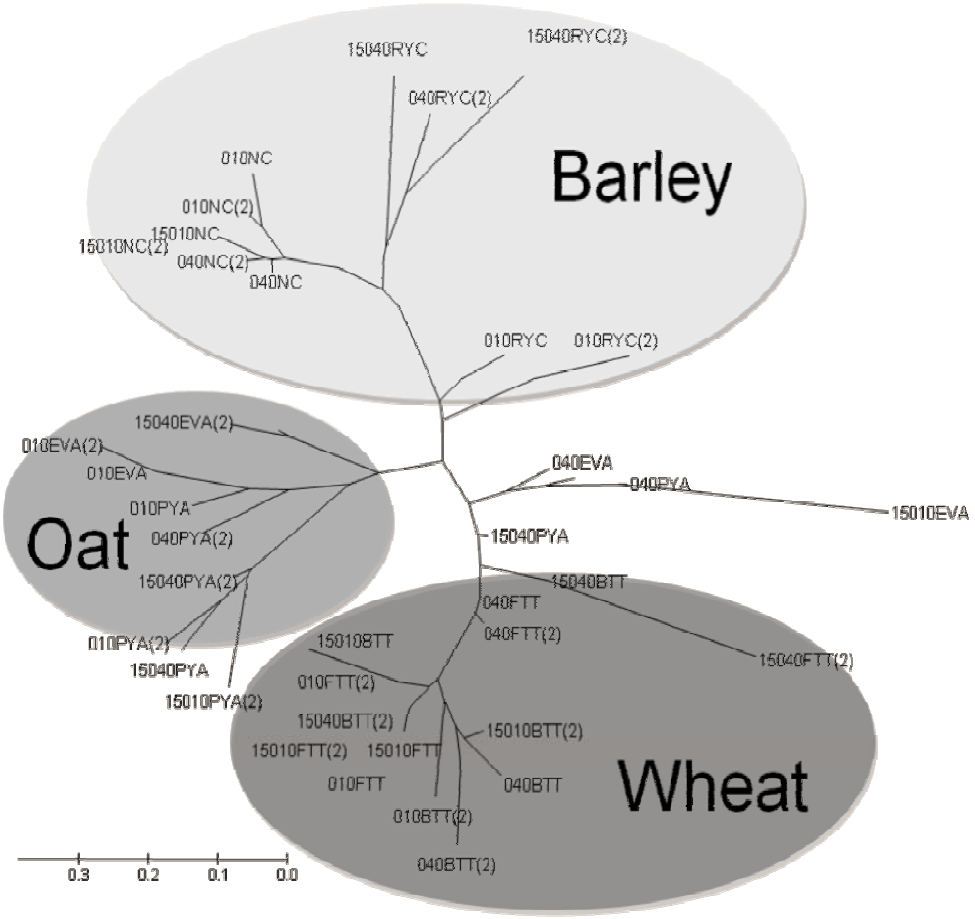
Moreover, the development of adequate and easily performable molecular tools to monitor the metabolic pathways associated with Se-nanospheres in cells plants need to be studied.

Finally, future research should be include toward the evaluate the Se-bioremediation process in copper mining waste water treatment for the obtaining of Se-enriched bacteria and utilize these bacteria as biofertilizer in the cereal biofortification process.

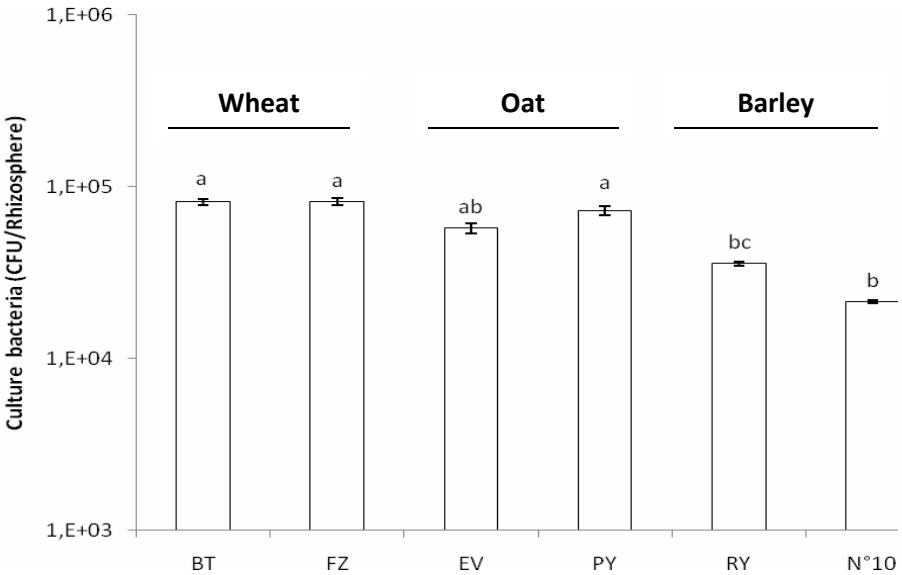
Appendices



Appendix i a). Banding pattern obtained by DGGE of bacterial communities associated with the rhizosphere of wheat, oat and barley on days 10 and 40 at doses of 0 and 150 g Se ha^{-1}.



Appendix i b) Tree based on the pattern of bands obtained by DGGE. Wheat (FTT, BTT), oat (PYA, EVA), barley (NC, RYC).

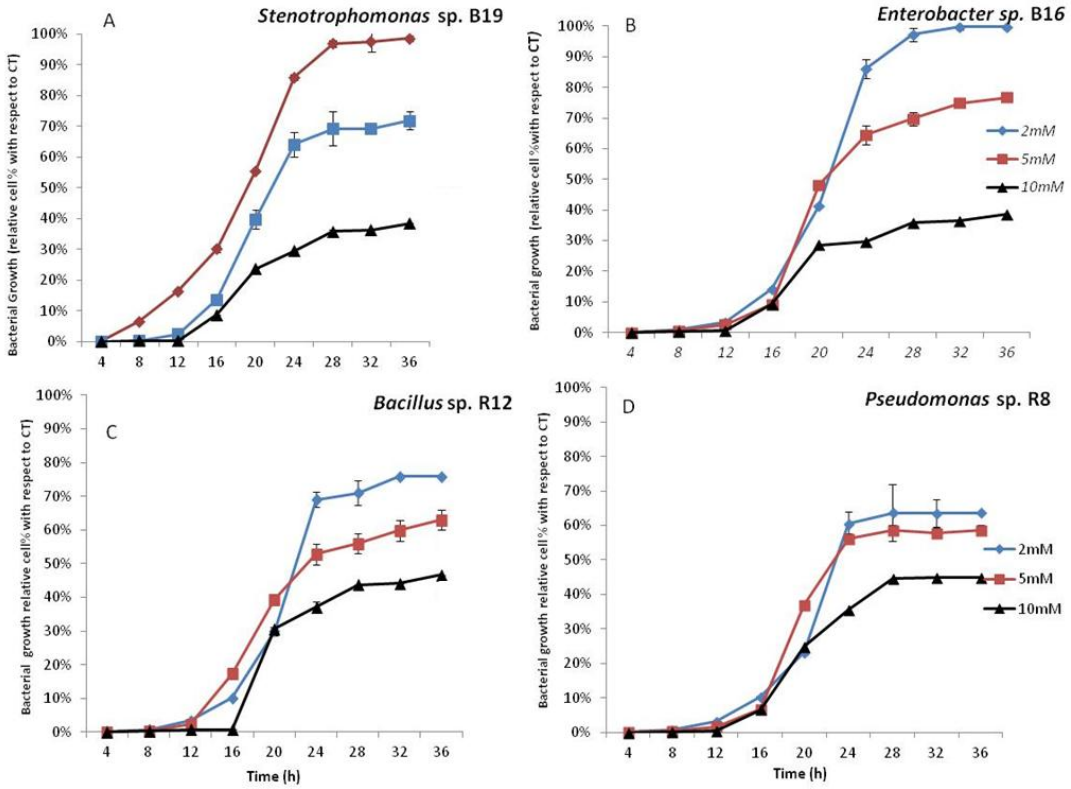


Appendix ii. Rhizobacteria populations capable to growth under selenite enrichment media at 2mM in Nutrient broth media from cereals crops (wheat, oat and barley) growing on Andisol.

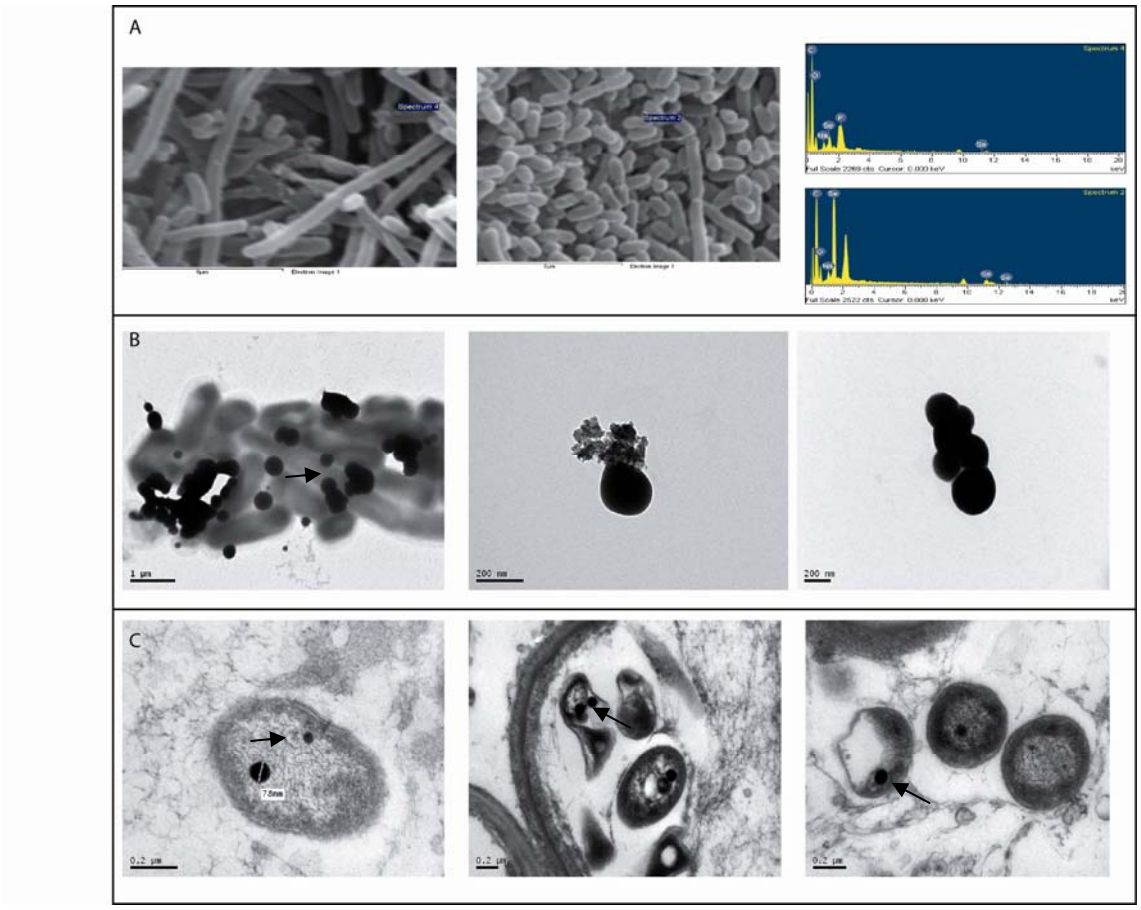
Appemdix iii. Identification and characterization of potential PGPR traits of isolated selenobacteria strains.

	Isolate			
	B19	B16	R12	R8
Source	Oat rhizosphere	Wheat rhizosphere	Wheat rhizosphere	Barley rhizosphere
Color colony[a]	White-orange centers	White-orange centers	Red	Red
IAA production (µg ml^{-1} medium)	8.0±0.023	12.2±0.017	10.2±0.029	10±0.001
Ca Phytate mineralization (PSM)	+	+	+	+
Ca Phosphate solubilization (NBRIP)	+	+	+	+
ACC desaminase activity	+	+	+	+
Siderophore production	+	+	+	+
Genus [b]	*Stenotrophomonas*	*Enterobacter*	*Bacillus*	*Pseudomonas*
Accession number	JN644923	JN644922	JN644921	JN644924

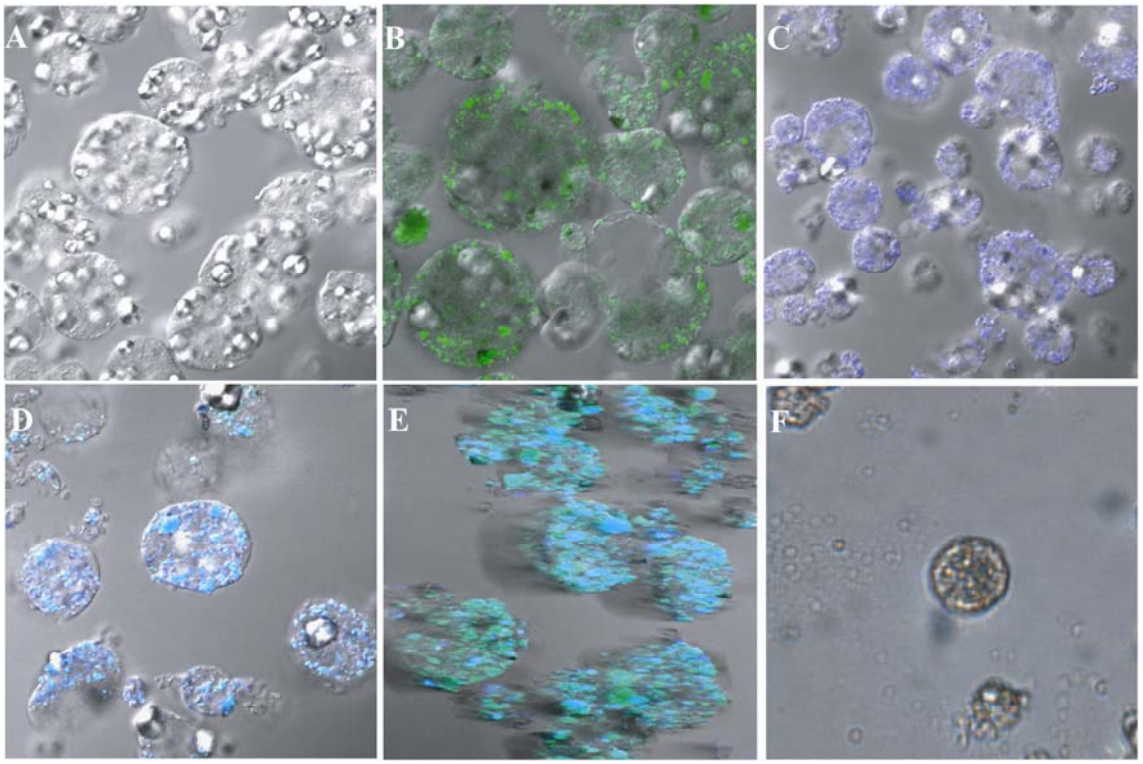
[a] on nutrient agar supplemented with 2 mM of sodium selenite. [b] based on partial sequencing of 16S rRNA gene and comparison with those present in GenBank from NCBI (http://www.ncbi.nlm.nih.gov). IAA: indole acetic acid. PSM: phytase–screening medium. NBRIP: national botanical research institute's phosphate growth medium.



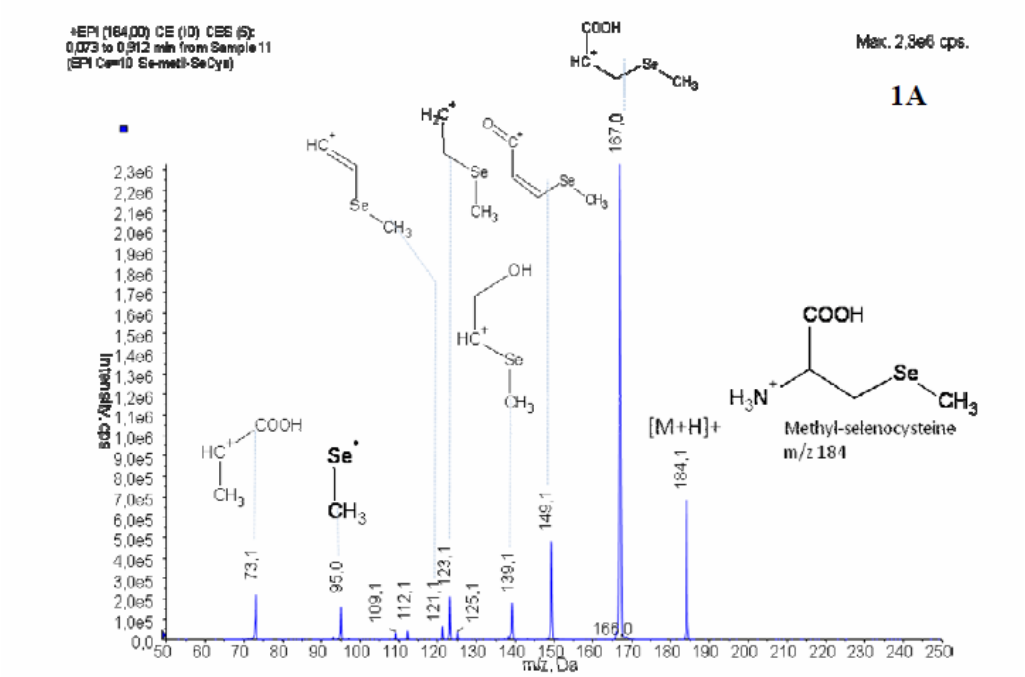
Appendix iv. Growth profile of selenobacteria strains in the presence of high concentrations of sodium selenite (2mM, 5mM and 10mM). (A) *Stenotrophomonas* sp. B19, (B) *Enterobacter* sp. B16, (C) *Bacillus* sp. R12 and (D) *Pseudomonas* sp R8.



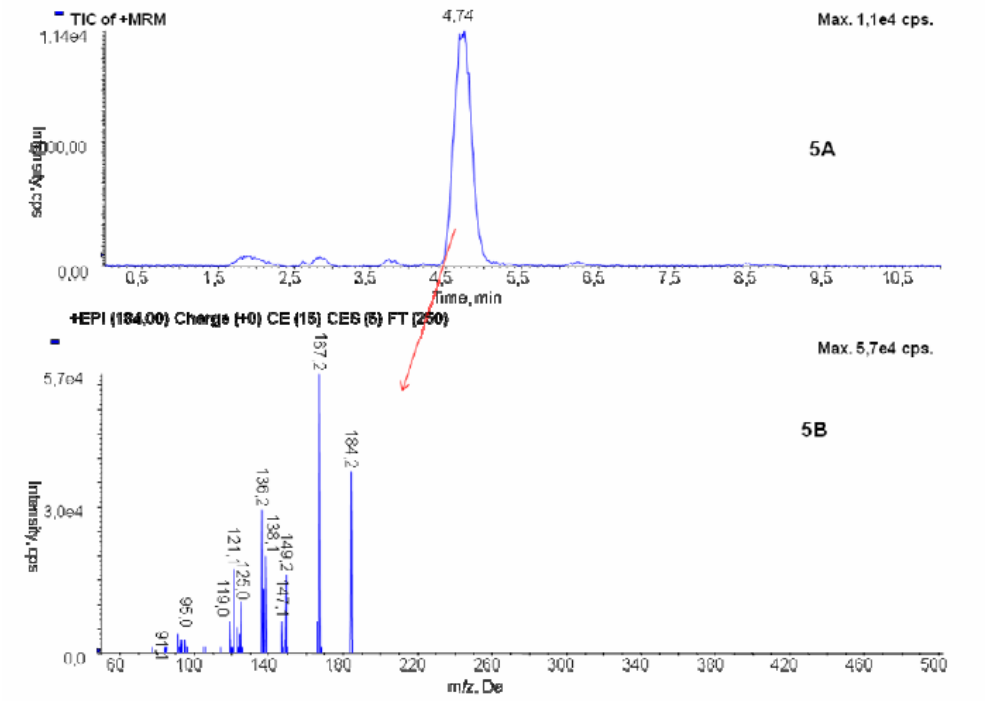
Appendix v. SEM-EDS analysis of selenobacteria cells grown in nutrient medium supplemented with 2 mM of selenite (A). TEM images of Se-nanospheres from selenobacteria (B) and (C) Se-nanospheres deposited inside the roots cells. The selenium deposits are indicated by black arrows.



Appendix vi. Confocal microscopy of the microcapsules (A) shows the production of microspheres of microcomposites, (B) fluorescence clays, (C) selenobacteria fluorescence stained with DAPI, (D) fluorescence clays and bacteria, (E) microspheres 3D projection fluorescence and optical microscopy (F) microcapsules selenobacteria selenium. (M.Calabi)



Appendix vii. MS-MS fragmentation pattern of the Se-methyl-Secysteine



Appendix vii. MRM-EPI experiment of selenobacteria samples *Enterobacter sp. B16* grown in nutrient enrichment medium supplemented with selenite (5mM) and peak fragmentation fingerprint 4.74 min.

Appendix viii. Comparisons of means between antioxidant activities under Se-biofertilizer by HSD the Tukey-Kramer test

Comparisons for all pairs using the test
Quantile Confidence

q*	Alpha
3,29108	0,05

LSD threshold matrix

Positive values show pairs of means that are significantly different.
Connecting letters report

SOD No Limed soil			
Nivel			**Media**
1	A		9,3596325
3	A		9,0313266
5	A		8,5654880
4		B	5,4455224
2		B	5,1277992

SOD Limed soil		
Nivel		**Media**
1	A	10,004898
2	A	9,123552
4	A	8,724760
3	A	7,589825
5	A	7,195440

Apx No limedsoil			
Nivel			**Media**
2	A		0,21446631
4	A		0,20768290
1	A	B	0,12345646
3		B	0,06433614
5		B	0,05661459

ApxLimedsoil				
Nivel				**Media**
4	A			0,43154941
1	A	B		0,27845001
2		B		0,22634281
3		B	C	0,10674393
5			C	0,04766693

CAT No Limedsoil		
Nivel		**Media**
2	A	0,19067469
3	A	0,17929218
4	A	0,16837755
5	A	0,16302689
1	A	0,11270091

CAT Limedsoil				
Nivel				**Media**
2	A			0,23140204
3		B		0,14214559
4		B		0,12871607
5		B		0,07942903
1			C	0,00647229

Levels not connected by the same letter are significantly different.