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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 ($P \leq 0.05$). 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 co-inoculated 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.

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 Se-fertilizers, 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).

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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.

2. Experimental

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.

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.

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.

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).

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 2.2).

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. (2012). 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) 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 by Yang et al. (2003).

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.

3. Results

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 1).

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 (Fig. 1).

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 non mycorrhizal plants (3.6–12.1 mg kg^{-1}) in both selenite and selenate (Table 2), while in SVP from plants inoculated with selenobacteria mixture, no significant differences were observed (Table 3).

Plants inoculated with selenobacteria grown in selenite showed higher Se content in tissues compared to those inoculated with selenobacteria grown in selenate (Table 2 and 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 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 3).

3.4. Selenium content in grain

Wheat plants inoculated with *Enterobacter* sp. B16 strain showed greatest Se concentration in grain (Table 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 non mycorrhizal 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 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 (Fig. 2).

Table 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	9.4.8	209.9	0.21
Bacillus sp. R12	1224.4	52.8	0.54
Pseudomonas sp. R8	722.5	357.5	0.63

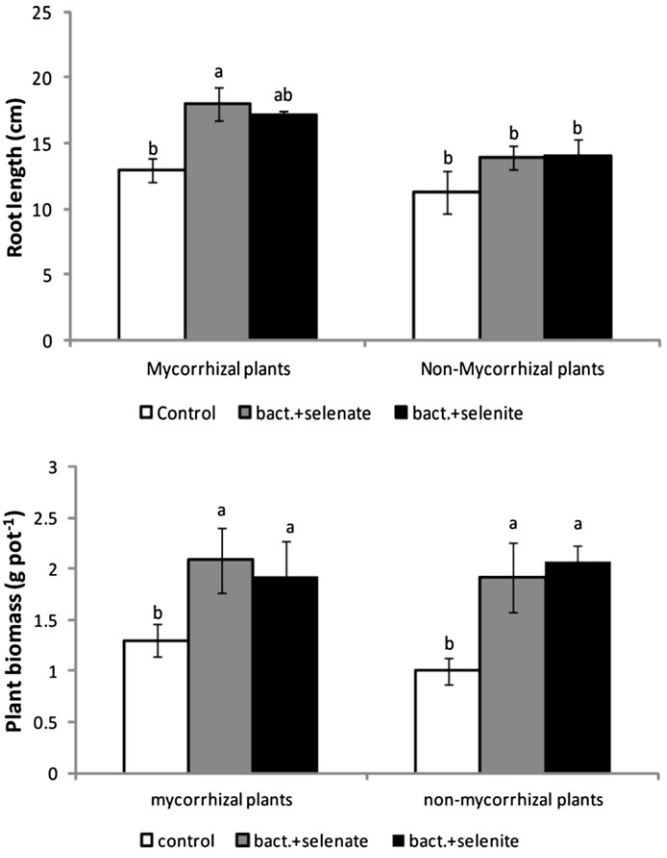

Fig. 1. Root length (cm) and plant biomass (g pot^{-1}) in mycorrhizal and non-mycorrhizal plants. Tukey test to compare treatments means, values followed by the same letter do not differ at $P \leq 0.05$ ($n = 3$).

3.5. PCR-DGGE

Analysis of DGGE profiles is shown in Fig. 3. In mycorrhizal and non mycorrhizal plants, the DGGE profiles showed predominant ribotypes similar to inoculated selenobacteria mixture (Fig. 3A). Moreover, differences between bacterial communities of mycorrhizal and non mycorrhizal plants were generally consistent as shown by hierarchical cluster analysis (Fig. 3B). In this context, Shannon–Wiener 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 (Fig. 3C).

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.,

Table 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	0.7 g	0.62 g	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 ($p \leq 0.05$; $n = 3$).

2012). 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 (Se^0) 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 + 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.,

Table 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 ($p \leq 0.05$; $n = 3$).

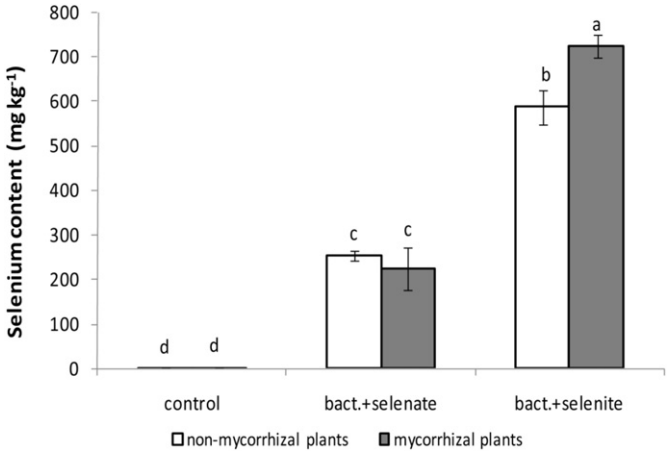

Fig. 2. Total Selenium content in grain of non-mycorrhizal and mycorrhizal plants inoculated with selenobacteria mixture grown in nutrient broth supplemented with 5 mM of selenite (Se IV) and selenate (Se VI). Tukey test to compare treatments means, values followed by the same letter do not differ at $P \leq 0.05$ ($n = 3$).

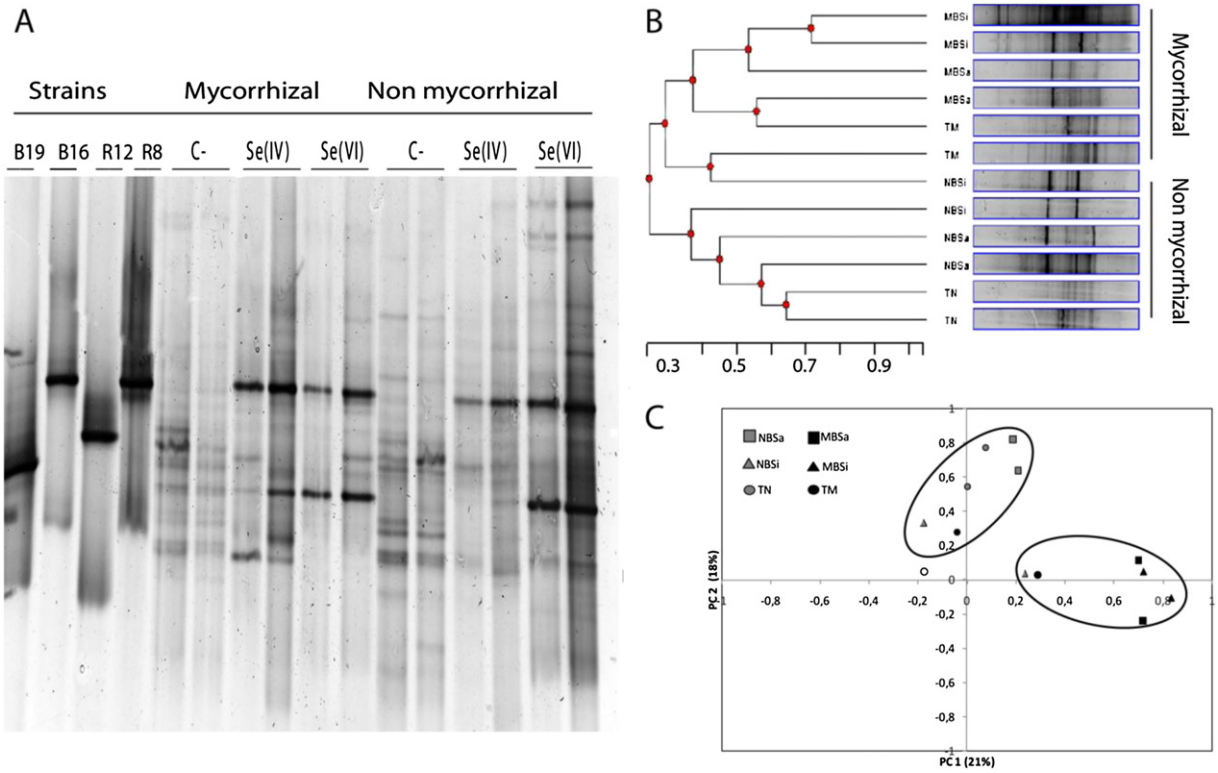

Fig. 3. A) Denaturing gradient gel electrophoresis (DGGE) analysis, B) dendrogram of DGGE profiles and C) principal component analysis (PCA) of bacterial communities in non mycorrhizal and mycorrhizal plants inoculated with selenobacteria mixture grown in nutrient broth supplemented with 5 mM of selenite [Se(IV)] and selenate [Se(VI)]. 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.

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 μg 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 co-inoculation 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.

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 of wheat 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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