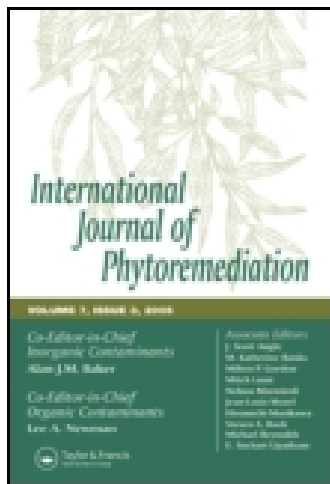


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Microbial-enhanced Selenium and Iron Biofortification of Wheat (*Triticum aestivum* L.) - Applications in Phytoremediation and Biofortification

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Selenium (Se) is an essential trace element for humans and other mammals. Most dietary Se is derived from crops. To develop a Se biofortification strategy for wheat, the effect of selenate fertilization and bacterial inoculation on Se uptake and plant growth was investigated. YAM2, a bacterium with 99% similarity to *Bacillus pichinotyi*, showed many plant growth promoting characteristics. Inoculation with YAM2 enhanced wheat growth, both in the presence and absence of selenate: YAM2-inoculated plants showed significantly higher dry weight, shoot length and spike length compared to un-inoculated plants. Selenate also stimulated wheat growth; Un-inoculated Se-treated plants showed a significantly higher dry weight and shoot length compared to control plants without Se. Bacterial inoculation significantly enhanced Se concentration in wheat kernels (167%) and stems (252%), as well as iron (Fe) levels in kernels (70%) and stems (147%), compared to un-inoculated plants. Inoculated Se-treated plants showed a significant increase in acid phosphatase activity, which may have contributed to the enhanced growth. In conclusion; Inoculation with *Bacillus sp.* YAM2 is a promising Se biofortification strategy for wheat and potentially other crops.

Keywords: *Bacillus pichinotyi*, wheat biofortification, ICP-AES, micronutrients

Introduction

Selenium (Se), which is ubiquitous in the environment, is an essential micronutrient that can potentially be toxic, depending on its chemical form and dose (Douron 2010; Lee and Jeong 2012). Unlike other trace elements that are also essential, such as manganese and iron, Se can be toxic for human health. Selenium has garnered strong consideration from the scientific community and the public as a potential anti-carcinogen and antioxidant. In human beings, almost 25 different selenoproteins have been identified, and many of them are involved in catalytic functions (Rayman 2012). In counties of the United States with high or intermediate Se levels, cancer mortality rates were significantly lower for total cancer and cancers of the lung, bladder, oesophagus, breast, pancreas, rectum, cervix, ovary and colon compared with low-Se counties (Clark *et al.* 1991). Several laboratory studies have shown that Se may interact with other elements, such as arsenic and mercury, and that this may reduce the toxicity of both the Se and the other

elements (Anan *et al.* 2011; Soudani *et al.* 2011; Zwolak and Zaporowska 2012).

Foods (i.e., vegetables, grains, cereals and other consumable plant products) can contain various chemical forms and concentrations of Se (Fang *et al.* 2003; Sanmartín *et al.* 2012). For human beings, an estimated adequate Se intake is 50–60 µg/day, while toxic levels of Se intake are 350–700 µg/day (Sanmartín *et al.* 2012). In different organisms, inorganic selenite can be converted into organic forms, which are considered safer (Sanmartín *et al.* 2012) and more effective dietary sources of Se. Selenium may also be complexed, via binding, with various polysaccharides and proteins. For human beings, Se consumption or supplementation with low doses has substantial health benefits, among which the prevention of cardiovascular disease and cancer is of special importance (Sanmartín *et al.* 2012).

Crops, which are major sources of dietary Se, derive their Se from the soil. Soil Se is very uneven in distribution as well as chemical availability. So, the Se content of the soil, from which consumable plant foods are derived, has major influence on dietary intake of Se. Around the world, the Se concentration in most of the soils is 0.1–2 mg Se/kg, with a mean of ~0.4 mg/kg; however, in seleniferous areas, higher concentrations (>10 mg/kg) can occur (Berrow and Ure 1989; Fordyce 2005). Selenium deficient soils in Australia, central Siberia,

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New Zealand, northeast to south central China, Denmark, and parts of Bangladesh and India, produce crops with very low Se content (Combs 2001).

A better understanding of Se uptake and metabolism in plants is highly necessary to help find a solution for nutritional Se deficiencies. This goal may be achieved by elevating soil Se levels or by enhancing uptake efficiency. Bacteria are known to affect elemental bioavailability and Se uptake by plants. In this study, we tested the hypothesis that a Se-tolerant strain (YAM2) would enhance growth and micronutrient levels (including Se) in wheat (*Triticum aestivum* L.). Moreover, we tested the effect of Se and strain YAM2 on acid phosphatase activity and soluble protein content of the plants.

Materials And Methods

Ammonia Production Test

For the ammonia (NH₃) production test, 10 mL peptone water (4%) in autoclaved glass tubes was inoculated with YAM2 fresh bacterial culture and incubated at 28°C for 72 h. Nessler reagent (0.5 ml) was added and the appearance of brown to yellow color monitored as indication of ammonia production (Cappuccino and Sherman 1992).

Auxin Biosynthesis

Auxin production was detected both in the presence and absence of tryptophan using a modification of the method described by Brick *et al.* (1991). 5 mL LB medium with and without L-tryptophan (1.0 g L⁻¹) was prepared and autoclaved in glass tubes, then inoculated with YAM2 fresh bacterial culture, and incubated at 37°C for 48 h. The cultures were centrifuged at 10,000 rpm for 5 minutes, and 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent (0.2 mL of 0.5 M FeCl₃ + 98.0 mL of 35% HClO₄). The reaction mixture was incubated in the dark for 30 min at room temperature and then monitored for pink color development.

Phosphate Solubilization

The method described by Gaur (1990) was used to test for phosphate solubilization. Pikovskaya's medium was prepared and poured in autoclaved petri plates. Fresh bacterial strains (24 h old) were streaked on the plates and incubated at 28°C for 7–10 days. The colonies were monitored for surrounding clear zone formation.

Hydrogen Cyanide Production

Hydrogen cyanide (HCN) activity of bacterial culture was determined as described by Lorck (1948). Overnight incubated bacterial culture was spread on modified agar plates (nutrient agar supplemented with glycine); at the top of the plate Whatman filter paper No.1 was placed, soaked in 2% sodium carbonate solution made in 0.5% picric acid. The plates were

incubated at 28°C for 3–4 days, and development of an orange to red color was observed.

Nitrate Reduction and Organic Acid Production

Nitrate reduction and organic acid production were measured as described in Cappuccino and Sherman (1992). To test for nitrate reduction activity, nitrate broth was used. For the mixed acids production test, MR/VP broth was used.

Plant Growth Experiment

The *Triticum aestivum* plants (variety Seher 2006), obtained from Punjab Seed Corporation Lahore, were cultivated at the University of the Punjab Lahore (Latitude: 31° 35' North, Longitude: 74° 18' East). The experiment started in January 2012, when the highest day temperature was 19°C, and the lowest night temperature was 2°C. Pots (12" in diameter and 14" in height) were filled with 8 kg of non-seleniferous field soil, taken from the Botanical Garden of the University of the Punjab.

The wheat seeds were surface-sterilized with 0.1% HgCl₂ for five minutes. To inoculate seeds with bacterial culture YAM2, the purified bacteria were grown overnight in L-broth at 37°C on a shaker at 150 rpm. The optical density (OD), at 600 nm, of the culture was adjusted to 1 with sterilized distilled water. Seeds were pre-inoculated with bacterial culture by immersing them in the bacterial suspension and sown in their respective pots. After sowing, 3 mL of bacterial culture (OD₆₀₀ = 1) was taken, and diluted to 100 mL with sterilized, distilled water, and then spread out over the respective pots also. The plants were watered with 600 mL water per pot two times weekly during the cold season (Jan–March) and as needed in the subsequent hot season. The environmental conditions were natural; the plants were grown in natural daylight in a wire house.

Selenium Treatment and Co-Cultivation

Sodium selenate (Uni-Chem) was used as a source of Se for the wheat plants. The Se treatment was given to the plant via watering with sodium selenate (Na₂SeO₄) solution (300 μM). The Se treatment was given in two doses. One liter of 300 μM sodium selenate solution per pot (3 mg Se kg⁻¹ soil) was given to the plants after five days post seed germination, and the second dose of one liter of 300 μM sodium selenate solution per pot (3 mg Se kg⁻¹ soil) was given to the plant at the time of spike formation. After inoculation and Se treatment, the plant and bacteria were co-cultivated for a period of 18 weeks before harvest.

Soluble Protein Content and Acid Phosphatase Activity

After 8 weeks of growth post germination, soluble protein content and acid phosphatase activity were determined for the wheat plants treated with or without Se and bacterial inoculum. For determination of total soluble proteins, the method described by Lowry *et al.* (1951) was used, and for this purpose

Table 1. Plant growth promoting characteristics of YAM2 strain. The properties were scored as positive (+) or negative (-)

Strain	HCN production	Phosphate solubilization	Auxin production	Organic acid production	Nitrate reduction	Ammonia production
YAM2	+	-	+ (12 $\mu\text{g mL}^{-1}$)	+	+	+

one gram of frozen plant material was crushed in phosphate buffer (0.1 M) with the help of a Heidolph SilentCrusher M at 16,000 rpm in an ice bath at a ratio of plant material to buffer of 1:4 (w/v). After following the protocol steps, the absorbance for soluble protein was taken at 750 nm with a Cecil Aquarius CE7200 double beam spectrophotometer.

The method described by Iqbal and Rafique (1987) was used for extraction and quantification of acid phosphatase enzyme. One gram of frozen plant shoot was crushed in cold 0.1 M Tris HCl buffer (pH 6.5) with the help of a Heidolph SilentCrusher M at 16,000 rpm in an ice bath with a ratio of buffer to plant material of 4:1 (v/w). To determine the activity of acid phosphatase, the temperature selected was 37°C, time duration was one hour, and the pH was 4.9. Following the chemical reactions, the absorbance was determined at 510 nm by a Cecil Aquarius CE7200 double beam spectrophotometer.

Elemental Analysis

After harvest, plant samples were oven dried at 50°C for 48 hours. Selenium and Fe content analysis in stem and kernel samples was done as described by Lindblom *et al.* (2012). Dried plant samples (0.1 g) were digested in 1 mL of concentrated nitric acid for 2 h at 60°C and then 6 h at 130°C. The acid digests were then diluted to 10 ml with double distilled water and analyzed for Se and Fe contents by inductively coupled plasma atomic emission spectroscopy (ICP-AES).

Statistical Analysis

Analysis of variance (one way and two-way) was performed using the statistical software package JMP V10.0 (SAS institute Inc.). A post-hoc Tukey's Honestly Significant Difference (HSD) test was used to determine significant differences between means. For comparison between two means, t-test was used. Before analysis, all data sets were checked for normal distribution and equal variance.

Results and Discussion

Strain YAM2 (Accession No: JX203257) was isolated from the sediments of the Roohi Nala drain, a sub-branch of the Hudaira drain that receives domestic as well as industrial effluents of Lahore city. Based on 16S rRNA sequencing, YAM2 shows 99% homology with *Bacillus pichinoty*, which was the closest known relative. Strain YAM2 showed extreme tolerance to selenite (>20 mg $\text{Na}_2\text{SeO}_3 \text{ mL}^{-1}$ of L-Agar) and selenate (>10 mM Na_2SeO_4 in LB medium) (results not shown).

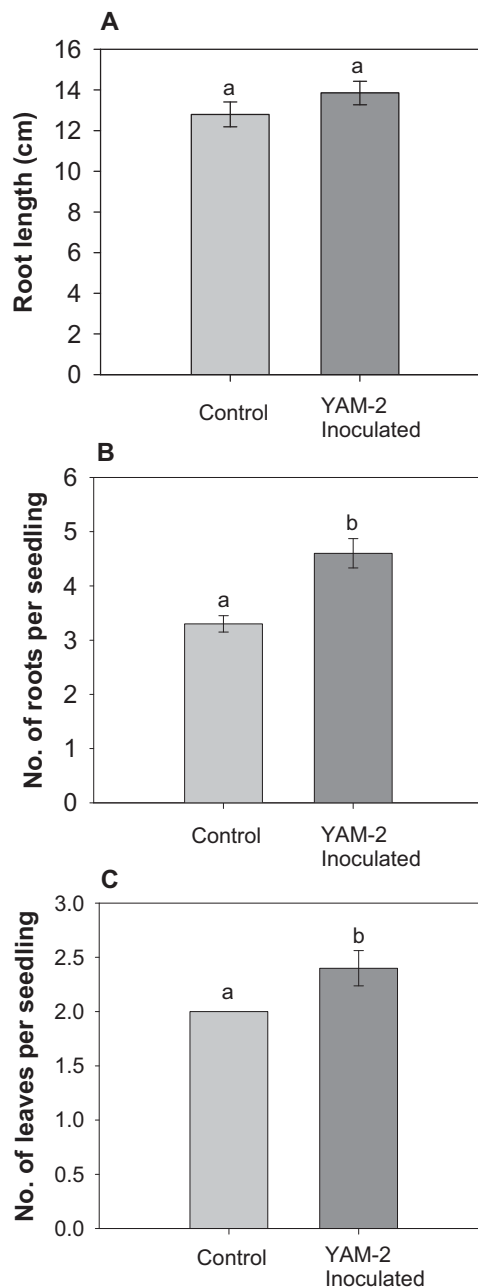


Fig. 1. Effect of YAM2 inoculation on root length (A) number of roots (B) and number of leaves (C) on *T. aestivum* seedlings. Bars labeled with different letters represent means that are significantly different, judged from Student's t-test at 95% confidence level. The data represent mean (n = 10) \pm SE of mean.

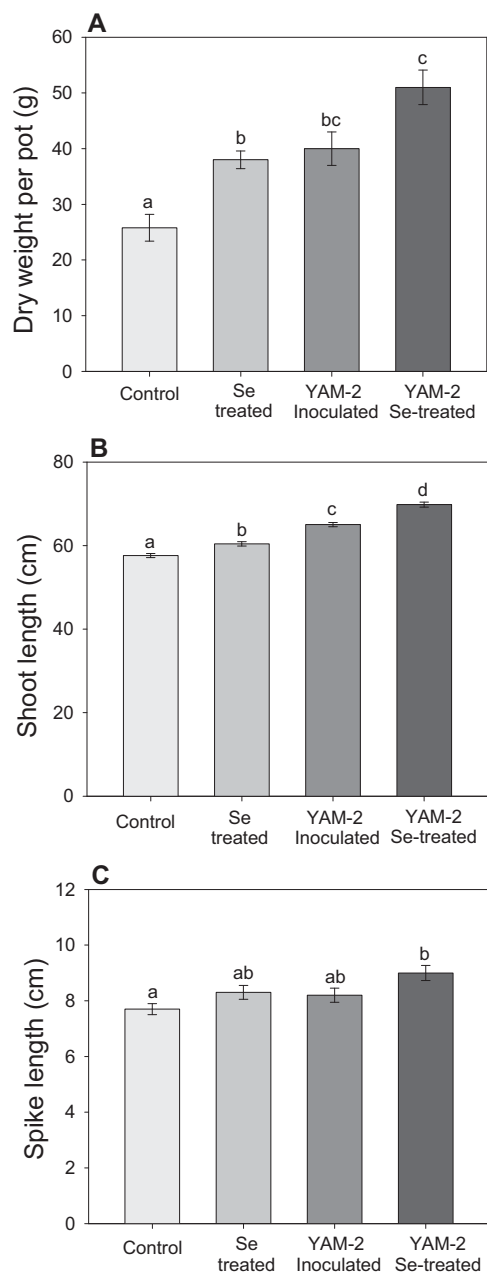


Fig. 2. Effect of YAM2 strain inoculation on physical growth parameters, (A) dry wt/ pot ($n = 3$), (b) shoot length ($n = 5$) and (c) spike length ($n = 5$) of *T. aestivum* grown with and without Se. The data shown are mean \pm SE of the mean. Bars labeled with different letters represent means that are significantly different, judged from one way ANOVA followed by Tukey's (HSD) test at 95% confidence level. Note: two-way ANOVA did not show significant interaction level between Se and YAM2 treatments.

Furthermore, YAM2 showed many plant growth promoting characteristics, including auxin production (Table 1). Inoculation of wheat (*T. aestivum*) seedlings with YAM2 resulted in significantly increased growth (Fig. 1). While root length was not different (Fig. 1A), there were more roots (Fig. 1B) and more leaves (Fig. 1C). Because of these qualities, strain YAM2 was tested for its capacity to promote growth of wheat until

maturity, as well as for its effects on Se uptake in wheat and plant nutrient content. Inoculation of *T. aestivum* with YAM2 resulted in a significant increase in dry weight production (41%) and shoot length (14%) compared with uninoculated control plants (Fig. 2A, B). Spike length was not statistically different (Fig. 2C).

In the absence of bacterial inoculum, Se treatment of the soil in a range normally found in seleniferous soils (3–6 mg Se kg^{-1} soil) yielded positive effects on *T. aestivum* growth parameters. Plants treated with Se showed significantly increased dry weight (38%) and shoot length (5%) compared to control plants that did not receive Se (Fig. 2A, B). Spike length was not significantly affected by Se (Fig. 2C).

In the presence of Se, YAM2 inoculation again positively affected *T. aestivum* growth: Se-treated inoculated plants showed an increase in dry weight (34.5%) and shoot length (16%) compared to un-inoculated Se-treated plants (Fig. 2A, B). Se-treated-YAM2 inoculated plants attained 32% more dry biomass and 7% taller shoots lengths compared to YAM2 inoculated plants grown without Se (Fig. 2A, B). This was almost statistically significant ($P = 0.06$). Spike length was not significantly affected. The positive effects of YAM2 inoculation and Se treatment on wheat growth were additive: plants treated with Se and YAM2 both attained higher biomass and were taller than plants treated with only Se or only YAM2 (Fig. 2A, B). Moreover, while spike length was not significantly

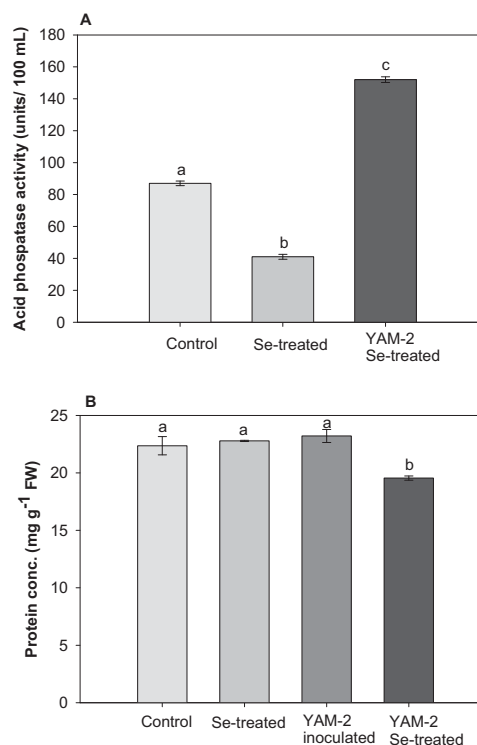


Fig. 3. Effect of YAM2 inoculation and Se treatment on acid phosphatase activity (A) and soluble protein content (B) of *T. aestivum* plants. The shown data are the mean ($n = 3$) \pm SE of the mean. Bars labeled with different letters represent means that are significantly different, judged from ANOVA followed by Tukey's (HSD) test at 95% confidence level.

affected by YAM2 or Se alone, it was significantly higher in YAM2 inoculated Se-treated plants as compared to plants not treated with either YAM2 or Se (Fig. 1C). The effects of Se and of YAM2 were independent, judged from two-way ANOVA which showed no significant interaction.

In the absence of bacterial inoculum, when the soil was treated with 3 mg Se kg⁻¹ soil, the wheat plants showed a significant decrease in leaf acid phosphatase activity (60%) compared to control plants not treated with Se (Fig. 3A). Plants that were inoculated with YAM2 and Se-treated showed a significantly higher acid phosphatase activity compared to control plants and Se-treated plants (Fig. 3A). Thus, YAM2 appeared to alleviate the negative effect of Se on this enzyme activity. The reason for the negative effect of Se on acid phosphatase activity is not clear. While it might be due to incorporation of Se into proteins in general, replacing S and thus negatively affecting protein function, this does not appear likely since the Se-treated plants showed no signs of toxicity and even grew better in the presence of Se. In a previous study, we also found that bacterial inoculation enhanced (by 40%) acid phosphatase activity in *T. aestivum* plants (Yasin *et al.* 2013). The mechanism underlying this phenomenon will require further study. Likely, the phosphatase enzyme activity measured was indeed plant-derived and not from YAM2 directly, since this strain tested negative for phosphate solubilization in the absence of plants (Table 1). It is possible that the bacteria stimulated the activity of this enzyme in the plant, not only in leaves but also in the root zone (rhizosphere). That would explain the enhanced plant growth, if phosphate supply was limiting plant growth and enhanced acid phosphatase activity in the root zone increased phosphate bioavailability.

Plants inoculated with YAM2 or treated with Se showed no difference with control plants with respect to soluble protein content in leaves (Fig. 3B). Plants that were YAM2 inoculated and Se treated, however, showed significantly lower leaf protein content compared to control plants (Fig. 3B). The reason for this effect is not clear.

Elemental analysis was carried out on Se-treated plants only, since Se was the primary element of interest. Selenium-treated YAM2 inoculated plants showed a significant increase in Se concentration in stem (252%) and kernel (167%) compared to Se-treated control plants (Fig. 4A, B). Thus, YAM2 stimulated plant growth and led to higher Se concentration. Total plant Se accumulation (biomass x concentration) was therefore doubly enhanced by this bacterium. YAM2 not only enhanced Se levels in *T. aestivum*, but it also significantly enhanced the iron (Fe) concentration in stems (147%) and kernels (70%) of Se treated wheat plants compared to the Se-treated control (Fig. 5A, B). There were no significant effects on the tissue concentrations of some other plant nutrient elements (not shown).

The mechanism by which YAM2 stimulated plant growth may have been its ability to produce auxin (Table 1). This plant growth promoting substance enhances root formation, which was indeed observed as a higher number of roots at the seedling level (Fig. 1). The effect of YAM2 on Se and Fe accumulation is less readily explained. In other studies, bacteria from various origins were also shown to affect plant Se accumulation and plant growth. For instance, Durana *et al.*

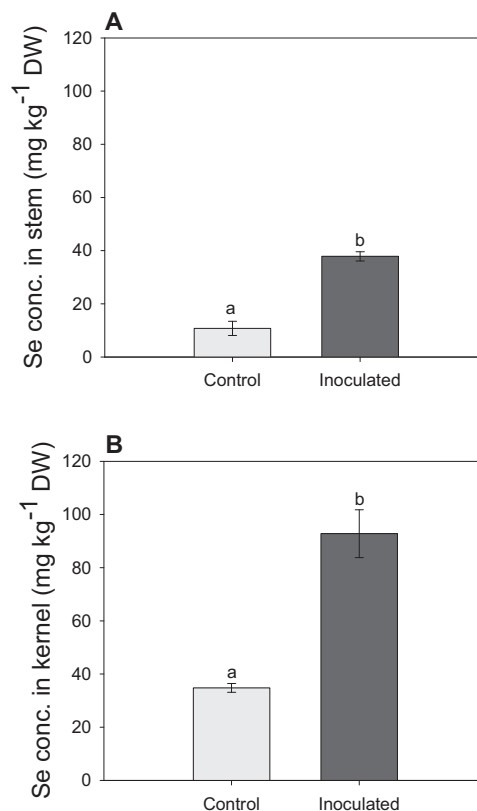


Fig. 4. Effect of YAM2 inoculation on Se concentration in *T. aestivum* stems (A) and kernels (B), treated with Se. Bars labeled with different letters represent means that are significantly different, judged from Student's t-test at 95% confidence level. The shown data represent mean (n = 5) ± SE of mean.

(2013) reported that inoculation with rhizospheric microorganisms significantly enhanced wheat Se content. Moreover, de Souza *et al.* (1999) found that rhizosphere bacteria from a seleniferous area enhanced plant uptake of selenate as well as Se volatilization in *B. juncea* and several aquatic plant species. In the study with *B. juncea*, it appeared that the mechanism included enhanced root hair formation induced by the bacteria, perhaps via a similar mechanism as found here for YAM2. Also, in the *B. juncea* study, there was a higher level of the amino acid serine (or O-acetylserine) in the growth medium, which may have upregulated the sulfate/selenate assimilation pathway. It is possible that YAM2 affected Se uptake in wheat via a similar mechanism. The mechanisms responsible for the positive effect of YAM2 on Fe uptake may include a general stimulation of root growth, as well as enhanced Fe bioavailability. The bacterium may help acidify the soil or produce chelators that bind soil Fe and bring it into solution. Many *Bacillus* species are known to produce siderophores that make soil Fe³⁺ bioavailable for plant uptake (Wilson *et al.* 2006).

The finding that YAM2 enhanced Se and Fe concentration in wheat kernels and stems is of significance for biofortification applications. For the purpose of Se biofortification of crops, it is important to note that in Se treated soil, the Se level in control plants was 34 mg Se kg⁻¹ dry weight (kernels) and 10.7 mg Se kg⁻¹ dry weight (stem), and in inoculated plants it

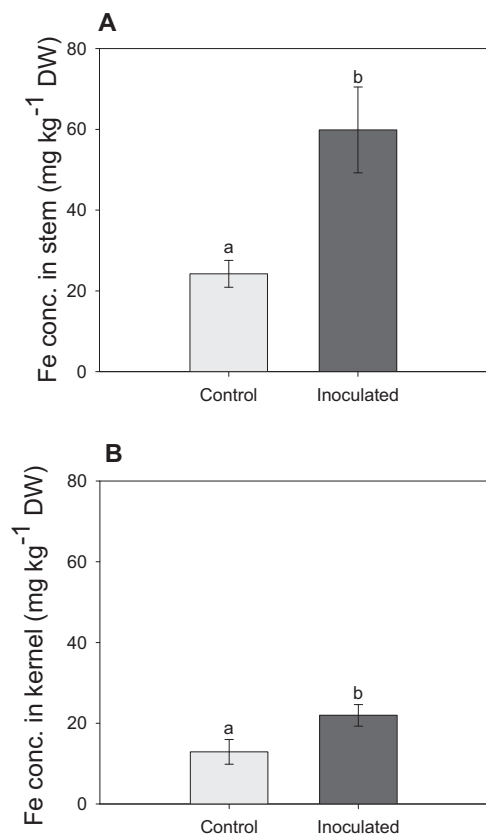


Fig. 5. Effect of YAM2 inoculation on Fe concentration in *T. aestivum* kernels (B) and stems (A), treated with Se. Bars labeled with different letters represent means that are significantly different, judged from Student's t-test at 95% confidence level. The data represent mean ($n = 5$) \pm SE of mean.

was 93 mg Se kg⁻¹ dry weight (kernels) and 38 mg Se kg⁻¹ dry weight (stem), higher than those obtained with Se-containing fertilizers (agronomic biofortification), (Galinha *et al.* 2012). Currently, according to the US National Academy of Sciences, an estimated adequate Se intake for humans is 55 μ g Se/day (Banuelos *et al.* 2012), and toxic levels for Se intake are 350–700 μ g Se/day (Sanmartín *et al.* 2012). With these Se levels, 1 g of kernels from Se-treated wheat or 0.5 g of kernels from YAM2-inoculated, Se-treated wheat would provide 34–47 μ g Se, which approaches the daily recommended intake of Se for an average human being. Since people who use wheat as a main source of starch typically eat almost 200–250 g of wheat kernels/flour per day, if these plants would have been used as biofortified food, it would be recommended to dilute the wheat kernels/ flour 200–400 fold with low-Se wheat kernels/ flour. Alternatively, a lower dose of Se could be supplied to the plants in the field so as to reach lower plant Se levels that could be eaten directly without dilution. Of course, one has to be careful with extrapolations of data obtained in pot experiments to field grown plants. Further experiments are needed to determine the optimal plant Se fertilization dose for straight consumption. For cows and cattle, the daily Se requirement is 100 μ g (NRC 2000). If the straw from these Se-fortified plants were to be consumed by cows, as is common in Pakistan and

India, then 2.5 g of inoculated Se treated wheat straw, mixed in green feed, would provide the daily Se requirement for cows. If livestock would feed on this Se-enriched straw, some of the Se will likely end up in the milk and meat (Banuelos *et al.* 2012). Thus, biofortification of Se to livestock will ultimately also benefit human consumers.

It is very interesting that this strain, YAM2, could enhance Fe levels in kernels (70%) and stems (147%), compared to uninoculated plants. More than 47% of all preschool aged children worldwide are suffering from Fe deficiency (Cakmak *et al.* 2010). Wheat is a dominant staple food making up >50% of the diet (Cakmak *et al.* 2010). It is notoriously difficult to enhance Fe levels in plants, especially in seeds. This has been a topic of intensive research for years, because iron deficiency limits plant growth and Fe is not readily available in terrestrial or aquatic environments (Guerinot 1994; Verta *et al.* 2002). Inoculation with YAM2 appears to be a promising strategy to improve biofortification efficiency.

Conclusion

The main finding of this study is that *Bacillus pichinoty*-like strain YAM2 enhanced wheat plant growth both in the presence and absence of Se and enhanced Se and Fe levels in Se-fertilized plants. Furthermore, this study showed that Se fertilization can effectively stimulate wheat productivity. Selenobacteria have been studied before for their potential for bioremediation of Se from polluted environments, but the use of selenobacteria as a tool for improving Se concentration in cereal crops is relatively new (Durana *et al.* 2013). The finding that this *Bacillus* strain can enhance Se and Fe uptake in wheat plants is significant because both Se and Fe are micronutrients for humans and livestock and both Se and Fe deficiency affect billions of people worldwide (Tan *et al.* 2002). Selenium-deficient soils in different parts of the world produce crops with low Se content (Combs 2001). Selenium-biofortified crops can be a cheap source of Se to fulfill the daily requirement and prevent disease in Se deficient areas, especially in low-income human populations (Bañuelos 2009; Finley 2006). Moreover, almost two billion people in the world are anemic, and many of these due to iron deficiency (WHO 2007). The YAM2 bacterium may be used for crop biofortification, not only of wheat but perhaps also other crops, to help alleviate these micronutrient deficiencies. Wheat plants grown on Se fertilized soil appear to be an excellent source of Se and Fe, and inoculation with YAM2 may further increase the health potential of the wheat, both of kernels and of straw, which will both ultimately benefit human consumers.

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