ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Effect of zinc solubilizing bioinoculants on zinc nutrition of wheat (*Triticum aestivum* L.)

***Sachin Kumar Vaid[1], Bipin Kumar Gangwar[2], Anita Sharma[1], P.C. Srivastava[3] and M.V. Singh[4]**

1. Department of Microbiology, G.B Pant University of Agriculture and Technology, Pantnagar-263145, India.
2. Department of Environmental Science, G.B Pant University of Agriculture and Technology, Pantnagar-263145, India.
3. Department of Soil Science, G.B Pant University of Agriculture and Technology, Pantnagar-263145, India.
4. Indian Institute of Soil Science, Berasia Road, Bhopal-462038, India.

Manuscript Info

Manuscript History:

Received: 11 October 2013
Final Accepted: 24 October 2013
Published Online: November 2013

Key words:
Zn solubilizing bacteria;
Phytic acid;
Methionine; Wheat

Corresponding Author

Sachin Kumar Vaid

Abstract

Three gram negative bacteria (*Burkholderia*, BC and two *Acinetobacter*, AB and AX strains) isolated from a Zn deficient rice-wheat field were tested for Zn solubilization in an *in vitro* plate assay, culture broth and also for gluconic acid and indole acetic acid (IAA) production. These bacteria were tested alone or in combination on yield attributes, yields and Zn nutrition of two wheat varieties; VL 804 (Zn responsive) and WH 1021 (Zn non-responsive) grown in a Zn deficient soil. Seed inoculation through these bacteria increased mean number of productive tillers (21.1%), number of grains per ear (15.7%), thousand grain weight (10.1%), grain yield (18.1%) and straw yield (3.1%) of both the wheat varieties significantly over the control. Bacterial inoculations especially with AX+AB in WH 1021 and AX or BC+AX in VL 804 resulted in a significant increase in Zn concentrations and its uptake in grains and straw over the control. These bacterial inoculations were also found effective in increasing methionine content and reducing phytic acid content (17.6%) in wheat grains of both varieties.

Introduction

Zn deficiency is widespread throughout the world, especially in rice cropland of Asia (Tisdale et al., 2009) and in soil orders of Aridisols, Alfisols, Mollisols and Vertisols (Srivastava and Gupta, 1996). According to an estimate made under All India Coordinated Research Project (AICRP) on Micro and Secondary Nutrients and Polluted Elements in Soils and Plants, 49% of tested soil samples were found deficient in Zn (Singh, 2001). According to Cakmak (2009) 50% of the Indian soils under intensive cultivation of wheat and rice are deficient in plant available Zn. The deficiency of Zn in soils is usually attributed to low solubility of Zn rather than low total content of Zn in most agricultural soils. P is also present in immobilized forms in soil. Inorganic P mostly forms insoluble mineral complexes in the soil which remained unavailable to plants (Rengel and Marschner, 2005). Organic matter is also an important reservoir of immobilized P that accounts for 20-80% of P in soils (Richardson, 1994).

As a result of prevalent Zn deficiency, the production of cereal crops suffers leading to the twin problems of low food production and Zn malnutrition in the population which uses cereals as their staple diet. According to a current statistics, overall undernourished people in the world are estimated to be 1.02 billion (FAO 2009). Hunger and malnutrition are the underlying causes of more than half of all the children's death, killing nearly 6 million children each year (FAO 2005).

Wheat is an important food grain crop of Asia. For example, in most of Central and West Asian countries, wheat provides nearly 50% of the daily calorie intake on average, likely increasing to more than 70% in the rural regions (Cakmak et al., 2004). Zn deficiency is currently listed as a major risk factor for human health and a prime cause of malnutrition globally. In a report published by WHO (2002), Zn deficiency was considered as the fifth most

important risk factor responsible for illness and death in the developing world (Cakmak, 2009). Zn has a number of critical functions in biological systems, including protection of structural and functional integrity of biological membranes, detoxification of highly toxic oxygen-free radicals and maintenance of gene expression and protein synthesis (Coleman, 1992; Marschner, 1995; Cakmak, 2000). Among all cations, Zn is required by the largest number of proteins in biological systems, and Zn-binding proteins make up nearly 10% of the proteome of eukaryotic organisms (Andreini et al., 2006; Bertini and Rosata, 2007). Human health complications of Zn deficiency include impairments in immune system together with incidence of infectious diseases such as diarrhea and pneumonia, especially in the developing world (Walker and Black, 2007; Gibson et al., 2008).

On the other hand in crops such as wheat and rice due to Zn deficiency the crop yield as well as its quality is severely affected. Generally, grain Zn concentration in commercial wheat cultivars are 20–35 mg/kg (Rengel et al., 1999; Cakmak et al., 2004). These concentrations are not adequate for human nutrition in diets with wheat constituting the main source of essential minerals. Hence, Zn fertilization is recommended to enhance availability of Zn in cereals through soil or foliar application. However, high cost of Zn fertilizers is often a deterrent for the low income farmers with small land holdings. Soil cultural practices capable of mobilizing enough amount of Zn to meet the crop requirement is very important for the low income farmers of small holdings in developing countries.

Role of microorganisms in soil fertility and plant health has been suggested by several authors. Plant growth-promoting bacteria (PGPR) have been reported to be the key elements for plant establishment under nutrient imbalance conditions. Puente et al. (2009) reported the growth of endophytic bacteria inoculated cardon seeds in pulverized rock for at least a year without fertilization. They found plant-bacteria association responsible for release of significant amounts of nutrients from the substrate and when they eliminated these endophytic bacteria from the seeds the development of seedlings stopped.

Several mechanisms have been proposed to explain that how plant growth-promoting rhizobacteria (PGPR) enhance the plant growth. Some of the mechanisms are categorized as direct while others as indirect. Direct mechanisms include production of plant hormones such as indole acetic acid (IAA), gibberellic acid (GA) and cytokinin, phosphate solubilization, Zn solubilization and nitrogen fixation. Indirect mechanisms include the plant growth promotion through suppression of soil-borne or foliar pathogens.

Application of huge amount of artificial fertilizers to replenish soil nutrients and micronutrients often results in high costs and environmental risks. Also added fertilizers convert to insoluble forms and remain unavailable to plants. Thus, the application of PGPR in agriculture not only reduces the use of expensive agro-chemicals but also supports ecofriendly crop production (Herrera et al., 1993; Requena et al., 1997; Glick, 1995).

Phytic acid is considered as an antinutrient in cereals due to its ability to bind minerals and proteins directly or indirectly and alters the solubility, functionality, digestibility and absorption of essential minerals in cereals and pulses (Latta et al., 1980; Rickard et al., 1997). Since Zn has variety of functions both in plants and in humans so in the present investigation, three bacterial strains, isolated from Zn-deficient soil were evaluated for their Zn solubilizing ability *in vitro* and their effectiveness, individually and in combinations on yield attributes, productivity and Zn nutrition of two wheat varieties of varying Zn response grown in a Zn deficient soil. Phytic acid and phytic acid: Zn ratio in grains was also examined to test their effect on bioavailability of Zn in wheat grains.

Materials and Methods

Isolation and characterization of Zn solubilizing bacteria

A total of 48 bacteria were isolated from the rhizosphere of rice plants, growing in a Zn deficient Typic Hapludoll at Crop Research Centre of Govind Ballabh Pant University of Agriculture and Technology, Pantnagar (India) using standard dilution plating on Nutrient agar, King's B medium and Tryptic soy agar. These bacteria were checked for their Zn solubilizing ability based on halo formation on solid Basal medium supplemented with 0.1% ZnO according to the method of Sarvanan et al. (2003) and on Bactotryptone medium supplemented with 5mM zinc phosphate by the method of Simine et al. (1998). The basal medium contains (per litre) glucose 10.0g; $(NH_4)_2SO_4$ 1.0g; KCl 0.2g; K_2HPO_4 0.1g; $MgSO_4.7H_2O$ 0.2g; ZnO (0.1%) and Agar (1.5%). The taxonomic identity of the isolated bacterial cultures was determined by sending the cultures to Chromous Biotech, Banglore, India for partial sequencing. The PCR amplification of gene-encoding 16S rRNA was performed using the bacterial universal primers: forward primer 5'-AGAGTRTGATCMTYGCTWAC-3' and reverse primer 5'-CGYTAMCTTWTTACGRCT-3'. The PCR mixture contained 4 µl of 2.5mM each dNTP, 400 ng of each primer, 10X PCR buffer 10 µl and 3 U of Taq DNA polymerase with a final reaction volume of 100 µl. All PCR reagents were of Chromous make. The DNA amplification was carried out by heating at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, with annealing at 55°C for 30 sec, with extension at 72°C for 2 min and with a final extension at 72°C for 5 min. The PCR product was sequenced by Big Dye Terminator version 3.1" Cycle

sequencing kit. The genetic analyzer was ABI 3130 (Applied Biosystems). The nucleotide sequences were compared by using BlastN program. Based on partial sequencing of the 16S rRNA gene using universal primers, three bacterial cultures were identified as *Burkholderia* sp. strain SG1 (BC), *Acinetobacter* sp. strain SG2 (AX) and *Acinetobacter* sp. strain SG3 (AB).

Zn solubilization potential of isolated strains in culture broth

Recovered bacteria were inoculated separately in 50 ml sterilized basal medium without agar supplemented with 0.1% ZnO in triplicate. Inoculated flasks were incubated in orbital shaker at 100 rpm for 20 d at 30 ˚C. A control flask (uninoculated) was also maintained under similar conditions. Samples were withdrawn at the interval of every 10 d and centrifuged to obtain the clear supernatant. The equilibrium pH of the supernatant was determined. Supernatants were filtered through Whatman No. 42 and directly used to quantify soluble Zn by atomic absorption spectrophotometer (GBC Avanta M) as per the procedure outlined by Saravanan et al. (2003). Percent soluble Zn was calculated by dividing the amount of soluble Zn in the medium calculated through AAS by the amount of total Zn added in the medium and multiplying this quantity by 100.

Organic acid analysis

Presence of organic acid(s) in the culture supernatant was tested in triplicate according to the method of Simine et al. (1998). Strain AB was grown in 300ml nutrient broth while for strains AX and BC 300ml Tryptic soy broth and King's B medium were used, respectively. These bacterial cultures incubated for 48 h were centrifuged at 15000 g for 15 min. Resultant pellet washed twice with NaCl (8 g L^{-1}), and cooled on ice (in order to prevent excessive heat generation during sonication) was subjected to sonication (6 cycles of 10 sec. with 20 sec. pause/ cycle). To separate cell debris from the extract, sonicated suspension was centrifuged at 15000 rpm for 20 min. The obtained supernatant (cell free extract) was divided into two fractions of 25 ml each and kept in 50 ml volumetric flasks. One flask contained 25 ml sterile Mineral salt medium (MSM) and the other flask received the same medium with 10 mM insoluble ZnO. Both the flasks were incubated at 30 ˚C on a rotary shaker at 120 rpm. Aliquots were withdrawn at 12, 24, 48 h and centrifuged. The obtained supernatant was stored at -20 ˚C until analyzed for identification and quantification of organic acids using HPLC (Dionex) attached with a C18 column and UV visible detector. Samples were filtered through 0.22 µm membrane filter prior to injection. Elution was carried out using a mobile phase consisted of 0.2 % phosphoric acid and 1.0 % acetonitrile in milliQ water with a flow rate of 1 ml min^{-1} at room temperature. Chromatograms were recorded over a period of 10 min.

These isolates were also checked for indole acetic acid (IAA) production in triplicate according to the method of Gordon and Weber, (1951).

Pot experiment

Two wheat varieties VL 804 (Zn responsive) and WH 1021 (Zn non-responsive) were selected for the pot experiment. A bulk surface (0-15 cm) sample of Zn deficient mollisol collected from Crop Research Centre of the University was used for the pot experiment. The experimental soil had a loam texture characterized by pH 7.0, electrical conductance at 25 ˚C 0.278 dS m^{-1}, organic C 10.3 g and DTPA extractable micronutrient content (mg kg^{-1}): Zn, 0.42, Cu, 1.24, Fe, 25.3 and Mn 5.79 (Lindsay et al. 1978). Plastic pots (4 kg capacity) were filled and a basal dose of 33.48 mg N as urea, 11.71 mg P and 14.82 mg K through potassium dihydrogen orthophosphate and potassium chloride kg^{-1} soil was applied to each pot. Various treatments used in triplicate were: Control, basal application of Zn at the rate of 2.5 mg Zn kg^{-1} soil as $ZnSO_4.7H_2O$, bacterial cultures *Burkholderia* sp. SG1 (BC), *Acinetobacter* sp. SG2 (AX), *Acinetobacter* sp. SG3 (AB) and their combinations (BC+AX), (BC+AB), (AX+AB) and (BC+AX+AB). Wheat seeds were surface sterilized with 0.1% $HgCl_2$ (v/v) for 5 min and then washed thoroughly with sterilized water. For treatments involving bacterial inoculation seeds were coated with bacterial suspensions (10^8 cfu ml^{-1}) using carboxymethyl cellulose (0.1 %). Control and basal application of 2.5 mg Zn kg^{-1} soil did not receive any bacterial treatment. Eight seeds were sown per pot. After emergence, only five plants were maintained per pot. Pots were regularly watered to maintain soil moisture content near to field capacity (22.2% w/w). Two top dressings of urea (16.74 mg N kg^{-1} soil) were applied at 22nd and 60th days after emergence. Before maturity, plant height, number of productive tillers per plant, mean ear length and average number of grains per ear were recorded.

At the maturity, plants were harvested close to the soil surface and washed thoroughly with tap water followed by 0.1 N HCl and distilled water. Harvested plants were separated into grains and straw, oven dried at 60 ˚C and weighed. Thousand grain weight was also recorded. Finely grounded grains and straw samples were digested in 10 ml diacid mixture (HNO_3:$HClO_4$, 4:1 v/v) for the analysis of micronutrients by atomic absorption spectrophotometry (GBC Avanta M). Zn uptake in grains was calculated by multiplying the Zn content in grain with

grain yield. Similarly the Zn uptake in straw was calculated. Total Zn uptake per pot was calculated by adding the Zn uptake in grains and Zn uptake in straw. Phytic acid and methionine contents in grains were estimated spectrophotometrically following the methods outlined by Latta et al. (1980) and Horn et al. (1946), respectively.

Statistical Analysis

The data were statistically analyzed for the analysis of variance using a factorial completely randomized design and examined for statistical significance at $p \leq 0.05$ (Snedecor and Cochran, 1967).

RESULTS

Efficiency of bacteria for Zn solubilization

Initially, laboratory investigations revealed that in Basal and Bactotryptone medium (BTG) supplemented with insoluble Zn as ZnO or $Zn_3(PO_4)_2$, only three cultures out of 48 showed zones of clearance of Zn solubilization after 48 h of incubation. Among the cultures, the strain SG2 (AX) showed biggest zones of clearance (1.5 cm) with ZnO and (1.25 cm) with $Zn_3(PO_4)_2$. The temporal changes in the equilibrium pH and soluble Zn (as percentage of total Zn supplied as ZnO) are presented in Fig 1. In broth assay, initial level of soluble Zn in the medium (0d of incubation without bacteria) was only 1.01% of the total Zn. After 20 d of bacterial inoculation, the level of soluble Zn in the medium increased to 2.64, 2.2 and 1.3% of total Zn in the case of AX, BC and AB, respectively. The solubilization of Zn was also closely related to the change in pH of the medium**.** The maximum decrease in the pH was recorded in AX (from pH 7.0 to 4.0 on 20th day) while changes observed with BC and AB were (7.2 to 6.8) and AB (7.2 to 6.7) respectively.

Analysis of organic acids in the culture broth

Gluconic acid was detected in the culture supernatant of all the bacterial isolates. The concentration of Gluconic acid in the medium without ZnO supplementation was much smaller and decreased with time (Fig 2). However, in the presence of ZnO supplement in the culture broth the concentration of gluconic acid increased during 48 h of incubation. The differences in the concentration of gluconic acid in medium with ZnO and without ZnO were statistically not significant at 10 h of incubation but the differences became significant at 24 and 48 h of incubation in all the three bacterial cultures. Among the three bacterial cultures, the highest gluconic acid (25.9 mM) production was recorded in the case of AX.

Indole acetic acid (IAA) production:

The range of IAA production in succinate broth of the three bacterial isolates after 48 h of incubation was 2 to 5.79 mg L^{-1} (Fig 3). The highest production of IAA (5.79 mg L^{-1}) was recorded in AX while the minimum (2.0 mg L^{-1}) was noted in BC.

Growth and yield attributing characters of wheat

The data on the effect of different treatments on the growth and yield attributing characters of both the wheat varieties are presented in Table 1. Wheat varieties of varying responsiveness to Zn application differed significantly in their yield attributes. Zn non-responsive variety (WH 1021) had significantly higher number of productive tillers per plant and thousand grain weight as compared to Zn responsive variety (VL 804) which maintained significantly higher number of average grains per ear as compared to WH 1021.

Effect of treatments averaged over wheat varieties showed that soil application of 2.5 mg Zn kg^{-1} soil increased the number of grains/ear significantly over the control but did not significantly influence number of productive tillers $plant^{-1}$ or thousand grain weight. Bacterial inoculations alone or in combination brought significant improvement in all yield attributes as compared to control. Averaged over the varieties, the highest number of productive tillers/plant and number of grains/ear were recorded under AX+AB inoculation while the highest value of thousand grain weight (40.3g pot^{-1}) was recorded under BC+AX and BC+AB inoculations.

The interaction effect of treatments × variety significantly influenced only the average number of grains/ear and thousand grain weight. In WH 1021, the highest average number of grains/ear (51.0) was recorded under AX+AB inoculation which was at par with values under BC+AX+AB (50.3) and BC+AB (50.0). In VL 804, the highest average number of grains ear^{-1} (57.7) was noted under AB inoculation which was also statistically at par with the values obtained with AX+AB and BC+AX inoculations. As regards the effect on the thousand grain weight, application of Zn fertilizer (2.5 mg Zn kg^{-1} soil) decreased it significantly in WH 1021 but had no significant effect in VL 804. In WH 1021, all the treatments except BC+AX+AB increased thousand grain weight significantly over the control and the highest value (41.7 g) was observed with AX+AB. In VL 804, BC+AX and BC+AB treatments significantly increased thousand grain weight over the control while rest of the treatments did not show any significant change. The highest increase in thousand grain weight was recorded under BC+AB inoculation which was 11.7% higher over the control and 12.9% higher over Zn fertilizer application, respectively. The observed effect

of different bacterial inoculations on the growth and yield attributes could be related partly to improve Zn nutrition of plants as well as production of IAA by these bacteria.

Grain and straw yield

In general, grain and straw yields were significantly higher in WH 1021 as compared to VL 804 (Table 2). Effect of treatments averaged over the varieties showed that all the treatments except application of Zn fertilizer and BC inoculation increased the grain yield significantly over the control. The highest grain yield (9.8 g) was recorded with AX+AB treatment which was 18.1 and 16.7% higher than the control and Zn fertilizer application, respectively. Treatment with BC+AB was found statistically similar to AX+AB. As regards the straw yields, none of the treatments except AX+AB significantly increased it. The percent increment in straw yield under AX+AB was 3.1 and 5.5 as compared to control and Zn fertilizer application, respectively. Application of BC, AX, AB and BC+AX+AB however, decreased the straw yield in comparison to control.

The interaction effect of variety × treatments influenced the yield of grain and straw of both the wheat varieties significantly. In WH 1021, all the treatments except AX inoculation and Zn fertilizer application increased the grain yield significantly over the control and the highest grain yield (12.2 g) was recorded with AX+AB treatment with an increase of 23.2 and 24.5% over the control and Zn fertilizer application, respectively. Seed inoculation with BC+AB and AX+AB was effective in increasing the straw yield of WH 1021 significantly over the control. The maximum increase was recorded by AX+AB treatment which was 12.3 and 16.3% over the control and Zn fertilizer application, respectively. However, in WH 1021 the straw yield decreased under the AX treatment. In VL 804, all the treatments except BC and Zn fertilizer increased grain yield significantly over the control and the highest grain yield (8.3 g) was recorded with BC+AX treatment with an increase of 22.1 and 18.6% over the control and Zn fertilizer application, respectively. Treatment with BC+AB was statistically similar to BC+AX. All the treatments except AX and BC+AX decreased straw yield of VL 804 in comparison to control. Application of Zn fertilizer and bacterial treatments AX and BC+AX could only maintain the straw yield equal to control.

Zn nutrition of wheat

The data on effect of different treatments on Zn concentration in grains and straw are presented in Table 3.

Zn concentration in grains

The mean concentration of Zn in the grains of WH 1021, variety was significantly higher than VL 804 (Table 3). The performance of different treatments averaged over wheat varieties indicated that all the bacterial treatments when used individually increased the Zn concentration in wheat grains significantly over the control. Inoculation with AX was most effective individually as it increased Zn concentration in grains by 44.4 and 13.0% over the control and Zn fertilizer treatment, respectively. Among the bacterial consortia, the highest Zn concentration in grains was recorded under BC+AX+AB inoculation; the observed increase was 51.7 and 18.7% over the control and Zn fertilizer treatment, respectively.

The interaction effect of treatments and varieties also influenced Zn concentration in wheat grains. In WH 1021, all the treatments except BC+AB increased Zn concentration significantly over the control. The highest concentration of Zn in grains (27.3 mg kg^{-1}) was recorded with AX+AB and the magnitude of increase was 54.5 and 29.5% over control and Zn fertilizer treatment, respectively. The performance of AX+AB inoculation was statistically similar to that of BC+AX+AB. In Zn responsive wheat variety VL 804, all the treatments significantly increased Zn concentration in grains and the highest increase was recorded with AX which was 56.3 and 12.6% over the control and Zn fertilizer application, respectively.

Zn concentration in straw

The mean concentration of Zn in wheat straw was significantly higher in Zn non-responsive variety WH 1021 than Zn responsive VL 804 (Table 3). Effect of different treatments averaged over wheat varieties revealed that in comparison to the control, the concentration of Zn in wheat straw decreased under all the treatments except AX+AB and BC+AX+AB which maintained Zn concentration of the straw equivalent to control.

The interaction effect of variety × treatments also influenced the concentration of Zn in wheat straw. Zn concentration in straw of WH 1021 decreased under all the treatments in comparison to control except BC+AX+AB which increased Zn concentration in straw by 12.6 and 48.6% over the control and Zn fertilizer treatment, respectively. In VL 804, all the treatments were ineffective in increasing the concentration of Zn in straw except AX+AB and the magnitude of increase was 15.3 and 62.7% over the control and Zn fertilizer application, respectively.

Zn uptake in grains and Straw

The data on Zn uptake in grain and straw under different treatments are presented in Table 4. Mean Zn uptake in grains was higher in WH 1021 than VL 804. Effect of different treatments averaged over wheat varieties showed that all the treatments increased Zn uptake in wheat grains significantly over the control. The highest increase was noted under (AX+AB) treatment which enhanced Zn uptake in grains by 69.8 and 34.2% over the

control and Zn treatment, respectively. Zn uptake in grains under AX+AB and BC+AX+AB treatments were statistically similar. The interaction effect of treatments and varieties had significant influence on Zn uptake in wheat grains. In Zn non-responsive variety; WH 1021, all the treatments increased Zn uptake in grains significantly over the control and the maximum increase was observed under AX+AB, which was 90.5 and 60.8% over the control and Zn fertilizer, respectively. In Zn responsive wheat variety; VL804, the highest increase in Zn uptake in grains was observed with AX treatment which increased Zn uptake in grains by 83.9 and 28.8% over control and Zn fertilizer, respectively.

Mean Zn uptake in straw of WH 1021 was significantly higher than VL 804 (Table 4). Effect of different treatments averaged over wheat varieties showed that in comparison to control, all the treatments decreased Zn uptake in straw except under AX+AB and BC+AX+AB inoculations which maintained Zn uptake in straw almost similar to that of control. The interaction effect of treatments and varieties revealed that only BC+AX+AB treatment increased Zn uptake in straw significantly over the control in WH 1021 and the magnitude of increase was 13.0 and 53.0% over control and Zn fertilizer application, respectively. In VL 804, none of the treatments enhanced Zn uptake in straw significantly except AX and AX+AB which could maintain Zn uptake in straw statistically similar to that of control.

Total Zn uptake

The data on effect of different treatments on total Zn uptake by wheat varieties are presented in Table 5. Total Zn uptake by Zn non-responsive variety; WH 1021, was significantly higher than that of VL 804 (Table 5). The effect of different treatments averaged over wheat varieties revealed that AX, AX+AB and BC+AX+AB inoculations increased total Zn uptake significantly over the control. The maximum total Zn uptake was observed under BC+AX+AB treatment; the magnitude of increase was 37.1 and 35.2% over the control and Zn fertilizer application, respectively. A significant interaction effect of treatments × variety revealed that in WH 1021, application of AX, AX+AB and BC+AX+AB treatments increased the total Zn uptake significantly over the control and the highest total Zn uptake was observed in AX+AB treatment which was 47.0 and 48.8% higher than the control and Zn fertilizer application, respectively. Treatment BC+AX+AB performed statistically similar to AX+AB treatment. In Zn responsive wheat variety; VL 804, all the treatments significantly increased the total Zn uptake except under Zn fertilizer application and BC inoculation. The maximum total Zn uptake was noted under BC+AB treatment; the increment in Zn concentration was 25.5 and 18.1% as compared to control and fertilizer Zn application, respectively.

Concentration of pro- and anti-Zn factors in grains of wheat varieties

The data on the effect of different treatments on the concentrations of methionine and phytic acid in grains of both the wheat varieties are presented in Table 6.

Methionine content in grains

Methionine content in grains of WH 1021 was significantly higher than that of VL 804 (Table 6). The performance of different treatments averaged over wheat varieties showed that different treatments increased methionine concentration significantly in wheat grains. The highest increase in methionine content of grains over the control and Zn fertilizer application was noted under treatment AX+AB; the magnitude of increase was 94.0 and 22.0 percent over the control and 2.5 mg Zn fertilizer application, respectively. The increase in methionine content in wheat grains under AX and BC+AB treatments was statistically similar to that under AX+AB inoculation. The interaction effect between the varieties and treatments had statistically significant effect on methionine content of wheat grains. In WH 1021, a significant increase in methionine concentration occurred under all the treatments except AB and the highest methionine concentration in grains was noted under AX+AB treatment, which was nearly 2.7 and 1.6 fold higher over the control and 2.5 mg Zn fertilizer application, respectively. Similar results were recorded in VL 804 variety except that AB and BC+AX+AB treatments did not change methionine content significantly in comparison to control and the highest methionine content was recorded with BC+AB treatment with an increase of 88.4 and 23.8 percent over the control and Zn fertilizer application, respectively.

Phytic acid content in grains

Both wheat varieties had statistically similar phytic acid contents (Table 6). The performance of different treatments averaged over wheat varieties indicated that phytic acid content in grains was significantly lowered by Zn fertilizer, BC, BC+AX, AX+AB and BC+AX+AB inoculations in comparison to the control. Inoculations with AX and AB maintained similar level of phytic acid in grains as observed under the control. The highest reduction in the concentration of phytic acid in grains was observed with AX+AB treatment which was 17.1 percent lower in comparison to the control. Treatments BC+AX and AX+AB had statistically similar content of phytic acid in grains.

Fig 1. The temporal changes in the equilibrium pH and soluble Zn (as percentage of total Zn supplied as ZnO) in culture broth of different bacterial strains. The vertical bars indicate LSD at ≤ 0.05.

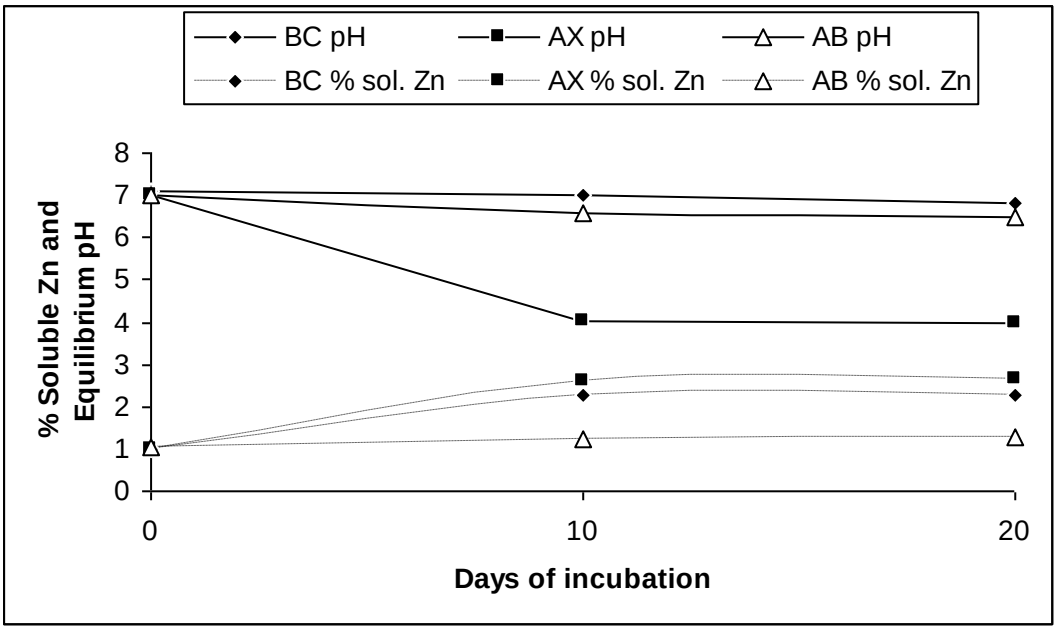


Fig 2. The concentration of Gluconic acid in the medium in the absence and presence of ZnO supplementation in culture supernatants of different bacterial strains. The vertical bars indicate LSD at ≤ 0.05.

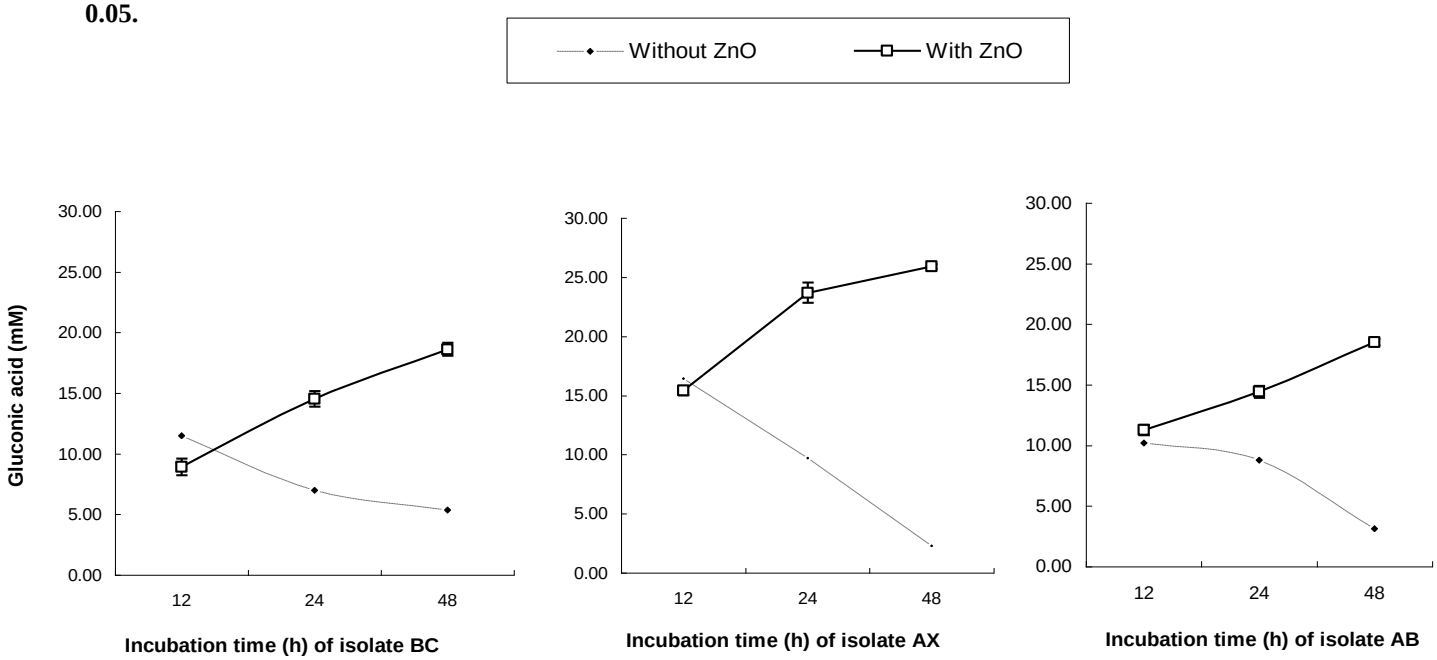


Fig 3. Indole acetic acid (IAA) production in succinate broth of the three bacterial isolates after 48 h of incubation. The vertical bars indicate LSD at ≤ 0.05.

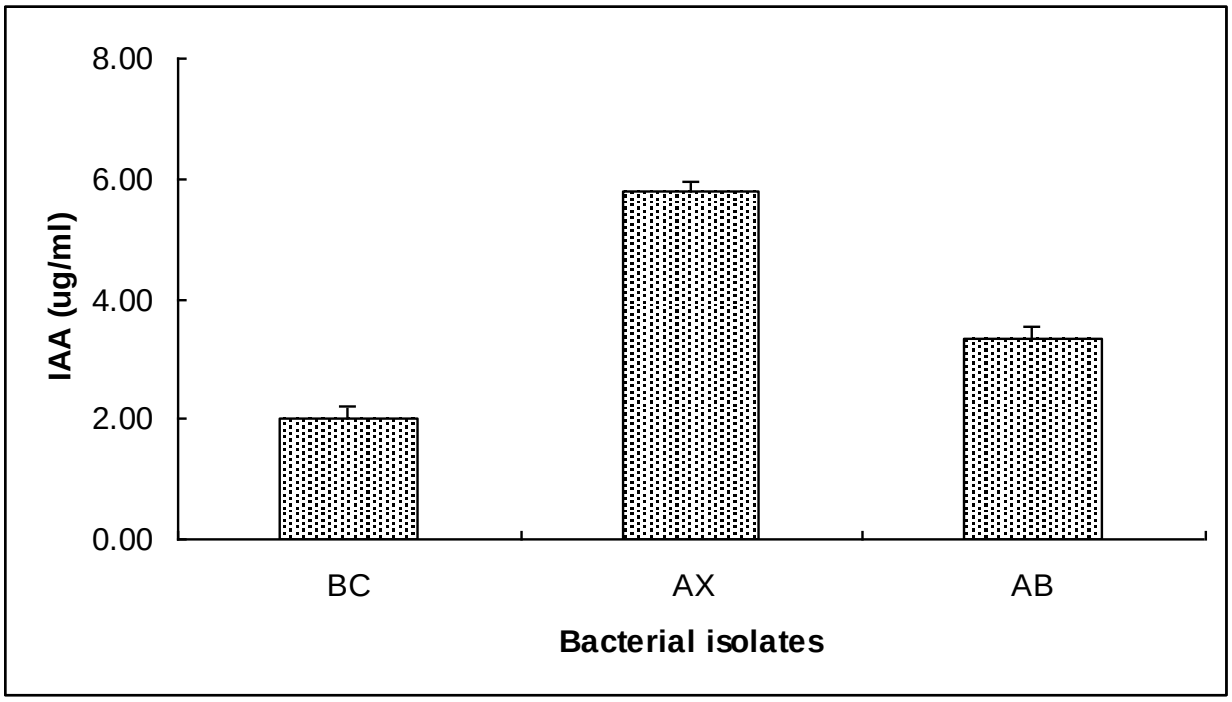
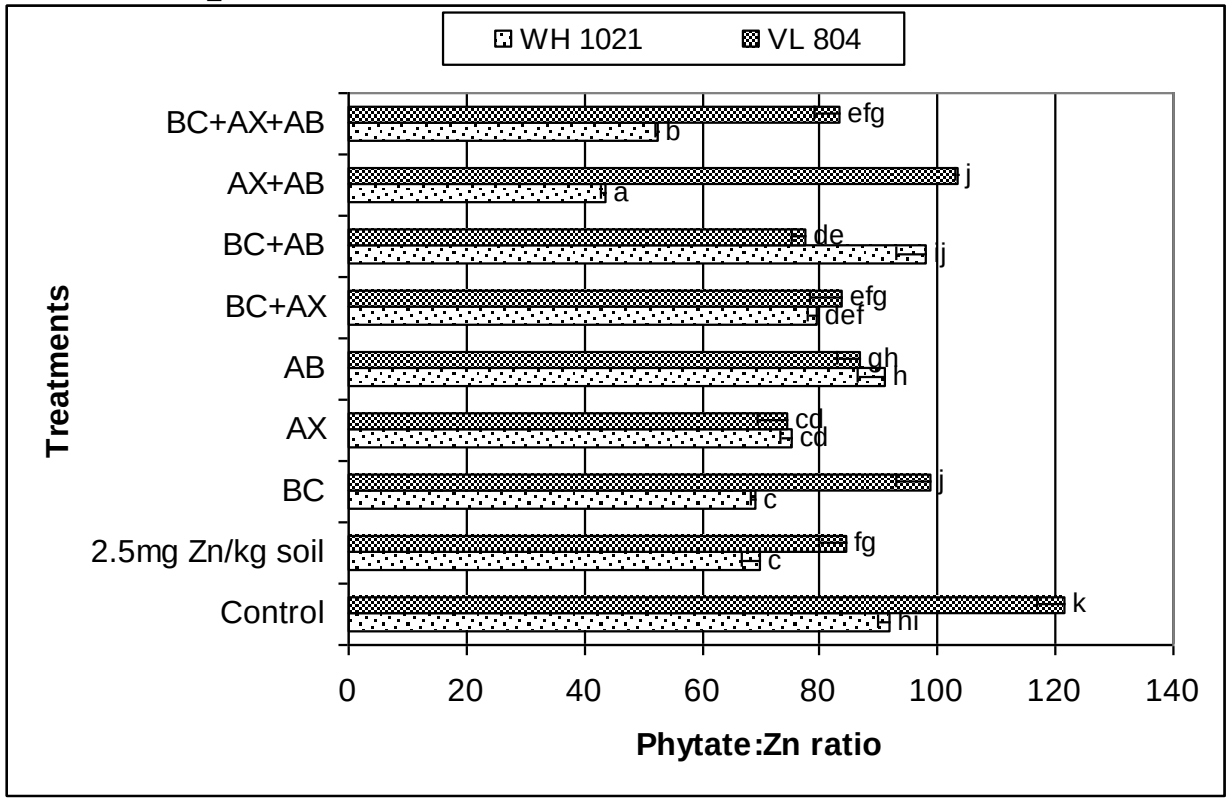


Fig 4. Effect of different treatments on phytate: Zn ratio in grains of two wheat varieties. The vertical bars indicate LSD at ≤ 0.05.



A significant interaction effect of treatments × variety revealed that in Zn non-responsive variety; WH 1021, soil application of Zn fertilizer, BC, BC+AX, AX+AB and BC+AX+AB treatments decreased phytic acid concentration in grains significantly as compared to the control. Inoculation with AX+AB decreased the phytic acid concentration in grains by 36.7% in comparison to the control while inoculation with AX alone increased phytic acid content significantly over the control. The concentration of phytic acid in grains under AB and BC+AB inoculations was statistically similar to that of control. In VL 804, the interaction effect of treatments × variety however, failed to influence phytic acid content of grains significantly except BC+AX inoculation which decreased the concentration of phytic acid significantly in comparison to the control; the magnitude of decline was 14.7 percent in comparison to the control.

Phytic acid: Zn ratio in wheat grains

In general, phytate: Zn ratio was lower in grains of WH 1021 as compared to VL 804 (Fig.4). The different treatments averaged over the wheat varieties significantly reduced this ratio, however none of the treatment was found effective in reducing this ratio to the ideal value (<15.0). The highest reduction was recorded under BC+AX+AB inoculation. As regards the interaction effect of variety x treatments, in WH 1021 grains the highest reduction in phytic acid: Zn ratio from 92 (Control) to 43 was observed under the treatment AX+AB. In VL 804 grains, all the treatments decreased the phytate: Zn ratio in grains in comparison to the control and the highest reduction was recorded under the treatment AX (75).

Discussion

The isolated bacteria; *Burkholderia* sp. SG1 (BC), *Acinetobacter* sp. SG2 (AX) and *Acinetobacter* sp. SG3 (AB), differed in their *in vitro* Zn solubilizing capability which could be related to the differences in their pH lowering ability mainly due to gluconic acid production (Simine et al., 1998). Gluconic acid production and resultant P solubilization had been reported in *Burkholderia* (Fernandez et al., 2007) and *Acinetobacter* (Ogut et al., 2010). Gluconic acid production was also observed for *Azospirillum* sp. by Rodriguez et al. (2004). Patrice et al. (2009) had also reported gluconic acid production by *Pseudomonas fluorescens* CHA0. In the present investigation, the highest gluconic acid production and solubilization of insoluble Zn was recorded with AX. Bacterial strains isolated in the present investigation also tested positive for IAA production; the highest *in vitro* IAA concentration was recorded in AX. Root is one of the plant's organs which is most sensitive to IAA fluctuations. IAA produced by plant growth promoting bacteria promotes plant growth by increasing the density and length of root hairs. Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their chances for survival (Volkmar and Bremer, 1998).

Considering 0.57 mg DTPA extractable Zn kg^{-1} soil as critical limit of Zn in soil for wheat crop (Srivastava et al., 2008), the experimental soil used in greenhouse experiment was deficient in Zn. However, no statistical response to soil application of 2.5 mg Zn kg^{-1} soil was recorded in terms of grain and straw yields of both varieties which could be ascribed to the non-responsive nature of WH 1021 variety while in case of a Zn responsive variety; VL804 the observed increase in the grain yield was statistically not significant and possible hinted at application of less than optimum dose in the experiment. In general, these bacterial isolates when used alone or in combination as seed inoculants brought significant improvement in yield attributes, yields, Zn concentration and uptake in grain and straw of both wheat varieties. The effect could be related to improved Zn acquisition in the case of inoculated plants and also production of indole acetic acid (IAA); a growth hormone by these inoculants. More pronounced effect was noted with the combinations of AX (AX + AB in WH1021 variety and AX or BC + AX in VL804 variety) which also had the highest gluconic acid and IAA production among the three bacterial strains. Mader et al. (2011) also observed 31% increase in wheat grain yield on inoculation with PGPR. Rosas et al. (2009) also reported 36% increase in wheat grain yield after inoculation with *Pseudomonas aurantiaca* on a sandy loam soil in Argentina.

Bioavailability of Zn in grains is controlled by anti-nutrient (phytic acid) and pro-nutrient (methionine) factors. A molar ratio of phytic acid and Zn; <15 indicate good Zn bioavailability in seed (Gibson, 2006). In the present investigation, though inoculation with AX+AB in WH1021 and with BC + AX in VL804 were most effective in significantly narrowing the phytic acid: Zn ratio in wheat grains as compared to control but could not reduce the ratio <15. A significant increase in the level of methionine (an essential amino acid) in grains of both the wheat varieties as compared to control was observed with these inoculations and the effect could be ascribed to the better expression of RNA polymerase enzyme which requires Zn. Price (1962) has also reported a sharp decline in RNA and ribosomes content of Zn deficient cells which resulted in reduced protein synthesis. Marschner (1995) have the similar findings, he also reported that Zn plays an important role in protein synthesis. A positive correlation between grain Zn and protein concentration was observed by Cakmak et al. (2010). Since these inoculations increased the concentration of methionine in grains of both wheat varieties significantly over the control, it is likely that these inoculations would help to produce grains with better Zn bioavailability.

Table 1. Effect of different treatments on yield attributes of two wheat varieties.

Treatments	Productive tillers plant^{-1}			No. Grains ear^{-1}			1000 grain weight (g)		
	WH 1021	VL 804	Mean	WH 1021	VL 804	Mean	WH 1021	VL 804	Mean
Control	2.1^{cde}	1.6^{ab}	1.9	44.3^{a}	48.7^{cd}	46.5	36.5^{ab}	36.7^{ab}	36.6
2.5 mg Zn kg^{-1} soil	1.9^{bcde}	1.3^{a}	1.6	48.3^{c}	53.0^{g}	50.7	34.5^{a}	36.3^{ab}	35.4
BC	2.1^{bcde}	2.0^{bcde}	2.0	46.7^{b}	51.0^{f}	48.8	39.8^{efg}	34.5^{a}	37.2
AX	1.8^{bcd}	1.7^{abc}	1.7	49.3^{cde}	56.0^{i}	52.7	39.2^{cdef}	37.8^{bcde}	38.5
AB	2.1^{cde}	2.1^{cde}	2.1	45.7^{ab}	57.7^{j}	51.7	39.3^{cdef}	36.8^{b}	38.1
BC+AX	2.2^{de}	2.2^{de}	2.2	46.0^{b}	56.3^{ij}	51.2	40.5^{fg}	40.0^{efg}	40.3
BC+AB	2.1^{bcde}	1.9^{bcde}	2.0	50.0^{def}	55.7^{ij}	52.8	39.5^{defg}	41.0^{fg}	40.3
AX+AB	2.3^{e}	2.3^{de}	2.3	51.0^{f}	56.7^{ij}	53.8	41.7^{g}	37.2^{bc}	39.4
BC+AX+AB	2.3^{de}	1.9^{bcde}	2.1	50.3^{ef}	54.3^{hi}	52.3	38.0^{bcde}	37.3^{bcd}	37.7
Mean	2.1	1.9	2.0	48.0	54.4	51.2	38.8	37.5	38.1
Effect	Var.	Treat	Var. × Treat.	Var.	Treat	Var. × Treat.	Var.	Treat	Var. × Treat.
SEm±	0.07	0.15	0.21	0.16	0.35	0.49	0.23	0.50	0.70
LSD($p \leq 0.05$)	0.2	0.4	NS	0.5	1.0	1.4	0.7	1.4	2.0

Pair of treatments that are not significantly different from one another share the same letters checked by the Duncan's test at 5% probability level.

Table 2. Effect of different treatments on grain and straw yields of two wheat varieties.

Treatments	Grain yield (g pot^{-1})			Straw Yield (g pot^{-1})		
	WH 1021	VL 804	Mean	WH 1021	VL 804	Mean
Control	9.9^{gh}	6.8^{ab}	8.3	14.6^{e}	11.6^{c}	13.1
2.5 mg Zn/kg soil	9.8^{g}	7^{bc}	8.4	14.1^{de}	11.4^{c}	12.8
BC	10.4^{i}	6.5^{a}	8.5	14.6^{e}	9.4^{a}	12
AX	10^{ghi}	8^{f}	9	13.8^{d}	11.1^{c}	12.5
AB	10.3^{hi}	7.4^{cd}	8.8	14.4^{e}	10.2^{b}	12.3
BC+AX	10.4^{i}	8.3^{f}	9.4	14.4^{e}	11.1^{c}	12.7
BC+AB	11.4^{j}	7.9^{ef}	9.7	15.7^{f}	11^{c}	13.4
AX+AB	12.2^{k}	7.5^{de}	9.8	16.4^{g}	10.5^{b}	13.5
BC+AX+AB	11.1^{j}	7.2^{bcd}	9.1	14.6^{e}	10.3^{b}	12.4
Mean	10.6	7.4	9	14.7	10.7	12.7
Effect	Var.	Treat.	Var. × Treat.	Var.	Treat.	Var. × Treat.
SEm±	0.1	0.1	0.2	0.1	0.1	0.2
LSD(p≤0.05)	0.1	0.3	0.4	0.2	0.4	0.5

Pair of treatments that are not significantly different from one another share the same letters checked by the Duncan's test at 5% probability level.

Table 3: Effect of treatments on Zn concentration in grain and straw of two wheat varieties.

Treatments	Zn concentration (mg kg^{-1})					
	Grain			Straw		
	WH 1021	VL 804	Mean	WH 1021	VL 804	Mean
Control	17.7^{d}	12.6^{a}	15.1	9.5^{g}	7.2^{de}	8.4
2.5 mg Zn/kg soil	21.1^{i}	17.5^{d}	19.3	7.2^{de}	5.1^{a}	6.1
BC	20.4^{h}	15.4^{c}	17.9	6.5^{bc}	5.0^{a}	5.7
AX	24.0^{j}	19.7^{g}	21.8	7.4^{de}	7.1^{cde}	7.2
AB	18.7^{ef}	18.1^{de}	18.4	7.6^{def}	7.1^{cde}	7.3
BC+AX	18.7^{ef}	15.9^{c}	17.3	7.1^{cde}	5.4^{a}	6.2
BC+AB	18.0^{de}	18.5^{ef}	18.2	7.8^{ef}	6.1^{b}	6.9
AX+AB	27.3^{k}	14.5^{b}	20.9	7.7^{ef}	8.3^{f}	8.0
BC+AX+AB	26.8^{k}	19.0^{f}	22.9	10.7^{h}	6.9^{cd}	8.8
Mean	21.4	16.8	18.7	7.9	6.4	7.2
Effect	Var.	Treat	Var. × Treat.	Var.	Treat	Var. × Treat.
SEm±	0.01	0.17	0.23	0.07	0.16	0.23
LSD(p≤0.05)	0.2	0.5	0.7	0.2	0.5	0.7

Pair of treatments that are not significantly different from one another share the same letters checked by the Duncan's test at 5% probability level.

Table 4: Effect of different treatments on Zn uptake in grain and straw of two wheat varieties.

Treatments	Zn uptake (µg pot^{-1})					
	Grain			Straw		
	WH 1021	VL 804	Mean	WH 1021	VL 804	Mean
Control	174.5^{g}	85.3^{a}	129.9	138.2^{j}	83.6^{ef}	110.9
2.5 mg Zn/kg soil	206.8^{j}	121.8^{c}	164.3	102.1^{gh}	57.8^{b}	79.9
BC	212.4^{j}	99.6^{b}	156.0	94.1^{fg}	47.1^{a}	70.6
AX	240.1^{k}	156.9^{f}	198.5	102.4^{gh}	78.7^{de}	90.6
AB	193.0^{h}	133.1^{d}	163.1	109.2^{h}	71.6^{cd}	90.4
BC+AX	195.1^{hi}	131.3^{cd}	163.2	101.9^{gh}	59.4^{b}	80.6
BC+AB	205.6^{ij}	145.3^{e}	175.5	122.0^{i}	66.6^{bc}	94.3
AX+AB	332.5^{m}	108.7^{b}	220.6	127.0^{j}	87.0^{ef}	107.0
BC+AX+AB	296.5^{l}	136.7^{de}	216.6	156.2^{k}	70.9^{cd}	113.5
Mean	220.4	124.3	172.3	117.0	69.2	93.1
Effect	Var.	Treat	Var. × Treat.	Var.	Treat	Var. × Treat.
SEm±	1.24	2.63	3.7	1.17	2.48	3.5
LSD(p≤0.05)	3.6	7.7	10.9	3.4	7.1	10.1

Pair of treatments that are not significantly different from one another share the same letters checked by the Duncan's test at 5% probability level.

Table 5: Effect of different treatments on total Zn uptake of two wheat varieties.

Treatments	Total Zn uptake (µg pot^{-1})		
	WH 1021	VL 804	Mean
Control	312.6^{gh}	168.9^{b}	240.8
2.5 mg Zn/kg soil	308.9^{gh}	179.5^{bc}	244.2
BC	306.5^{g}	146.7^{a}	226.6
AX	342.5^{i}	235.6^{f}	289.0
AB	302.2^{g}	204.7^{de}	253.5
BC+AX	297.0^{g}	190.7^{cd}	243.8
BC+AB	327.6^{hi}	211.9^{e}	233.0
AX+AB	459.5^{j}	195.7^{cde}	327.6
BC+AX+AB	452.7^{j}	207.6^{de}	330.1
Mean	337.4	193.5	265.4
Effect	Var.	Treat	Var. × Treat.
SEm±	2.21	4.70	6.63
LSD(p≤0.05)	6.3	13.5	19.0

Pair of treatments that are not significantly different from one another share the same letters checked by the Duncan's test at 5% probability level.

Table 6: Effect of bacterial treatments on methionine and phytic acid concentration in grains of two wheat varieties.

Treatments	Methionine concentration (g kg^{-1})			Phytic acid concentration (g kg^{-1})		
	WH 1021	VL 804	Mean	WH 1021	VL 804	Mean
Control	2.4^{a}	3.0^{a}	2.7	21.3^{gh}	20.1^{defg}	20.7
2.5 mg Zn/kg soil	4.1^{bcd}	4.5^{cde}	4.3	19.3^{cde}	19.4^{cde}	19.3
BC	4.8^{ef}	4.7^{def}	4.8	18.4^{bc}	20.0^{defg}	19.2
AX	5.3^{fg}	4.9^{ef}	5.1	23.6^{i}	19.2^{cde}	21.4
AB	2.8^{a}	3.6^{b}	3.2	22.3^{h}	20.6^{efg}	21.5
BC+AX	4.3^{cde}	4.8^{ef}	4.6	19.5^{cdef}	17.5^{b}	18.5
BC+AB	4.4^{cde}	5.6^{g}	5.0	21.2^{gh}	18.8^{cd}	20.0
AX+AB	6.6^{h}	3.9^{bc}	5.2	15.6^{a}	19.7^{cdef}	17.6
BC+AX+AB	5.6^{g}	2.6^{a}	4.1	18.5^{bc}	20.8^{fg}	19.6
Mean	4.5	4.2	4.3	20.0	19.6	19.8
Effect	Var.	Treat	Var. × Treat.	Var.	Treat	Var. × Treat.
SEm±	0.07	0.15	0.21	0.14	0.30	0.43
LSD($p \leq 0.05$)	0.2	0.4	0.6	NS	0.9	1.2

Pair of treatments that are not significantly different from one another share the same letters checked by the Duncan's test at 5% probability level.

Conclusions

The bacteria isolated from a Zn deficient soil namely *Burkholderia* sp. SG1, *Acinetobacter* sp. SG2 and *Acinetobacter* sp. SG3 showed Zn solublizing activity by lowering down the pH due to gluconic acid production and were also found positive for indole acetic acid production. These bacteria when used either individually or in combination augmented Zn nutrition of wheat crop (cvs. WH 1021 and VL 804) and increased the concentration and uptake of Zn both in grains and straw. These inoculations decreased the concentration of phytic acid and phytic acid: Zn ratio but increased the concentration of methionine in grains of wheat varieties. Similar findings were obtained when the net house experiment with same strains was repeated on rice (unpublished data). Performance of these isolates was also checked on wheat and rice in field for further validation of results (unpublished data).

Acknowledgement:

Authors are grateful for the financial support provided by NAIP, New Delhi.

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