INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–0360/2018/20–8–1779–1786
DOI: 10.17957/IJAB/15.0690
http://www.fspublishers.org

Full Length Article

Bacillus Strains as Potential Alternate for Zinc Biofortification of Maize Grains

Muhammad Zahid Mumtaz[1,2], Maqshoof Ahmad[1], Moazzam Jamil[1], Saeed Ahmad Asad[3] and Farhan Hafeez[4*]
[1]*Department of Soil Science, University College of Agriculture and Environmental Sciences, the Islamia University of Bahawalpur, Bahawalpur, Pakistan*
[2]*Institute of Molecular Biology and Biotechnology, University of Lahore, Defence Road Lahore, Pakistan*
[3]*Centre for Climate Research and Development (CCRD), COMSATS University, Park Road Islamabad-45550, Pakistan*
[4*]*Department of Environmental Sciences, COMSATS Institute of Information Technology (CIIT), Abbottabad, Pakistan*
[*]For correspondence: drfarhan@ciit.net.pk

Abstract

Decreased efficiency of Zn fertilization upon formation of insoluble zincate complex is a serious threat to soil-plant nutrition. Zn solubilizing bacteria are recently reported to be a potential alternate to combat this issue but evaluation of their bioaugmentation potential is direly required. In present study, four promising Zn solubilizing strains; *Bacillus* spp. (ZM20), *Bacillus aryabhattai* (ZM31 and S10) and *B. subtilis* (ZM63) were selected to evaluate their ability for Zn biofortification of maize grains. The results revealed that inoculation/co-inoculation of Zn solubilising *Bacillus* strains significantly improved plant growth and yield attributes of maize. Co-inoculation of *B. aryabhattai* ZM31 and *B. subtilis* ZM63 yielded the best results in terms of growth and yield of studied plant. The inoculation/co-inoculation of strains also enhanced the macro and micronutrients in plant roots and shoots and successfully biofortified the maize grains with Fe and Zn. Co-inoculation significantly improved the N (171%) and Fe (78%) concentration in maize grains over uninoculated control. Maximum improvement in P (48%), K (81%) and Zn concentration (68%) in maize grains were observed with co-inoculation of *B. aryabhattai* (ZM31) and *B. subtilis* (ZM63) compared with uninoculated counterparts. The study infers that reported *Bacillus* strains showed their potential to improve nutrient concentrations in maize grains along with improvement in maize growth and yield parameters in pot experiment. These strains, therefore, should be evaluated for their potential to biofortify maize grains under field conditions before their recommendations as potential bio-inoculants for Zn biofortification under nutrient deficient soils.

Keywords: Zn solubilisation; *Bacillus* spp.; Biofortification; Malnutrition; Soil plant health

Introduction

Nutrients deficiency is a serious health problem around the world that induces malnutrition and can cause death especially in women and children (Pipero *et al*., 2015). Zinc (Zn) malnutrition in human is widespread across the globe, especially in resource-poor populations where daily calories intake is narrowed predominantly to staple cereals. It is estimated that one-third of global population is at high risk of Zn deficiency (FAO, 2014). In Pakistan, Zn malnutrition was reported in 37% of children with age of five years or less (Khalid *et al*., 2014). Efforts are being carried out to control Zn malnutrition but its mitigation is imposing a big challenge for researchers.

Zinc plays important role in various biochemical reactions of plant through influencing the production of phytohormones and formation of starch and chlorophyll contents (Hirschi, 2008; Rehman *et al*., 2018a). It enhances the quality of cereal grains through stimulating the synthesis of nucleic acid, proteins, and lipids (Hussain *et al*., 2013; Sharma *et al*., 2013). Maize is main cereal crop used for staple food in many parts of the world (Menkir, 2008). It is typically low in Zn contents when grown on Zn-deficient soils (Noulas *et al*., 2018). According to an estimate, half of the world soils are deficient in Zn which is due to several agronomic, soil, and environmental factors (Alloway, 2009). The important soil factors which are responsible for bioavailability of Zn to plants include total Zn contents in soil, pH, calcium carbonate concentration, organic matter contents, and high concentration of basic cations (Na, Ca and Mg) and anions bicarbonate and phosphate and soil solution or plant available forms (Alloway, 2009; Rehman *et al*., 2018a). In alkaline calcareous soils, Zn makes complexes with calcite and becomes unavailable to plants while under anaerobic conditions, it becomes reduced with sulphur that renders its availability to plants. The high bioavailable phosphorous also reduces the availability of Zn to crop plants under arid and semi-arid conditions

To cite this paper: Mumtaz, M.Z., M. Ahmad, M. Jamil, S.A. Asad and F. Hafeez, 2018. Bacillus strains as potential alternate for zinc biofortification of maize grains. *Int. J. Agric. Biol*., 20: 1779–1786

(Alloway, 2009; Noulas *et al*., 2018). Inorganic form of unavailable Zn naturally present in soil in the form of oxides and sulfides but the most dominant unavailable Zn minerals are silicates (Weil and Brady, 2017).

Increasing the Zn contents of staple foods can reduce the problem of malnutrition. Zinc contents in grains can be increased through various approaches such as dietary intervention, plant breeding, agronomic practices, and transgenic approaches. Among agronomic practices, application of Zn fertilizers has been used for various crops but these fertilizers are transformed into various unavailable forms depending upon soil types and chemical reactions (Cakmak, 2008; Rehman *et al*., 2018a). The unavailable Zn compounds can be converted back to available form through bioaugmentation of plant growth promoting rhizobacteria (PGPR) inoculants having the ability to solubilize insoluble Zn compounds, may be called as Zn solubilizing bacteria (ZSB) (Saravanan *et al*., 2007; Rehman *et al*., 2018b). Application of ZSB strains is a novel biotechnological approach through which biofortified grains of cereals can be attained by enhancing the uptake of micronutrient concentration in crops. They are beneficial for plants as they increase the root functions, decrease disease impact and increase plant growth and development (Rana *et al*., 2012; Ramesh *et al*., 2014; Abaid-Ullah *et al*., 2015).

Several ZSB strains have been documented for their ability to solubilize unavailable forms of Zn thus improving plant growth, yield and grain quality. Among these, genus *Bacillus* is one of the most studied as they are found to be ubiquitous in nature which possesses multiple growth-promoting traits (Ramirez and Kloepper, 2010; Zhao *et al*., 2011). Zinc solubilizing *Bacillus* strains solubilize unavailable Zn compounds through the production of chelating ligands, secretion of organic acids, amino acid, vitamins and phytohormones, and through oxidoreductive systems and proton extrusion (Wakatsuki, 1995; Saravanan *et al*., 2003). Production of organic acids by microbial strains is a major Zn solubilizing mechanism. Among these organic acids, production of 2-ketogluconic acid and gluconic acid by PGPR is responsible for Zn solubilization (Fasim *et al*., 2002).

Zinc solubilizing *Bacillus* strains have the ability to increase uptake and translocation of Zn similar to chemical fertilizers (Tariq *et al*., 2007; Abaid-Ullah *et al*., 2015). These strains have the ability to biofortify cereals due to their solubilizing command and induction of systemic resistance against pathogens (Lucas *et al*., 2014). According to previous reports, inoculation of *B. aryabhattai* (MDSR14) and *B. thuringiensis* (FA-4) in wheat, biofortified the grains with Zn up to 38 and 46%, respectively (Ramesh *et al*., 2014; Abaid-Ullah *et al*., 2015). Shakeel *et al*. (2015) also confirmed the Zn biofortification of rice grains with inoculation of *Bacillus* spp. and *B. cereus*. Soybean seeds were biofortified with inoculations of *B. firmus* (KHBD-6), *B. amyloliquefaciens* (KHBAR-1), *Bacillus* spp. (BDN-5), and *B. cereus* (ATCC 13061) (Sharma *et al*., 2012). Biofortification of grains through inoculation of these strains might be due to diverse mechanisms; it may stimulate biological N_2 fixation, produce phytohormones such as; IAA, ABA and GA, mineral solubilization, production of siderophores, HCN, ammonia, ACC deaminase and antifungal activity (Nelson, 2004; Abaid-Ullah *et al*., 2015; Mumtaz *et al*., 2017). Biofortification through inoculation of Zn solubilizing rhizobacteria is an emerging approach that can improve nutritional status of crops and human health. Despite of this fact need is to evaluate the bioaugmentation of Zn solubilizing bacterial strains so that it can be used as sustainable intervention to increase bioavailability of Zn in soil ultimately helping plants to accumulate more Zn in grains. Here, we hypothesize that Zn-solubilizing rhizobacteria can biofortify Zn contents in maize grains through solubilizing insoluble Zn compounds. Current study was conducted to evaluate the potential of Zn solubilizing *Bacillus* strains to improve Zn concentration in maize grains along with improvement in growth and yield attributes in a pot experiment.

Materials and Methods

Zinc Solubilizing *Bacillus* Strains

Thirteen Zn solubilizing rhizobacterial isolates were screened for plant growth promoting traits e.g., phosphate solubilization, siderophores production, indole acetic acid (IAA) production, and some other miscellaneous characteristics in an earlier study (Mumtaz *et al*., 2017). Four promising growth promoting strains identified as *Bacillus* sp. (ZM20), *B. aryabhattai* (ZM31), *B. subtilis* (ZM63) and *B. aryabhattai* (S10) through 16S rRNA sequence analysis were selected to test their ability to promote growth, yield and quality of maize grains. Compatibility test of strains was performed *in vitro* by visual examination of growth at 48 and 96 h through cross streaking.

Experimental Conditions

A pot study was conducted to evaluate the effect of Zn solubilizing *Bacillus* strains along with their co-inoculation treatments on growth, yield and nutrient uptake of maize. The experiment was conducted in the wire house of Department of Soil Science, University College of Agriculture and Environmental Sciences, the Islamia University of Bahawalpur, Pakistan, located at Lat: 29.40N, Lon: 71.68E and 116 meters elevation above the sea level. The non-sterile soil was used from field of research farm of University for filling the pots and the physico-chemical properties of soil were recorded before start of experiment by following the standard protocols as described by Ryan *et al*. (2001). The characteristics of soil before sowing are as follows: sandy loam textural class, pH 8.1, EC 0.47 dS m^{-1}, organic matter 0.29%, total nitrogen 0.02%, available phosphorus 6 mg kg^{-1}, extractable potassium 176 mg kg^{-1}, available Zn 3.0 mg kg^{-1} and total Zn 43 mg kg^{-1}.

Earthen pots of 12" were filled with 10 kg of soil. Bacterial cultures were prepared by growing the strains in DF minimal media modified with insoluble Zn source (ZnO) in refrigerating incubator at 30 ± 1ºC with 100 rpm shaking conditions for 48 h.

Inoculum of selected strains containing approximately 10^{11} cells mL^{-1} were coated on maize seeds along with sterilized peat, and sugar solution (10%) in 4:5:1 ratio as a carrier. For co-inoculation, bacterial cultures of respective strains were used in 1:1 ratio. In uninoculated control, sterilized peat with sugar solution in 5:1 ratio was used for seed coating. A set of 11 treatments used in this study were as: uninoculated control, *Bacillus* spp. (ZM20), *B. aryabhattai* (ZM31), *B. aryabhattai* (S10), *B. subtilis* (ZM63), *Bacillus* spp. (ZM20) × *B. aryabhattai* (ZM31), *Bacillus* spp. (ZM20) × *B. aryabhattai* (S10), *Bacillus* spp. (ZM20) × *B. subtilis* (ZM63), *B. aryabhattai* (ZM31) × *B. aryabhattai* (S10), *B. aryabhattai* (ZM31) × *B. subtilis* (ZM63), and *B. aryabhattai* (S10) × *B. subtilis* (ZM63). Six seeds of maize variety Poineer 30Y87 were sown in each pot in the mid of July-2015 with equal spacing. Pots were arranged according to completely randomized design (CRD) and experiment was replicated three times at ambient light and temperature. Recommended dose of fertilizer for maize was used as 1.08 gram pot^{-1} of N applied in two split doses of urea, 0.81 gram pot^{-1} P in the form of diammonium phosphate (DAP) and 0.54 gram pot^{-1} K in the form of sulfate of potash (SOP). The DAP and SOP were applied at the time of sowing. The split doses of N were given at V2 stage (collar of second leaf) and anthesis stage with equal distribution. Good quality irrigation water was applied to each pot to meet the water requirement of crop (Ayers and Westcot, 1994). Thinning was done after germination to maintain three plants per pot. The insecticides were applied to crop at an economic threshold level of the pest and repeated twice at an interval of two weeks. At physiological maturity, plant biometrical parameters and yield components were recorded and maize root, shoot, and grain samples were analysed to determine nutrient uptake and biofortification of grains.

SPAD Value and Biometrical Observation

For determination of SPAD value, three mature leaves (3rd, 4th and 5th from top of each plant) were selected from each pot. Three measurements from each leaf were noted by using SPAD chlorophyll meter model CL-01 (Hansatech Instruments Ltd., England) and averaged for each treatment. The plant parameters e.g., plant height, root length, shoot and root dry weight, cob length, number of rows and grains cob^{-1}, 100-grains weight, and cob grain yield were recorded at the time of harvesting.

Analysis of NPK and Micronutrients

The root, shoot and grain samples of maize were oven dried and digested as described by Wolf (1982). Digestion was done through pouring 1 mL of H_2O_2 in digestion tube containing overnight incubated plant samples with 2 mL conc. H_2SO_4 and heated up to 350ºC in digestion block until fumes appeared. The addition of 1 mL of H_2O_2 repeated after every 20 min until the material became colourless. The volume of extracts was made 50 mL with distilled water. The total nitrogen of plant samples was determined by Kjeldahl method (Jackson, 1973). The phosphorus concentration was measured through adopting the procedure as described by Jackson (1973). For determination of potassium concentration, the samples were analysed with flame photometermodel BWB-XP (BWB Technologies Ltd.'s, UK) and compared with standard solutions ranging from 0 to 100 ppm of KCl. Flame photometer readings of samples were compared with calibration curve and the actual K concentration in samples was computed.

Commercial service of Central Hi Tech Laboratory, University of Agriculture Faisalabad- Punjab, Pakistan was used for Fe and Zn analysis. The digested samples were analysed by using Atomic Absorption Spectrophotometry (Agilent Technologies, Australia) at the most sensitive wave lengths of 248.7 nm for Fe and 213.9 nm for Zn and readings were compared with respective standards.

Statistical Analysis

The set of data in triplicate for various attributes was analysed for analysis of variance technique (ANOVA) in accordance with CRD design by using Statistix 8.1 statistical package. The means were compared by least significant difference (LSD) test to quantify and evaluate the source of variation at 5% probability (Steel *et al*., 2007).

Results

SPAD Value

Data showed that chlorophyll intensity (SPAD value) of maize in different inoculation treatments ranged from 41.8 to 57.4 µg cm^{-2} (Table 1). Inoculation as well as co-inoculation treatments caused increase in SPAD value that varied from 15 to 37% as compared to uninoculated control. Maximum SPAD value with an increase up to 37% were recorded from co-inoculation treatment where *B. aryabhattai* ZM31 and *B. subtilis* ZM63 were used in combination and it showed 35% more SPAD value as compared to uninoculated control.

It is revealed from the data (Table 1) that ZSB strains showed a significant effect on relative water contents of maize leaf. Most of the treatments were non-significant to each other but significantly different as compared to uninoculated control. Co-inoculation was more effective in improving the relative water contents in maize leaves as compared to sole inoculation and the maximum relative water contents with an increase up to 34.6% as compared to

Fig. 1: Comparison of root length of maize plants of uninoculated control (left side), sole inoculation of *Bacillus* spp. ZM20 (centre) and co-inoculated combination *Bacillus aryabhattai* ZM31 × *Bacillus subtilis* ZM63 (right side) under pot conditions.

Fig. 2: Comparison of cob of uninoculated control (on left side) and co-inoculation combination *Bacillus aryabhattai* ZM31 × *Bacillus subtilis* ZM63 (on right side) grown under pot conditions

uninoculated control was obtained from co-inoculation of *B. aryabhattai* ZM31 and *B. subtilis* ZM63. The increase in relative water contents with sole inoculation was ranged from 19.9 to 26.4% over uninoculated control.

Growth Parameters

Growth attributes were recorded in terms of plant height, root length, and shoot and root dry weight (Table 1). These growth attributes were found to be significantly higher with inoculation of *Bacillus* strains compared to uninoculated control but most of the inoculation treatments were non-significant to each other. Co-inoculation of strains caused more increase in growth parameters than individual inoculation. Significant improvement in root length was observed due to combined use of bacterial strains as compared to un-inoculated control (Fig. 1 and Table 1). Highest increase up to 32.0% in plant height and 67.0% in root length was observed due to co-inoculation with *B. aryabhattai* ZM31 and *B. subtilis* ZM63 followed by the combination of *B. aryabhattai* S10 and *B. subtilis* ZM63 that gave 26.7 and 58.5% more plant height and root length, respectively, as compared to uninoculated control. Maximum shoot dry weight and root dry weight with increase up to 30 and 36%, respectively, was observed from treatment with combined use of *B. aryabhattai* ZM31 and *B. subtilis* ZM63 as compared to uninoculated control.

Yield Components

Yield components i.e., cob length, number of rows cob^{-1}, 100-grains weight and grain yield cob^{-1} were recorded (Table 2). Inoculation increased the yield components significantly except the combined use of *B. aryabhattai* ZM31 and *B. aryabhattai* S10 in case of 100-grain weight and co-inoculated combination of *Bacillus* spp. ZM20 and *B. aryabhattai* S10 in case of grain yield cob^{-1} as compared to uninoculated control. Co-inoculation with Zn solubilizing *Bacillus* strains yielded more values for yield components than sole inoculation. Co-inoculation with *B. aryabhattai* ZM31 and *B. subtilis* ZM63 gave significant improvement in cob length (Fig. 2 and Table 2) that gave the maximum cob length and rows number cob^{-1} with increase up to 39% as compared to uninoculated control. Maximum 100-grain weight and grain yield cob^{-1} resulted from treatment with combined use of *B. aryabhattai* ZM31 and *B. subtilis* ZM63 that caused an increase of up to 29 and 41%, respectively, over the uninoculated control.

Nutrients Concentration in Root and Shoot

Inoculation of Zn solubilizing *Bacillus* strains enhanced the concentration of macronutrients in maize roots and shoots (Supplementary Table 1). Maximum concentration of N in maize roots and shoots was recorded due to combined use of *Bacillus* spp. ZM20 and *B. aryabhattai* ZM31. The combined use of *B. aryabhattai* ZM31 and *B. subtilis* ZM63 showed highest increase in P concentration in maize roots (up to 71%) and shoots (up to 72%) as compared to uninoculated control. The same treatment also caused the maximum increase in K concentration in maize roots and shoots as compared to uninoculated control.

Inoculated plants also showed significant increase in concentration of micronutrients (Fe and Zn) in maize roots and shoots as compared to uninoculated control (Table 3). Maximum concentration of Fe in both organs was observed from treatment where *B. aryabhattai* S10 and *B. subtilis* ZM63 were used in combination.

Table 1: Effect of Zn solubilizing *Bacillus* strains inoculation/co-inoculation on growth attributes of maize seedlings

Treatments*	SPAD value	Relative water contents (%)	Plant height (cm)	Root length (cm)	Shoot dry weight (g)	Root dry weight (g)
T1 = Control	41.8 ± 0.60^{f}	55.3 ± 0.88^{f}	143.3 ± 0.88^{e}	67.9 ± 0.52^{g}	35.1 ± 0.26^{d}	8.93 ± 0.09^{g}
T2 = *Bacillus* sp. ZM20	51.0 ± 1.56^{cde}	68.0 ± 1.73^{cde}	169.7 ± 0.67^{cd}	98.0 ± 1.00^{c}	41.0 ± 1.15^{b}	10.57 ± 0.15^{bcd}
T3 = *B. aryabhattai* ZM31	53.5 ± 1.82^{bc}	66.3 ± 1.45^{de}	171.0 ± 1.00^{cd}	106 ± 1.53^{b}	39.0 ± 1.53^{bc}	10.21 ± 0.12^{d}
T4 = *B. aryabhattai* S10	49.9 ± 1.07^{cde}	69.6 ± 0.87^{bc}	169.0 ± 1.06^{d}	87.0 ± 2.62^{de}	37.3 ± 1.86^{bcd}	10.50 ± 0.15^{cd}
T5 = *B. subtilis* ZM63	52.8 ± 1.60^{bcd}	69.9 ± 1.10^{bc}	171.0 ± 0.58^{cd}	77.3 ± 2.33^{f}	40.3 ± 1.45^{bc}	10.32 ± 0.27^{d}
T6=*Bacillus* spp. ZM20 × *B. aryabhattai* ZM31	52.8 ± 2.05^{bcd}	65.5 ± 0.35^{e}	180.3 ± 1.45^{b}	77.0 ± 0.58^{f}	39.7 ± 1.44^{bc}	9.44 ± 0.06^{fg}
T7=*Bacillus* spp. ZM20 × *B. aryabhattai* S10	48.1 ± 0.57^{e}	69.4 ± 1.11^{cd}	167.0 ± 0.58^{d}	92.3 ± 2.03^{d}	37.0 ± 1.15^{cd}	9.46 ± 0.09^{ef}
T8=*Bacillus* spp. ZM20 × *B. subtilis* ZM63	49.7 ± 0.73^{de}	68.7 ± 0.89^{cde}	167.0 ± 1.73^{d}	105.3 ± 0.33^{b}	45.0 ± 1.53^{a}	11.20 ± 0.15^{b}
T9=*B. aryabhattai* ZM31 × *B. aryabhattai* S10	50.6 ± 1.69^{cde}	67.3 ± 0.67^{cde}	173.7 ± 1.86^{c}	82.3 ± 1.45^{ef}	40.6 ± 1.20^{bc}	10.17 ± 0.19^{de}
T10=*B. aryabhattai* ZM31 × *B. subtilis* ZM63	57.4 ± 0.31^{a}	74.5 ± 0.29^{a}	189.3 ± 1.20^{a}	113.5 ± 0.33^{a}	45.6 ± 0.38^{a}	12.04 ± 0.42^{a}
T11=*B. aryabhattai* S10 × *B. subtilis* ZM63	56.4 ± 0.35^{ab}	72.8 ± 0.44^{ab}	181.6 ± 1.22^{b}	107.6 ± 0.88^{b}	41.0 ± 1.53^{b}	11.01 ± 0.29^{bc}
LSD ($p \leq 0.05$)	3.7489	3.3002	4.302	5.5416	3.8535	0.6027

*Treatments = inoculation/co-inoculation of Zn solubilising *Bacillus* strains; LSD = least significant difference; Means ± standard error, data are mean values of three replicates; superscripts indicate the same letter (s) do not differ significantly according to least significant difference test.

Table 2: Effect of Zn solubilizing *Bacillus* strains inoculation/co-inoculation on yield components of maize

Treatments*	Cob length (cm)	Cob row numbers	100 grains weight (g)	Grain yield cob^{-1} (g)
T1 = Control	11.3 ± 0.167^{f}	10.2 ± 0.17^{f}	14.6 ± 0.21^{e}	40.5 ± 0.29^{g}
T2 = *Bacillus* spp. ZM20	14.8 ± 0.167^{b}	13.7 ± 0.33^{ab}	18.5 ± 0.17^{a}	54.2 ± 0.70^{b}
T3 = *B .aryabhattai* ZM31	12.7 ± 0.333^{e}	12.6 ± 0.33^{c}	16.3 ± 0.15^{c}	48.2 ± 0.60^{e}
T4 = *B. aryabhattai* S10	12.8 ± 0.441^{e}	11.3 ± 0.33^{e}	17.7 ± 0.38^{b}	52.7 ± 0.40^{bc}
T5 = *B. subtilis* ZM63	13.2 ± 0.167^{de}	13.0 ± 0.00^{bc}	15.0 ± 0.03^{de}	48.9 ± 0.46^{de}
T6 = *Bacillus* spp. ZM20 × *B. aryabhattai* ZM31	14.3 ± 0.165^{bc}	11.0 ± 0.58^{ef}	17.3 ± 0.32^{b}	44.9 ± 1.12^{f}
T7 = *Bacillus* spp. ZM20 × *B. aryabhattai* S10	14.2 ± 0.167^{bc}	11.0 ± 0.00^{ef}	15.5 ± 0.24^{d}	41.2 ± 0.15^{g}
T8 = *Bacillus* spp. ZM20 × *B. subtilis* ZM63	13.3 ± 0.163^{de}	11.6 ± 0.33^{de}	17.7 ± 0.44^{b}	51.0 ± 1.11^{cd}
T9 = *B. aryabhattai* ZM31 × *B. aryabhattai* S10	13.2 ± 0.145^{de}	12.3 ± 0.33^{cd}	15.3 ± 0.15^{de}	47.7 ± 0.95^{e}
T10 = *B. aryabhattai* ZM31 × *B. subtilis* ZM63	15.8 ± 0.145^{a}	14.2 ± 0.15^{a}	18.8 ± 0.17^{a}	57.2 ± 0.72^{a}
T11 = *B. aryabhattai* S10 × *B. subtilis* ZM63	13.8 ± 0.441^{cd}	13.2 ± 0.17^{bc}	18.6 ± 0.23^{a}	54.0 ± 0.29^{b}
LSD ($p \leq 0.05$)	0.7445	0.8719	0.7351	2.5883

*Treatments = inoculation/co-inoculation of Zn solubilizing *Bacillus* strains; LSD = least significant difference; Means ± standard error, data are mean values of three replicates; superscripts indicate the same letter (s) do not differ significantly according to least significant difference test

Table 3: Effect of Zn solubilizing *Bacillus* strains on Fe and Zn in root and shoot of maize

Treatments*	Fe in roots (mg kg^{-1})	Zn in roots (mg kg^{-1})	Fe in shoot (mg kg^{-1})	Zn in shoot (mg kg^{-1})
T1 = Control	63.5 ± 2.31^{f}	45.3 ± 0.71^{f}	42.5 ± 0.45^{e}	42.2 ± 0.60^{d}
T2 = *Bacillus* spp. ZM20	108.4 ± 0.19^{ab}	52.8 ± 0.30^{c}	46.4 ± 0.72^{c}	47.4 ± 0.70^{b}
T3 = *B. aryabhattai* ZM31	60.5 ± 0.52^{f}	46.2 ± 0.70^{ef}	43.5 ± 0.36^{de}	42.4 ± 0.25^{d}
T4 = *B. aryabhattai* S10	75.8 ± 2.32^{e}	50.1 ± 0.10^{d}	46.4 ± 0.82^{c}	51.6 ± 0.24^{a}
T5 = *B. subtilis* ZM63	59.4 ± 1.12^{f}	47.4 ± 0.40^{e}	43.5 ± 0.49^{de}	44.1 ± 0.73^{c}
T6 = *Bacillus* spp. ZM20 × *B. aryabhattai* ZM31	86.9 ± 2.40^{d}	54.0 ± 0.09^{b}	44.4 ± 0.41^{d}	44.4 ± 0.47^{c}
T7 = *Bacillus* spp. ZM20 × *B. aryabhattai* S10	99.8 ± 1.54^{c}	49.9 ± 0.07^{d}	47.7 ± 0.03^{bc}	44.3 ± 0.19^{c}
T8 = *Bacillus* spp. ZM20 × *B. subtilis* ZM63	89.9 ± 2.90^{d}	54.0 ± 0.16^{b}	51.1 ± 0.60^{a}	47.4 ± 0.06^{b}
T9 = *B. aryabhattai* ZM31 × *B. aryabhattai* S10	101.7 ± 1.66^{bc}	47.0 ± 0.56^{e}	47.9 ± 0.11^{bc}	44.0 ± 0.26^{c}
T10 = *B. aryabhattai* ZM31 × *B. subtilis* ZM63	100.0 ± 2.49^{c}	55.4 ± 0.54^{a}	48.5 ± 0.48^{b}	51.9 ± 0.61^{a}
T11 = *B. aryabhattai* S10 × *B. subtilis* ZM63	110.9 ± 1.14^{a}	46.8 ± 0.57^{e}	52.5 ± 0.89^{a}	48.3 ± 0.71^{b}
LSD ($p \leq 0.05$)	6.7278	0.0655	1.7799	1.2266

*Treatments = inoculation/co-inoculation of Zn solubilizing *Bacillus* strains; LSD = least significant difference; Means ± standard error, data are mean values of three replicates; superscripts indicate the same letter (s) do not differ significantly according to least significant difference test

This treatment showed 74.7 and 23.4% more Fe concentration in maize roots and shoots, respectively, as compared to uninoculated control. Inoculation of *Bacillus* strains resulted in improvement in Zn concentration in maize roots and shoot. Maximum Zn concentration of 55.4 mg kg^{-1} in roots and 51.9 mg kg^{-1} in shoots were observed due to combined use of *B. aryabhattai* ZM31 and *B. subtilis* ZM63 with increase up to 22.5 and 22.9%, respectively, as compared to uninoculated control.

Nitrogen, Phosphorus and Potassium Concentration in Maize Grains

The improvement in NPK concentration was observed due to inoculation/co-inoculation treatments (Table 4). Co-inoculation treatments showed more NPK concentration in maize grains than sole inoculation of different *Bacillus* strains. Most of the treatments were non-significant in improving the N concentration in maize grains as compared to uninoculated control.

Table 4: Effect of Zn solubilizing *Bacillus* strains on N, P and K concentration in maize grains

Treatments*	N in grains (%)	P in grains (%)	K in grains (%)
T1 = Control	1.12 ± 0.015^{d}	0.60 ± 0.018^{d}	0.42 ± 0.004^{e}
T2 = *Bacillus* spp. ZM20	1.22 ± 0.015^{d}	0.86 ± 0.007^{a}	0.74 ± 0.005^{a}
T3 = *B. aryabhattai* ZM31	1.26 ± 0.020^{d}	0.70 ± 0.002^{bc}	0.55 ± 0.005^{cd}
T4 = *B. aryabhattai* S10	1.22 ± 0.005^{d}	0.70 ± 0.015^{bc}	0.56 ± 0.004^{cd}
T5 = *B. subtilis* ZM63	1.25 ± 0.035^{d}	0.73 ± 0.028^{b}	0.46 ± 0.029^{e}
T6 = *Bacillus* spp. ZM20 × *B. aryabhattai* ZM31	3.02 ± 0.030^{a}	0.62 ± 0.005^{d}	0.56 ± 0.026^{cd}
T7 = *Bacillus* spp. ZM20 × *B. aryabhattai* S10	2.63 ± 0.080^{b}	0.66 ± 0.001^{c}	0.55 ± 0.019^{e}
T8 = *Bacillus* spp. ZM20 × *B. subtilis* ZM63	1.66 ± 0.025^{c}	0.69 ± 0.008^{bc}	0.59 ± 0.012^{c}
T9 = *B. aryabhattai* ZM31 × *B. aryabhattai* S10	1.15 ± 0.010^{d}	0.66 ± 0.014^{c}	0.54 ± 0.004^{d}
T10 = *B. aryabhattai* ZM31 × *B. subtilis* ZM63	2.90 ± 0.015^{a}	0.89 ± 0.004^{a}	0.76 ± 0.020^{a}
T11 = *B. aryabhattai* S10 × *B. subtilis* ZM63	2.87 ± 0.020^{a}	0.87 ± 0.021^{a}	0.69 ± 0.001^{b}
LSD ($p \leq 0.05$)	0.1823	0.0421	0.0417

*Treatments = inoculation/co-inoculation of Zn solubilizing *Bacillus* strains; LSD = least significant difference; Means ± standard error, data are mean values of three replicates; superscripts indicate the same letter (s) do not differ significantly according to least significant difference test

Table 5: Effect of Zn solubilizing *Bacillus* strains on Fe and Zn concentration in maize grains

Treatments*	Fe in grain (mg kg^{-1})	Zn in grains (mg kg^{-1})
T1 = Control	41.4 ± 0.20^{d}	39.0 ± 0.87^{g}
T2 = *Bacillus* spp. ZM20	55.6 ± 0.01^{b}	42.1 ± 0.93^{de}
T3 = *B. aryabhattai* ZM31	40.9 ± 0.36^{d}	41.2 ± 0.52^{ef}
T4 = *B. aryabhattai* S10	47.4 ± 0.23^{c}	46.3 ± 0.41^{c}
T5 = *B. subtilis* ZM63	42.5 ± 0.24^{d}	39.3 ± 1.16^{fg}
T6 = *Bacillus* spp. ZM20 × *B. aryabhattai* ZM31	43.0 ± 0.70^{d}	40.9 ± 0.60^{efg}
T7 = *Bacillus* spp. ZM20 × *B. aryabhattai* S10	41.2 ± 0.03^{d}	39.3 ± 1.25^{fg}
T8 = *Bacillus* spp. ZM20 × *B. subtilis* ZM63	55.8 ± 1.01^{b}	47.5 ± 0.01^{bc}
T9 = *B. aryabhattai* ZM31 × *B. aryabhattai* S10	41.5 ± 0.01^{d}	43.9 ± 1.22^{de}
T10 = *B. aryabhattai* ZM31 × *B. subtilis* ZM63	54.5 ± 0.52^{b}	50.6 ± 0.34^{a}
T11 = *B. aryabhattai* S10 × *B. subtilis* ZM63	58.15 ± 1.87^{a}	48.4 ± 0.22^{b}
LSD ($p \leq 0.05$)	2.2276	1.9777

*Treatments = inoculation/co-inoculation of Zn solubilizing *Bacillus* strains; LSD = least significant difference; Means ± standard error, data are mean values of three replicates; superscripts indicate the same letter (s) do not differ significantly according to least significant difference test

The combined use of *Bacillus* sp. ZM20 and *B. aryabhattai* ZM31 showed maximum concentration of N in grains with increase up to 171% as compared to uninoculated control. The combination of strains *B. aryabhattai* ZM31 and *B. subtilis* ZM63 also showed significantly maximum concentration of P and K in grains with increase up to 48 and 81%, respectively, as compared to uninoculated control.

Zinc and Fe Concentration in Maize Grains

Maize plants treated with co-inoculation combination *B. aryabhattai* S10 × *B. subtilis* ZM63 showed the maximum Fe concentration in grains with increase up to 40.5% as compared to uninoculated control (Table 5). Maximum Zn concentration of 50.6 mg kg^{-1} was recorded with combined use of *B. aryabhattai* ZM31 and *B. subtilis* ZM63 with increase up to 27.7% over the uninoculated control (Table 5). Co-inoculation treatments showed more concentration of Fe and Zn in grains than sole inoculation treatments.

Discussion

Zinc is one of the essential micronutrients thus several Zn fortification interventions are used to improve the Zn dietary consumptions (White and Broadley, 2005; Cakmak, 2008). The Zn solubilization ability of rhizobacterial isolates along with their plant growth promoting characteristics were recently assessed in a series of experiments under axenic conditions (Mumtaz *et al*., 2017). In the current study, four most promising Zn solubilizing *Bacillus* strains i.e., *Bacillus* sp. (ZM20), *B. aryabhattai* (ZM31, S10) and *B. subtilis* (ZM63) were tested for their ability to biofortify maize grains along with improvement in maize growth and yield attributes.

In the present study, inoculation/co-inoculation of Zn solubilizing *Bacillus* strains caused significant increase in SPAD value of maize leaf which is due to their ability to solubilize nutrients which in turn increased the photosynthetic leaf area of inoculated plants. Inoculation/co-inoculation showed variable SPAD value and the effect of co-inoculation treatments was more as compared to sole inoculation. Similarly, Nadeem *et al*. (2007) and Ahmad *et al*. (2013) reported the increase in chlorophyll intensity with co-inoculation of bacteria in maize and mungbean plants.

Inoculation of Zn solubilizing *Bacillus* strains in present study proved to be effective to increase the plant growth and yield of maize as compared to uninoculated control. Co-inoculation treatments were better to increase growth parameters (plant height, root length, shoot and root dry weight) and yield components (cob length, rows and grain numbers cob^{-1}, 100-grain weight and grain yield cob^{-1}) as compared to uninoculated control. Co-inoculation

with *B. aryabhattai* ZM31 and *B. subtilis* ZM63 showed maximum improvement in grwoth and yield components as compared to other inoculation/co-inoculation treatments. Increase in growth and yield component due to inoculation of Zn solubilizing *Bacillus* spp. In present study could be due to greater nutrient bioavailability through beneficial growth promoting characteristics like solubilization of P and Zn, production of siderophores and phytohormones that inoculated strains possess which is evident from *in vitro* characterization study (Mumtaz *et al*., 2017). The increased yield components due to inoculation with these Zn solubilizing *Bacillus* spp. may also be due to higher enzyme activities, drop in rhizophore pH and increased microbial biomass-C which increases the Zn bioavailability as reported by Ramesh *et al.* (2014).

In present study, co-inoculation of Zn solubilizing *Bacillus* spp. enhanced the uptake and accumulation of macro and micronutrients i.e., N, P, K, Fe, and Zn in maize roots and shoots as compared to uninoculated control. The improvement in uptake of nutrients may be due to stimulation of root proliferation by the synergistic effect of bacterial strains (Ahmad *et al*., 2013), nutrient solubilization especially Zn and P by production of organic acids (Mumtaz *et al*., 2017) and other growth promoting substances which might have enhanced the nutrient uptake (Saravanan *et al*., 2007). The higher concentration of nutrients in plant vegetative parts reveals its positive effect on their translocations to grains. More nutrient concentration in plant vegetative parts cause more nutrients to accumulate in grains thus enhancing the quality of cereals grains (Goteti *et al*., 2013; Tripathi *et al*., 2014).

This work showed the potential of *Bacillus* strains to increase the iron contents in grains. Such increase in Fe contents in maize grains might be due to solubilization of Fe by neutralizing the pH of alkaline soil through production of organic acids (Mumtaz *et al*., 2017) that enhanced the translocation of Fe from root and shoot to grains. Higher contents of Fe in grains due to co-inoculation with *B. aryabhattai* S10 and *B. subtilis* ZM63 may be due to ability of these strains to produce more siderophores under condition of Fe scarcity as reported in earlier characterization study (Mumtaz *et al*., 2017) that may result in higher available iron content in root zone and ultimately increased uptake and accumulation of Fe ingrains. It might also be due to the over expression of ferritin genes which increase the capacity for iron storage in plants (Curie and Briat, 2003).

In current study, maize grains were biofortified with Zn due toinoculation with Zn solubilizing *Bacillus* strains. The combined use of *Bacillus* spp. (ZM20) and *B. subtilis* (ZM63) gave even better results than sole inoculation regarding the improvement in Zn concentration in grains. The improvement in Zn concentration in maize grains due to co-inoculation has also been reported by Vaid *et al*. (2014). They observed significant increase in Zn accumulation in grains due to co-inoculation with *Burkholderia* spp. SG1 (BC) + *Acinetobacter* spp. SG2 (AX). The precise mechanism of action for Zn biofortification upon inoculation is still not known but it may be related to the modulation of root morphology that improves Zn acquisition and accumulation in maize and the ability of strains to solubilize insoluble mineral Zn compounds present in soil through secretion of organic acids which decreased the rhizospheric pH and enhanced its translocation from roots to grains.

In our study, amount of Zn concentration in maize grains ranged from 39.3 to 50.6 mg kg^{-1} due to inoculation/co-inoculation with *Bacillus* strains that clearly shows the Zn accumulation in grains and should be evaluated at farmer fields. The increased Zn concentration in maize grains found in this study might have large implications in terms of remediation of malnutrition in rural population. Thus, *Bacillus* strains benefited the maize in present work through improving plant physiology, growth, yield and biofortification of grains.

Conclusion

We conclude that Zn solubilizing *Bacillus* strains showed their potential to improve nutrient concentrations in maize grains along with improvement in maize growth and yield parameters either inoculated with single strain or co-inoculated where later yield more promising results in a pot experiment. Among co-inoculated combinations, the combined use of *B. aryabhattai* (ZM31) and *B. subtilis* (ZM63) significantly enhanced the growth, yield and quality of the maize grains. Our findings clearly demonstrate that the studied *Bacillus* spp. have potential to be evaluated as biofortifying strategy for maize grains under field conditions before their recommendations as potential bio-inoculants for Zn biofortification under nutrient deficient soils.

Acknowledgements

The authors acknowledge the financial support by the Endowment Fund Secretariat, University of Agriculture Faisalabad and Department of Soil Science, The Islamia University Bahawalpur-Pakistan.

References

Abaid-Ullah, M., M.N. Hassan, M. Jamil, G. Brader, M. Kausar, N. Shah, A. Sessitsch and F.Y. Hafeez, 2015. Plant growth promoting rhizobacteria: an alternate way to improve yield and quality of wheat (*Triticum aestivum*). *Int. J. Agric. Biol.*, 17: 51–60

Ahmad, M., Z.A. Zahir, M. Khalid, F. Nazli and M. Arshad, 2013. Efficacy of *Rhizobium* and *Pseudomonas* strains to improve physiology, ionic balance and quality of mung bean under salt-affected conditions on farmer's fields. *Plant Physiol. Biochem.*, 63: 170–176

Alloway, B.J., 2009. Soil factors associated with zinc deficiency in crops and humans. *Environ. Geochem. Health*, 31: 537–548

Ayers, R.S. and D.W. Westcot, 1994. *Water Quality for Agriculture*, pp: 1-11. FAO irrigation and drainage papers 29 (Rev.1), Food and Agriculture Organization of the United Nations, Rome, Italy

Cakmak, I., 2008. Zinc deficiency in wheat in Turkey. *In: Micronutrient Deficiencies in Global Crop Production*, pp: 181–200. Alloway, B.J. (ed.). Springer, Dordrecht, The Netherlands

Curie, C. and J.F. Briat, 2003. Iron transport and signaling in plants. *Annu. Rev. Plant. Biol*., 54: 183–206

FAO, 2014. Food and Agriculture Organization. Undernourishment around the world in 2014. *In: The State of Food Insecurity in the World*, pp: 8-12. Food and Agriculture Organization of the United Nations, Rome, Italy

Fasim, F., N. Ahmed, R. Parsons and G.M. Gadd, 2002. Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. *FEMS Microbiol. Lett*., 213: 1–6

Goteti, P.K., L.D.A. Emmanuel, S. Desai and M.H.A. Shaik, 2013. Prospective zinc solubilising bacteria for enhanced nutrient uptake and growth promotion in maize (*Zea mays* L.). *Int. J. Microbiol*., 2013: 1-7

Hirschi, K., 2008. Nutritional improvements in plants: time to bite on biofortified foods. *Trends Plant Sci*., 13: 459–462

Hussain, S., M.A. Maqsood, Z. Rengel, T. Aziz and M. Abid, 2013. Estimated zinc bioavailability in milling fractions of biofortified wheat grains and in flours of different extraction rates. *Int. J. Agric. Biol*., 15: 383–388

Jackson, M.L., 1973. *Soil Chemical Analysis*. Prentice Hall Inc. Englewood Cliffs, New Jersey, USA

Khalid, N., A. Ahmed, M.S. Bhatti, M.A. Randhawa, A. Ahmad and R. Rafaqat, 2014. A question mark on zinc deficiency in 185 million people in Pakistan-possible way out. *Crit. Rev. Food Sci. Nutr*., 54: 1222–1240

Lucas, J.A., J. García-Cristobal, A. Bonilla, B. Ramos and J. Gutierrez-Manero, 2014. Beneficial rhizobacteria from rice rhizosphere confers high protection against biotic and abiotic stress inducing systemic resistance in rice seedlings. *Plant Physiol. Biochem*., 82: 44–53

Menkir, A., 2008. Genetic variation for grain mineral content in tropical-adapted maize inbred lines. *Food Chem.*, 110: 454–464

Mumtaz, M.Z., M. Ahmad, M. Jamil and T. Hussain, 2017. Zinc solubilizing *Bacillus* spp. potential candidates for biofortification in maize. *Microbiol. Res*., 202: 51–60

Nadeem, S.M., Z.A. Zahir, M. Naveed and M. Arshad, 2007. Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. *Can. J. Microbiol*., 53: 1141–1149

Nelson, L.M., 2004. Plant growth promoting rhizobacteria (PGPR): Prospect for new inoculants. *Crop Manage*., 3: 1-12

Noulas, C., M. Tziouvalekas and T. Karyotis, 2018. Zinc in soils, water and food crops. *J. Trace Elem. Med. Biol*., 49: 252-260

Pipero, P., G. Bejtja, K. Rjepaj, E. Mersini, M. Pipero and A. Ylli, 2015. Malnutrition in Albania, related problems and flour fortification as a solution. *Int. J. Nutr. Metab*., 7: 29–32

Ramesh, A., S.K. Sharma, M.P. Sharma, N. Yadav and O.P. Joshi, 2014. Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in vertisols of central India. *Appl. Soil Ecol*., 73: 87–96

Ramirez, C.A. and J.W. Kloepper, 2010. Plant growth promotion by *Bacillus amyloliquefaciens* FZB45 depends on inoculum rate and P-related soil properties. *Biol. Fert. Soils*, 46: 835–844

Rana, A., M. Joshi, R. Prasanna, Y.S. Shivay and L. Nain, 2012. Biofortification of wheat through inoculation of plant growth promoting rhizobacteria and cyanobacteria. *Eur. J. Soil Biol*., 50: 118–126

Rehman, A., M. Farooq, L. Ozturk, M. Asif and K.H.M. Siddique, 2018a. Zinc nutrition in wheat-based cropping systems. *Plant Soil*, 422: 283–315

Rehman, A., M. Farooq, M. Naved, A. Nawaz and B. Shahzad, 2018b. Seed priming of Zn with endophytic bacteria improves the productivity and grain biofortification of bread wheat. *Eur. J. Agron*., 94: 98-107

Ryan, J., G. Estefan and A. Rashid, 2001. *Soil and Plant Analysis Laboratory Manual*, 2nd edition, p: 172. International Centre for Agriculture in Dry Areas (ICARDA), Syria

Saravanan, V.S., M. Madhaiyan and M. Thangaraju, 2007. Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium *Gluconaceto bacterdiazotrophicus*. *Chemosphere*, 66: 1794–1798

Saravanan, V.S., S.R. Subramoniam and S.A. Raj, 2003. Assessing *in vitro* solubilization of different zinc solubilizing bacterial (ZBS) strains. *Braz. J. Microbiol*., 34: 121–125

Shakeel, M., A. Rais, M.N. Hassan and F.Y. Hafeez, 2015. Root associated *Bacillus* spp. improves growth, yield and zinc translocation for basmati rice (*Oryza sativa*) varieties. *Front. Microbiol.*, 6: 1286

Sharma, A., B. Patni, D. Shankhdhar and S.C. Shankhdhar, 2013. Zinc - an indispensable micronutrient. *Physiol. Mol. Biol. Plants*, 19: 11–20

Sharma, S.K., M.P. Sharma, A. Ramesh and O.P. Joshi, 2012. Characterization of zinc-solubilizing *Bacillus* isolates and their potential to influence zinc assimilation in soybean seeds. *J. Microbiol. Biotechnol*., 22: 352–359

Steel, R.G.D., J.H. Torrie and D.A. Dickey, 2007. *Principles and Procedures of Statistics: A Biometrical Approach*, 3rd edition. McGraw Hill Book Co, New York

Tariq, M., S. Hameed, K.A. Malik and F.Y. Hafeez, 2007. Plant root associated bacteria for zinc mobilization in rice. *Pak. J. Bot*., 39: 245–253

Tripathi, D.K., V.P. Singh, D.K. Chauhan, S.M. Prasad and N.K. Dubey, 2014. Role of macronutrients in plant growth and acclimation: recent advances and future prospective. *In*: *Improvement of Crops in the Era of Climatic Changes*, pp. 197–216. Ahmad, P., M. Wani, M. Azooz and L.S. Phan Tran (eds.). Springer, New York, USA

Vaid, S.K., B. Kumar, A. Sharma, A.K. Shukla and P.C. Srivastava, 2014. Effect of zinc solubilizing bacteria on growth promotion and zinc nutrition of rice. *J. Soil Sci. Plant Nutr*., 14: 889–910

Wakatsuki, T., 1995. Metal oxidoreduction by microbial cells. *J. Ind. Microbiol. Biotechnol*., 14: 169–177

Weil, R.R. and N.C. Brady, 2017. *The Nature and Properties of Soils*. Fifteen ed. Harlow, Essex, UK

White, P.J. and M.R. Broadley, 2005. Biofortifying crops with essential mineral elements. *Trends Plant Sci*., 10: 586–593

Wolf, B., 1982. The comprehensive system of leaf analysis and its use for diagnosing crop nutrient status. *Commun. Soil Sci. Plant Anal*., 13: 1035–1059

Zhao, Q., Q. Shen, W. Ran, T. Xiao, D. Xu and Y. Xu, 2011. Inoculation of soil by *Bacillus subtilis* Y-IVI improves plant growth and colonization of the rhizosphere and interior tissues of muskmelon (*Cucumis melo* L.). *Biol. Fert. Soils*, 47: 507–514

(Received 08 march 2018; Accepted 26 March 2018)