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Original Research

Potential of Integrated Use of *Bacillus* sp. AZ6 and Organic Waste for Zinc Bio-Activation to Improve Physiological Attributes of Maize

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Abstract

Zinc (Zn) is an important micronutrient for plant physiology, including the activation of various enzymes, nitrogen metabolism, cell-membrane integrity, and carbonic anhydrase activity. Its deficiency is reported in plants grown in poor soils worldwide including Pakistan. Exogenous application of Zn fertilizers is not efficient due to fixation into the soil. Thus, Zn solubilizing bacteria have a stronger power to chelates the insoluble Zn compounds which may increase Zn availability for plant uptake. Therefore, a pot trial was conducted to evaluate the effects of four products of bio-activated zinc oxide (ZnO) formulated using bio augmented-organic matter coated with zinc oxide (BOZ) on the physiological attributes of maize. Four different products viz. BOZ1, BOZ2, BOZ3, and BOZ4 were prepared using *Bacillus* sp. AZ6 strain augmented orange peel and ZnO. The results revealed that BOZ4 formulation performed outclass by exhibiting 85%, 31%, and 108% higher photosynthetic rate, stomatal conductance, and transpiration rate, respectively, compared to control. Likewise, it showed a maximum increase in carbonic anhydrase activity, vapor pressure deficit, electrolyte leakage, and SPAD value by 74%, 44%, 45%, and 66%, respectively, over control. Hence, it is concluded that bio-activation of ZnO by *Bacillus* sp. AZ6 and orange-peel waste could serve as an effective strategy to improve the physiology of maize.

Keywords: *Bacillus* sp., bio-activation, carbonic anhydrase, zinc solubilizing bacteria, zinc oxide, maize crop, bioaugmented-organic matter

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Introduction

Zinc (Zn) is an important micronutrient needed by all the organisms including microorganisms, humans, animals, and plants. It is an important component of various enzymes which catalyze many metabolic reactions in plants [1]. It is essential for nitrogen (N) metabolism, uptake of N, synthesis of protein, chlorophyll, carbonic anhydrase (CA) activity, and resistance against biotic, abiotic, and oxidative stresses [2]. Zn also plays a significant role in photosynthesis, cell membrane integrity, pollen formation and enhances the antioxidant enzymes activity as well as chlorophyll contents within plant tissues [3, 4]. It constitutes more than 300 enzymes as a core co-factor for their activation [5]. The catalytic activities of all the enzymes is badly affected by the Zn deficiency in addition to their metabolic reactions [6]. The CA is a metalloenzyme and participates in pH adjustment, transfer of CO_2, ion exchange, respiration, photosynthetic CO_2 fixation, and closure of stomata. Zn catalysis CA as a cofactor by binding with water and activating the catalytic site of the enzyme. Zn deficiency lessens the CA content in plants [7].

Maize is counted as one of the imperative cereal crops worldwide and its total production is greater than other cereal grains [8]. It is most susceptible to Zn deficiency thus shows stunted growth under Zn deficiency. In Pakistan, Zn is deficient due to its precipitation in soils, which results in limited Zn availability to plants consequently leading to poor roots development [9]. Therefore, the application of inorganic fertilizers could be a good source of Zn for enhancing crop production; however, due to its fixation in alkaline and calcareous soils, it is not economical practice [10]. It is well-understood that 90% of applied Zn fertilizer in calcareous soils is precipitated and adsorbed on soil colloids [11]. Two sources of Zn including zinc oxide (ZnO) and zinc sulfate ($ZnSO_4$) having 80% and 33% of Zn are mostly applied as Zn fertilizers. Plants uptake Zn in its soluble form, Zn^{2+}, but a significant amount of Zn in the soil that is insoluble. Overall, lower Zn contents, soil alkalinity, high calcite content, high bicarbonate ion levels, and high phosphorus levels are the primary physicochemical soil factors that cause Zn deficiency in staple foods [12, 13].

The efficiency of ZnO (an insoluble and cost-effective source) can be enhanced through bio-activation with bacteria and organic amendments [14, 15]. The bio-activation of Zn through Zn solubilizing bacteria (ZSB) strains had been previously reported by Hussain et al. [16], Hussain et al. [17], Nazir et al. [18], Ain et al. [19]. Generally, the ZSB plant growth-promoting rhizobacteria (PGPR) bio-activate Zn through chelating the metal compounds by protonation and production of organic acids [20]. Lowering the soil pH through the production of organic acid by bacteria can increase the bioavailability of Zn [21]. In general, small changes in soil pH can dramatically change the release of different micronutrients in the soil, particularly the bioavailability of Zn increases by a factor of 100 as the pH decreases by 1 unit and conversely. Previously, the inoculation with organic acid-producing bacteria showed improvement in bioavailable Zn contents in rhizospheric soil and plant tissues [22]. The bacterial genus *Bacillus* and *Pseudomonas* showed their potential to dissolve insoluble Zn sources such as ZnO and $ZnCO_3$ [23]. Such bacteria are multifaceted having characteristics of N_2 fixation, solubilization of minerals, production of phytohormones, siderophores, organic acids, and are involved in an antagonistic activity that can improve plant growth even under biotic and abiotic stresses [14, 24, 15]. The use of Zn-solubilizing plant growth-promoting microbial inoculants in agriculture has been related to a substantial increase in crop yield [25, 26].

Therefore, the current pot study was conducted to demonstrate the hypothesis that integrating ZSB strain with organic material and chemical Zn source could improve maize physiology. In the present study, various formulations of *Bacillus* sp. AZ6 strain, organic matter, and ZnO were produced and applied in a pot trial to investigate their effects on maize physiology. The formulated products showed promising results and improved the maize physiology in terms of gas exchange attributes, carbonic anhydrase activity, leaf chlorophyll contents, and electrolytes leakages.

Materials and Methods

Production of Bioaugmented-Organic Matter Coated with ZnO

The bioaugmented-organic matter coated with ZnO (BOZ) products were formulated using the method of Hussain et al. [16], Hussain et al. [17]. Pre-isolated PGPR strain *Bacillus* sp. AZ6 having Accession No. KT221633 [14] was selected for the preparation of BOZ. The strain was grown in DF (Dwarkin and Foster) minimal media for 48 h at 30±1ºC. Orange peel collected locally was inoculated with strain AZ6 at 90:10 ratio (w/v; orange peel: bacterial culture) and kept in an incubator at 30±1ºC for 72 h. The ZnO powdered bulk was mixed with different ratios of inoculated orange peel to achieve different BOZ formulations *viz*. BOZ1 (90:10 ratio; w/w; bioaugmented orange peel: ZnO ratio), BOZ2 (80:20 ratio; w/w; bioaugmented orange peel: ZnO ratio), BOZ3 (70:30; w/w; bioaugmented orange peel: ZnO ratio), and BOZ4 (60:40; w/w; bioaugmented orange peel: ZnO ratio). To achieve chelation of Zn with strain AZ6 and orange peel, the prepared formulations were further stored at 30±1ºC for 3 days. Zn concentration in BOZ products was determined through atomic absorption spectrophotometer (240 FS) after the acid digestion method [27]. The BOZ formulations showed a Zn concentration of 9.8% in BOZ1, 19.7% in BOZ2, 29.6% in BOZ3, and 39.5% in BOZ4. The pH of formulated products BOZ1, BOZ2, BOZ3, and BOZ4 were 5.1, 5.1, 4.9, and 4.8, respectively [16, 17].

Pot Experiment

In the wire house of the Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad, Pakistan, a pot experiment was performed to assess the potential for the combined use of strain AZ6 and organic waste for bio-activation of Zn to improve the physiological characteristics of maize. The soil used in the pot experiment was collected locally from the research farm. The pots were filled with air-dried, ground, sieved (2 mm) soil and analyzed for different physicochemical properties [16]. The treatments including control (without Zn and strain AZ6), ZnO, $ZnSO_4$, BOZ1, BOZ2, BOZ3, BOZ4, and ZSB were applied in pot soil and mixed thoroughly with an upper layer at the time of pot filling. The application rates for BOZ1, BOZ2, BOZ3 and BOZ4 were 6.80, 7.65, 8.74, and 10.20 kg ha^{-1}, respectively. Total Zn in all the BOZ products was maintained up to 4.91 kg ha^{-1}. In the case of ZSB, surface disinfected maize seeds of cultivar Syngenta-NT662 were inoculated with strain AZ6 along with a mixture of sugar, clay, and grounded orange peel as reported by Baig and Verma [28]. The seeds were shaken well until the seeds emerged with a fine coating. The nutrient source of N, phosphorus (P), and potassium (K) were applied at the rate of 175, 160, and 125 kg ha^{-1}, respectively, using Urea, Diammonium phosphate, and Sulfate of Potash, respectively. P and K fertilizer were applied at the time of sowing, whereas, N was applied in two splits i.e., half at the time of sowing and the remaining half after three weeks of sowing. As and where necessary, the plants were irrigated with tap water. After the two weeks of germination, only two comparatively healthy plants were maintained per pot. All the standard agronomic as well as insect and pest management strategies were performed. After two-months of germinations, the data regarding physiological parameters were recorded.

Determination of Gas Exchange Attributes and Carbonic Anhydrase Activity

Infrared gas analyzer model CIRAS-3 (PP System, Amesbury, MA, USA) with PLC 3 universal leaf cuvette was used to determine photosynthetic rate, transpiration rate, stomatal conductance, and vapor pressure deficit. The measurements were made in the morning from a fully expanded second top leaf with similar conditions as described by Mumtaz et al. [29]. The activity of carbonic anhydrase was calculated using the [30] methods. The second top-leaf samples were cut into small pieces (1 cm^2) at a temperature below 25ºC. Leaf fragments of 200 mg were suspended in a 0.2 M hydrochloride solution and incubated for 20 min at 40ºC. After blotting, the pieces were moved into phosphate buffer test tubes (pH 6.8), followed by the addition of alkaline bicarbonate solution and 0.002 percent blue bromothymol indicator. The tubes were incubated at 50ºC for 20 minutes. Titration was performed with a mixture of reactions and 0.05 N HCl using methyl red indicator. The results were expressed as fresh weight s^{-1} in terms of µmol (CO_2) kg^{-1}.

Determination of Leaf Chlorophyll Contents and Electrolyte Leakage

In terms of SPAD value, the leaf chlorophyll contents of the top second completely expanded leaves from each plant were calculated using the chlorophyll meter (SPAD-502, Konica Minolta, Japan). As reported by Lutts et al. [31] electrolyte leakage has been used to test membrane permeability. Samples of leaves were washed three times with distilled water to extract contaminants from the surface debris. By cutting the young leaves, the leaf discs were prepared and placed in a closed vial containing 10 mL of distilled water, incubated for 24 h on a rotary shaker and autoclaved for 20 min at 120ºC. As reported by Lutts et al. [31] the electrolyte leakage was measured.

Growth and Grain Yield Parameters

The data of maize growth, yield, and grain quality of the current pot trial had already published in our previous study [16].

Statistical Analysis

The reported data was subject to one-way variance analysis (ANOVA) using Statistix v. 8.1 computer tools (Analytical Software, USA). The means of treatment is compared at a 5 percent probability level with the least significant difference (LSD) test [32].

Results

Improvement in Gas-Exchange Attributes

The application of various BOZ formulations showed a significant improvement in gas-exchange attributes including photosynthetic rate, transpiration rate, and stomatal conductance. The current study showed the photosynthetic rate in the range of 12.47 to 23.23 µmol CO_2 m^{-2} s^{-1} through achieving the highest photosynthetic rate in BOZ4 and the lowest in control (Table 1). Among applied BOZ formulations, BOZ4 showed the highest increase in photosynthetic rate by 85% over control. This treatment also showed increased photosynthetic rate by 66%, 41%, and 55% as compared to ZnO, $ZnSO_4$, and ZSB, respectively.

Table. 1. Effect of bioaugmented-organic matter coated with zinc oxide (BOZ) formulations on vapor pressure deficit, electrolyte leakage, SPAD value of maize leaves.

Treatments	Photosynthetic rate (µmol CO_2 m^{-2} s^{-1})	Transpiration rate (mmol H_2O m^{-2} s^{-1})	Stomatal conductance (mmol m^{-2} s^{-1})	Vapor pressure deficit (kPa)	Electrolyte leakage (%)	SPAD value
Control	12.47 e	5.41 e	185.3 e	2.93 d	60.33 a	30.20 f
ZnO	14.00 de	6.01 e	190.3 de	3.06 cd	58.00 ab	37.09 e
$ZnSO_4$	16.50 c	8.12 c	212.6 bcd	3.45 bc	52.33 c	41.00 cd
BOZ1	15.08 cd	7.75 cd	203.0 cde	3.41 bc	48.00 d	40.38 cd
BOZ2	17.23 bc	8.94 bc	219.3 abc	3.82 ab	38.67 e	42.41 c
BOZ3	19.40 b	9.64 b	233.0 ab	3.97 a	35.67 f	45.29 b
BOZ4	23.23 a	11.28 a	242.3 a	4.21 a	33.33 f	50.08 a
ZSB	15 cd	6.58 de	197.6 cde	3.10 cd	56.33 b	38.12 de

*Means followed by the same letters are not statistically different at $P<0.05$ according to the least significant difference (LSD) test

The sole application of ZnO, $ZnSO_4$, and ZSB also showed increase in photosynthetic rate by 12%, 32%, and 20%, respectively, over control. The similar treatments increased the stomatal conductance by 3%, 15%, and 7%, respectively, as compared to control (Table 1). The control showed the lowest stomatal conductance of 185.3 mmol m^{-2} s^{-1}. The application of BOZ4 showed a maximum increase in stomatal conductance of 242.3 mmol m^{-2} s^{-1} by 31%, 27%, 23%, and 14% as over control, ZnO, ZSB, and $ZnSO_4$, respectively, and was statistically similar to BOZ3, however, showed highly significant stomatal conductance over control. On an average basis, the application of BOZ4 showed a maximum increase in transpiration rate up to 108%, 88%, 71%, and 39% as compared to control, ZnO, ZSB, and $ZnSO_4$, respectively (Table 1). The sole application of ZnO, $ZnSO_4$, and ZSB also promoted a transpiration rate up to 12%, 32%, and 20%, respectively, as compared to control.

Effect on Electrolyte Leakage and SPAD Value

The application of BOZ formulation resulted in a significant reduction in electrolyte leakage as demonstrated in Table 1. Maximum reduction in electrolyte leakage was observed with the application of BOZ4 (45%) followed by BOZ3, BOZ2, and BOZ1 that showed 41%, 36%, and 21%, respectively, over control. BOZ4 application demonstrated 43%, 36%, and 41% reduction in electrolyte leakage than ZnO, $ZnSO_4$, and ZSB, respectively. The application of BOZ4 resulted in highest increase in SPAD value up to 66%, 35%, 22%, and 31%, respectively, as compared to control, ZnO, ZnSO4, and ZSB, respectively (Table 1). While, the sole application of ZnO, $ZnSO_4$, and ZSB increased the SPAD value up to 23%, 36%, and 26%, respectively, as over control.

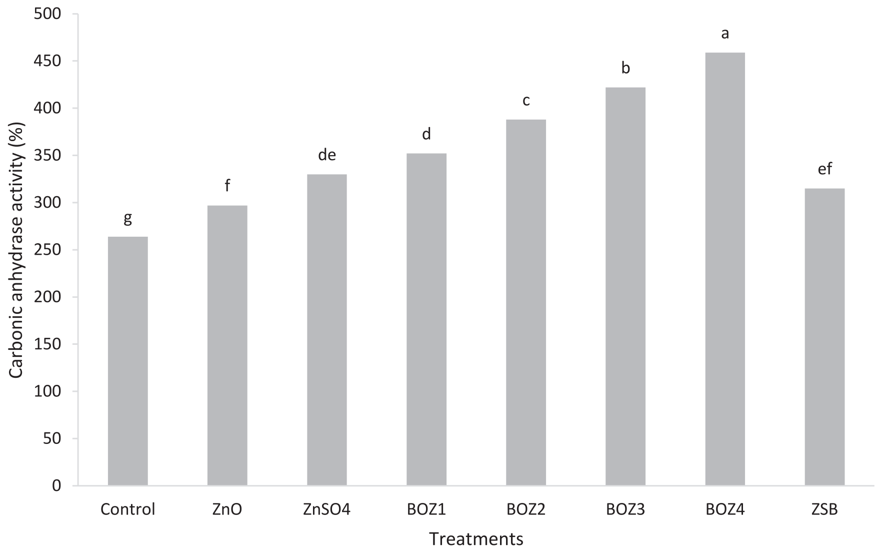

Fig. 1. Effect of bioaugmented-organic matter coated with zinc oxide (BOZ) formulations on carbonic anhydrase activity in maize.

Increase in Carbonic Anhydrase Activity and Vapor Pressure Deficit

A significant increase in carbonic anhydrase activity was observed with the application of BOZ formulations (Fig. 1). Results revealed that the application of BOZ4, BOZ3, BOZ2, BOZ1 resulted in 74%, 60%, 47%, and 33%, respectively, increase in carbonic anhydrase activity as compared to control. Further, the application of BOZ4 showed increase by 55%, 39%, and 46% over ZnO, $ZnSO_4$, and ZSB, respectively. The application of ZnO, $ZnSO_{4,}$ and ZSB also promoted carbonic anhydrase activity up to 13%, 25%, and 19%, respectively, over control. These treatments also showed increase in vapor pressure deficit by 4%, 18%, and 6%, respectively, as compared to control (Table 1). The increase with BOZ formulation was ranked BOZ4>BOZ3>BOZ2>BOZ1 by 44%, 36%, 30%, and 16%, respectively, higher as over control. The increase in vapor pressure deficit due to BOZ4 was also higher than ZnO, $ZnSO_{4,}$ and ZSB by 38%, 22%, and 36%, respectively.

Discussion

Zinc is the most important micronutrient for crop physiology. Its deficiency affects cereals crop physiology as reported by several researchers [33, 34, 35]. Moreover, Zn deficiency in cereals is observed due to the limited availability of Zn in the soil. Mostly, Zn the applied chemical fertilizers become fixed in alkaline soils due to the higher soil pH. An increase in plant available Zn content in soils by the inoculation of different ZSB was reported by Mumtaz et al. [36]. In the current study, four BOZ formulations were prepared by using orange peel augmented with *Bacillus* sp. AZ6 and mixed with bulk ZnO powder and their effectiveness to improve maize physiology was observed as compared to control. The results revealed that the use of biologically activated Zn can significantly increase the physiological attributes of maize as compared to the chemical Zn sources and the inoculation with ZSB strain. Among the biologically activated Zn formulations, BOZ4 showed a better increase in physiological attributes of maize as compared to BOZ1, BOZ2, and BOZ3 formulations. The same products were previously applied in pot and field trial conditions by our research group and reported their effectiveness to improve maize growth, yield, and biofortification [16, 17]. Similarly, Nazir et al. [37] and Nazir et al. [18] also reported an increase in rice and wheat productivity and biofortification through the application of urea coated with bio-activated ZnO formulations. Ain et al. [19] also reported the alleviation of salinity stress in wheat through the application of urea coated with bioaugmented ZnO.

In the current study, strain *Bacillus* sp. AZ6 was used to augment organic matter (orange peel) and to chelate the ZnO. This strain is Zn solubilizing and possesses multiple plant growth-promoting characteristics as reported by Hussain et al. [14]. Zn deficiency in soils results in human deficiency. The ZSB solubilizes the insoluble Zn in the soil, making it available to plants and, finally, to humans. Inoculation of Zn-solubilizing bacteria results in an increase in plant height in a variety of crops. In rice, *Acinetobacter* sp. and *Serratia* sp. inoculation increased plant height and root volume, while *Enterobacter cloacae*, *Rhizobium* sp., and *Pantoea agglomerans* increased wheat shoot and root biomass and Zn content [38, 39]. Plant height, number of branches per plant, stem girth, and total biomass were all increased after inoculation with Zn-solubilizing *Bacillus* sp. Vidyashree et al. [40] reported the chelation of insoluble Zn with ethylenediaminetetraacetic acid produced by *Pseudomonas* sp., *Azospirillum lipoferum*, and *Agrobacterium* sp. that promoted the sustainable Zn availability in rice. The ZSB strains increase root growth in terms of root length, surface area, and root hair that causes increase in nutrient utilization. In the present study, strain AZ6 could endorse plant physiology through producing auxin, ACC-deaminase activity, siderophores production, and solubilization of phosphate and Zn as reported by Hussain et al. [14]. The chelating complexes are synthesized or unconstrained into the rhizosphere by plant roots and potential rhizosphere microflora to chelate Zn and increase its utilization in the plant root zone. Bacterial metabolites form a complex with Zn^{2+} and reduce its reaction with soil. In combination with $ZnSO_4$, Zn-solubilizing bacteria and Arbuscular mycorrhizal (AM) fungi improve Zn transformation, nutrient absorption, yield, and Zn content of stover. The insoluble nutrient in the soil was being solubilized by zinc-solubilizing bacteria. The immobile nutrient in the soil was being mobilized by AM fungi. The plant's growth, yield, dry matter production, and Zn content were all improved by microbial inoculation alone or in combination with Zn fertilizer [41]. Similarly, Othman et al. [38] reported that inoculation with ZSB strains promoted root weight, length, and root volume, and the biofortification of Zn.

In the present study, we attempted to increase the availability of Zn through the application of BOZ4 formulation as reported in our earlier study [16, 17]. The application of BOZ4 formulations significantly promoted the gas exchange characteristic of maize that might be due to bioactivation of ZnO through strain AZ6 and organic matter. The bioactivation of ZnO could be attained through reaction with organic acid and chelating agents produced from bacterial strains and the decomposition of organic acids. ZnO bioactivation dissolves the compounds and increases Zn bioavailability that has a potential role to increase gas exchange attributes and CA activity. In the present study, the application of BOZ4 products significantly promoted CA activity, photosynthesis, transpiration rate, stomatal regulation, and chlorophyll contents. The increase in photosynthetic activity due to the application of BOZ4 formulation might due to the effect of higher CA activity. Moreover, the increase in net photosynthesis may be attributed to an increase in chlorophyll contents that might be true in the current study that application of BOZ4 product promoted Zn uptake [16, 17] that causes an increase in net photosynthesis, CA activity, and chlorophyll contents. Zinc solubilizing bacteria play an important role in plant growth, owing to the direct or indirect mechanisms that involve the production of Indole Acetic Acid (IAA), organic acids, Hydrocyanic acid (HCN), ACC deaminase, siderophores, mineralization of various minerals such as P liquefaction and Zn solubilization, all of which are important for plant development [42, 43]. The increase in gas exchange attributes, CA activity, chlorophyll contents due to increased uptake of Zn was previously supported by Jan et al. [44], Cao et al. [45], Aslam et al. [46], Wang et al. [47] and documented the improvement of wheat's physiological qualities under drought stress through the application of endophytic bacteria containing CA activity. Thus, ZnO bio-activation with ZSB may serve as an effective and cost-effective technique to improve the physiological parameters of maize.

Conclusion

Bio-activated Zn formulations potentially enhanced the physiological attributes of maize over control, $ZnSO_4$, ZnO, and ZSB strain. Among formulated bio-activated Zn products, BOZ4 was a more effective treatment as compared to BOZ3, BOZ2, BOZ1 formulations. The combined use of organic matter supplemented with inexpensive Zn source in terms of ZnO and ZSB strain in the BOZ4 formulation could be a novel approach to enhance Zn availability, organic matter contents, and to enrich the rhizosphere with beneficial rhizobacteria. Simultaneously, it is also a cost-effective and environmentally friendly approach for sustainable crop production.

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Conflict of Interest

The authors declare no conflict of interest.

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