"In the name of ALLAH, the most merciful, the most gracious"

O Lord Open my Eyes
That I can see the glimpsing Blessing sprinkled on us,
And show me the right path,
The path of those who got success here in life

And will be rewarded on

ROZ-E-JAZA
Efficacy of bio-activated Zn for improving yield and quality of maize

By

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Regd. No. 2010-ag-531
M.Sc. (Hons.) Soil Science

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INSTITUTE OF SOIL & ENVIRONMENTAL SCIENCES
FACULTY OF AGRICULTURE, UNIVERSITY OF AGRICULTURE,
FAISALABAD-PAKISTAN
(2015)
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I hereby declare that the contents of the thesis “Efficacy of bio-activated Zn for improving yield and quality of maize” are product of my own research and no part has been copied from any published source (except the references, standard mathematical or genetic models/equations/formulae/protocols, etc. I further declare that this work has not been submitted for award of any diploma/degree. The university may take action if the information provided is found inaccurate at any stage. In case of any default the scholar will be proceeded against as per HEC plagiarism policy.

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TO

MY AFFABLE FATHER (GHULAM HUSSAIN ANJUM)

A symbol of success for me!
Always behaved me like a friend
Whose mature, valuable guidance,
Financial assistance,
Enabled me to perceive and pursue
High ideas in life

MY ADORABLE MOTHER (MAQBOOL ELAHI)

A minerate of love, affection and kindness
Who enlightened me
A learning spirit
I am learning much
From her lap till now

MY BROTHER (ENGINEER TAJAMMAL HUSSAIN)

AND

SISTERS (SAMREEN ANJUM, SUMERA ANJUM)

Whose prayers, sympathies,
Steer my way towards success
Whatever am I
Due to the efforts of all my family
I am for them, they are for me
Made me perfect
For my family
I have a great gratitude and pride
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Finally, I apologize if I have caused annoyance or offence to anybody and the errors that remain in the manuscript are mine alone.

May ALLAH bless all these people with long, happy and peaceful lives (Ameen)

Azhar Hussain
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ABSTRACT

Zinc (Zn) is an important micronutrient and its adequate supply is considered indispensable for growth, development and normal functioning of plants. In plants, it plays a significant role in photosynthesis, DNA replication, cell division, membrane permeability and integrity, protein synthesis and enzymatic activity, specifically in carbonic anhydrase. Zinc is equally important for human diet and its deficiency affects the immune system, disrupts normal cell growth and reproductive system, and causes skin disorders. Being a co-factor for more than 100 enzymes in human beings, it also provides protection against cancer. Strategies must be employed to increase Zn contents of cereal grains to overcome the Zn deficiency in human beings. Recent reports depict that more than 70% of Pakistani soils are categorized as zinc deficient due to Zn deficient parent material, high soil pH, high calcareousness, more salts and waterlogged conditions and the deficiency passes on to crops as well. Decent amount of zinc is believed to be indispensable for growth and development of maize which is one of the important cereal crops of Pakistan. It is crucial to increase bioavailability of Zn in maize. Among the principal sources used for this purpose, ZnSO\(_4\), containing 33% of Zn, is commonly used, but only 4-8 % of the total applied zinc is available to plants while other gets fixed into soil. Furthermore, being expensive, it has economic implications for farmer’s community. Contrarily, zinc oxide (ZnO) is a cheaper and insoluble source which contains 80% of Zn. Bio-activation of insoluble source (ZnO) could be a cost effective method to improve Zn availability from it. Keeping in view the above said problem, the present study was conducted to formulate and evaluate bio-activated zinc for improving yield and quality of maize. For bio-activation purpose, several zinc solubilizing bacteria were isolated from rhizosphere of maize grown soil and quantified on the basis of zinc solubilizing potential and maximum pH reduction in broth medium by the bacterial isolates, finally selecting ten potential zinc solubilizing bacteria. The selected bacterial isolates, capable of solubilizing ZnO, were further screened for their plant growth promoting activity under axenic conditions. Out of ten bacterial isolates, AZ6 was selected for further experiments on the basis of maximum zinc solubilization potential, pH reduction and improved growth of maize seedlings. The selected bacterium was later identified as *Bacillus* sp. AZ6 (Accession No. KT221633), on 16S rRNA gene sequence analysis. The *Bacillus* sp. AZ6 was characterized for its plant growth promoting attributes. The results implied that *Bacillus* sp. AZ6 had 1-aminocyclopropane-1-carboxylate deaminase activity and produced siderophores for the biocontrol purpose. Auxins production was also observed by inoculation of *Bacillus* sp. AZ6 in the presence and absence of L-tryptophan. *Bacillus* sp. AZ6 also has the ability to produce organic acids like cinamic, ferulic, caffeic, chloroggenic , syriric and gallic acids, which were detected on HPLC. These acids solubilized the insoluble source of zinc (ZnO) by lowering pH of broth media. *Bacillus* sp. AZ6 was used with the organic material (grinded orange peel) to bio-activate the insoluble ZnO with different formulations. Efficient formulations (BOZ1, BOZ2, BOZ3, & BOZ4) were evaluated for temporal release of zinc. With the application of bio-activated zinc formulations, zinc bioavailability was increased significantly as compared to available form (ZnSO\(_4\)), on the 60th day of incubation. BOZ4 was the most efficient among all for the whole sampling duration (0, 12\(^{th}\), 24\(^{th}\), 36\(^{th}\), 48\(^{th}\), 60\(^{th}\) and 72th day). Different combinations varied in their potential for enhancing Zn bioavailability in soil and they were further evaluated in pot and field trials. The pot experiment was conducted to evaluate and compare the different formulations of bio-
activated zinc with the ZnSO$_4$ on maize crop. Data were analyzed following completely randomized design. Sole application of insoluble source of zinc (ZnO) did not influence the growth, physiological, yield and quality parameters of maize whereas ZnSO$_4$ improved these parameters significantly. Inoculations with zinc solubilizing bacteria also promoted these parameters in most of the cases as compared to control (without zinc) but results were at par with the control. Application of bio-activated zinc formulations had a significant effect on growth (fresh and dry root and shoot biomass), physiology (photosynthetic rate, transpiration rate, stomatal conductance, chlorophyll contents and carbonic anhydrase activity), yield and quality (crude protein, crude fiber, ash, oil contents, dry matter and grain zinc concentration) of maize as compared to ZnSO$_4$ in pot conditions. Among the bio-activated zinc formulations, application of BOZ4 and BOZ3 significantly promoted most of parameters as compared to available form of zinc (ZnSO$_4$). BOZ4 improved grain yield 11% more as compared to ZnSO$_4$. The results of pot trial were confirmed under field conditions by conducting experiments on maize in two seasons (Field trial I in March, Field trial II in July). The results implied that growth, physiology, yield and quality parameters were significantly improved by BOZ4 and BOZ3 formulations as compared to ZnSO$_4$. Data were analyzed following randomized complete block design for field experiments. BOZ4 improved the grain yield in field trial I and II by 10% and 12% respectively as compared to ZnSO$_4$. The combined use of organic material enriched with zinc source (ZnO) and zinc solubilizing bacteria seems to be an effective approach, yet, cost effective, less time consuming and environmental friendly as compared to other zinc sources. Concluding, bio-activation of ZnO is an effective strategy for economical supply of Zn for improving yield and quality of maize, ultimately the farmer’s community can get the maximum profit from their limited resources in addition to biofortification of crop produce with respect to Zn.
CHAPTER 1

INTRODUCTION

Population of the developing world is increasing day by day with growth rate near to 1.8% (United Nations, 2012). Due to drastic increase in population the demand of food is also very high but the land is same, about 65 % of the developing world is starving (Food Security Statistics, 2008). Inadequate nutrition is common among poor communities; malnutrition is also common due to deficiency of micronutrients in foods such as cereals. Due to dependence on cereals for food, humans are also suffering the deficiency of micronutrients.

Crop nutrition describes the interrelationship of mineral elements in the soil and solution phase and their function in plant development. These interrelationships hold a multifaceted symmetry of important and valuable mineral elements for healthy plant growth. Nutrient imbalance is one of the major causes of low yield of crops in the country. Continuous cropping with high yielding crop varieties, continuous growing of same crop and fewer interest on integrated nutrient management has resulted in reduction of organic matter in the soil leading to deficiencies of micronutrients (Rakkiyappan and Thangavelu, 2000).

The use of micronutrients in soil nutrition is the pillars of agriculture in developed countries. Proper plant nutrition is one of the most important factors in improving the quality and quantity of plants product (Mousavi et al., 2013). Micronutrients application can enhance plants resistance to environmental stresses such as drought and salinity (Cakmak, 2000). To compensate the micronutrients deficiency in soil, supplementing of these nutrients is of supreme importance. A balanced fertilization not only guarantees optimal production of crop but also gives the higher profit to the growers, and is the best option to minimize the risk of nutrient losses to the environment. Plant growth and development is determined by the accessibility of some specific mineral nutrients which are extremely crucial for the completion of plant growth stages (Marshner, 1995). Because of this reason above mentioned problems, application of essential micronutrients to the plants as a chemical fertilizer is compulsory for intensive crop rising.

Zinc (Zn) is an important micronutrient needed by humans, animals as well as crops. Zn is an important component of different enzymes catalyzing many metabolic reactions in
all crops. Growth and development would hamper if specific enzymes were not there in the plant tissue (George and Schmitt, 2002). The essential processes of life in plants are influenced by zinc, such as (a) nitrogen metabolism, i.e., nitrogen and protein uptake quality; (b) photosynthesis, i.e., synthesis of chlorophyll and carbon anhydrase activity resistance against biotic and abiotic stresses, i.e., resistant against oxidative damage (Alloway, 2004). Zinc also plays a significant role in plant resistance against diseases, photosynthesis, cell membrane integrity, protein synthesis, pollen formation (Gurmani et al., 2012) and enhances the level of antioxidant enzymes and chlorophyll within plant tissues (Sbartai et al., 2011). Moreover, Zn is critical as a co-factor for the activity of more than 300 enzymes (Mccall et al., 2000). In addition, Zn is required for the production of phytohormones such as abscisic acid, auxin, gibberellins and cytokinins and its deficiency results in an impairment growth of plant cells. Therefore, Zn deficiency in plants seriously affects various vital processes occurring within plants (Imran et al., 2014).

Due to soil and plant factors Zn deficiency exists in humans. Zinc deficiency is fifth largest cause of death among developing world (WHO, 2002). According to the Ministry of health (2009) every third child and 40 % mothers are zinc deficient. The average Zn requirement for males and females are 12 and 10 mg d$^{-1}$ respectively. Zinc deficiency can be overcome either by genotype selection/genotype improvement in plants or fertilizer management (Bouis and Welch, 2010). Zinc biofortification of cereals is an effective techniques to alleviate deficiency in humans. Zinc biofortification of cereals like rice, wheat and maize through fertilizer is considered the best technique because people of developing world rely on cereals for food and suffering from severe zinc deficiency due to alkaline calcareous soils of arid and semi-arid soils (Alloway, 2008). Zinc contents in the edible parts of the plant can be increased either by increasing the required element or by decreasing its interaction with complexing agents, e.g. phytate in case of zinc. (Frossard et al., 2000). Phytate binds with the zinc and form complexes in the human intestine and hinders its absorption (Brown et al., 2001).

Pakistani soils are generally alkaline and calcareous in nature and are more prone to Zn deficiency, because these soils are intrinsically low in available Zn (Tinker and Lauchli, 1984). In Indo-gagnetic plains, 50-70% agricultural soils are Zn deficient (Alloway, 2008). Moreover, in these soils Zn also precipitates or sorbs and becomes unavailable to plants
(Khoshgoftarmanesh et al., 2004). Zn deficiency also becomes a major problem due to salt stress in arid and semi-arid regions of Pakistan. It is assumed the most common deficient element of alkaline calcareous soils (Rashid and Ryan, 2008). Factors contributing in the unavailability of Zn in Pakistani soils are clayey, alkaline and calcareous nature of soils (Tahir et al., 1991; Kapoor et al., 2002). Zn deficiency also significantly affects the root system including poor development of roots (Fageria, 2004). Usually in rice crop, flooding of soil reduces Zn availability most probably due to dissolution of indigenous phosphorus (Neue and Lantin, 1994), formation of insoluble compounds with manganese, iron, carbonate and sulfide under strictly anaerobic conditions (Alloway, 2004).

Inorganic fertilizers are recommended as good sources of Zn but they are quickly fixed in soil, causing poor availability to plants (Zia et al., 2000). Application of zinc sulfate (ZnSO₄) in the form of fertilizer decreases Zn deficiency and increases plant yield. But ZnSO₄ becomes transformed into different insoluble forms depending on the type of soil and entirely becomes unavailable within seven days of application (Rattan and Shukla, 1991). In calcareous soils, up to 90% of applied Zn fertilizer is adsorbed on soil colloids and precipitated (Saeed and Fox, 1977).

There are two common sources of Zn, which are ZnO and ZnSO₄. The most commonly used source is ZnSO₄. Zinc contents in ZnSO₄ and ZnO are 33 and 80% respectively. Zinc in ZnSO₄ is in soluble form and easily available to plants but is sparingly soluble in ZnO (Slaton et al., 2005). ZnSO₄ is an expensive source of Zn and more than 90% of it is quickly fixed in soil after application (Saeed and Fox, 1977). In this way the farmers get economic loss instead of benefit. On the other hand, ZnO is insoluble source of zinc containing 80% Zn and is a cheaper source than ZnSO₄. It would be effective if we bio-activated this insoluble source through bacteria and organic amendments.

There are many techniques through which we can enhance the fertilizer use efficiency of ZnO, because it has more amount of Zn as compared to ZnSO₄. By using nano technology, coating technique and zinc solubilizing bacteria the solubility of ZnO can be increased (Bremner and Douglas, 1971). The coating of zinc solubilizing bacteria on ZnO is called bio-activation of ZnO. A number of scientists tried to solubilize indigenous zinc of soil; some of them apply zinc solubilizing bacteria with soluble zinc source or inoculated with organic amendments. But no work has been done to solubilize insoluble zinc source with the organic
amendment. This will help to solubilize indigenous zinc in soil as well. So, it is crucial to increase bioavailability of Zn to plants by solubilizing fixed Zn and/or by reducing fixation of the applied Zn fertilizers (Imran et al., 2014). This can be achieved either by using organic amendments or potential Zn solubilizing bioinoculants alone or in combination (consortium).

Generally plant growth promoting rhizobacteria solubilize the nutrients through acidification, release of organic acids, chelation and by exchange reactions (Chang et al., 2005). Reduction in pH and availability of micronutrients in soil is very much sensitive to soil. A little change in soil pH may have a great impact on micronutrient mobility/solubility in soil. It has been reported that availability of Zn decreases 100 times with one unit increase in pH (Havlin et al., 2005). Thus by decreasing the pH of alkaline soil, bioavailable fraction of Zn can be enhanced to an appreciable level. Rhizosphere microflora has been reported to lower the soil pH to a good extent (Wu et al., 2006), which may occur due to secretion of some organic acids and protons extrusion (Fasim et al., 2002). For instance, *Pseudomonas fluorescens* secreted gluconic acid and 2-ketogluconic acid in culture medium during solubilization of Zn phosphate.

Since these microbes play a key role in improving food quality, thus they would be given key importance in future while devising approaches to alleviate Zn malnutrition in humans through food, especially where diverse food is not available to common people and they cannot afford food supplements. Among microbes, both bacteria and fungi have shown terrific ability to improve plant Zn availability in the rhizosphere and also enhance Zn in plant parts (Subramanian et al., 2009).

Bacteria have also shown high mobilization of soil Zn (Tariq et al., 2007). *Glomus diazotrophicus* PA15 has Zn solubilizing potential (Saravanan et al., 2007). Similarly *Pseudomonas aeruginosa* has a potential to solubilize ZnO in liquid medium (Fasim et al., 2002). Bacterial inoculation has also ability to increase bioavailable Zn in rhizosphere soil (Whiting et al., 2001) and improves plant Zn content (Whiting et al., 2001; Biari et al., 2008).

Plant growth promoting rhizobacteria produced siderophores (Saravanan et al., 2011), gluconate, or the derivatives of gluconic acids, e.g., 2-ketogluconic acid (Fasim et al., 2002), 5-ketogluconic acid (Saravanan et al., 2007), and various other organic acids (Tariq et al., 2007) for the mobilization of Zn and iron. These bacteria can be used to solubilize
insoluble sources of Zn such as ZnO and ZnCO$_3$ because most of the soils are rich in Zn contents but less in soluble Zn. *Bacillus* and *Pseudomonas* spp. have much potential to solubilize these sources in soil system for taking economically efficient Zn (Saravanan, 2003). The rhizosphere microorganism may benefit plants through different mechanisms including mobilization of nutrients, fixation of N$_2$, production of phytohormones, enhancing plant stress tolerance against salinity, drought, toxicity of metals, and pesticide burden and also acts as a biocontrol agent (Khalid *et al*., 2009). PGPR have achieved worldwide fame for their agricultural benefits. These are the potential trend for the future research as well as tools for sustainable agriculture (Podile and Kishore, 2006). Their mechanisms of action includes nitrogen fixation, mineralization and solubilization of nutrients, production of growth substances e.g. phyto-hormones, release of ACC-deaminase to lower the elevated ethylene levels in plant roots. PGPR are also helpful in increasing root length and density, production of siderophores like β-1-3-glucanase, fluorescent pigments, chitinases, antibiotics and cyanide against production of water soluble vitamins riboflavin, niacin, biotin, pantothenic acid and thiamine resulting in the enhanced resistance to oxidative stresses and drought. (Lugtenberg and Kamilova, 2009; Vessey, 2003; Marulanda *et al*., 2010).

Soil organic matter is considered a very crucial factor of nutrient mobility in soil. Various organic amendments such as compost, farmyard manure (FYM), poultry manure, olive husk etc., are applied to soil to improve soil health, fertility and crop yields (Imran *et al*., 2014). Moreover, application of organic amendments improves biological properties of soil. These properties may increase soluble fraction of Zn in soil for plant uptake (Tejada *et al*., 2006). For instance, microbial biomass and soil enzyme activities are substantially increased with the application of organic amendments (Liang *et al*., 2003). Organic matter along the ZnO provides carbon source to the zinc solubilizing bacteria. This increase in microbial population and activities is considered as an important indicator of soil health and soil productivity.

In most agricultural countries of the world many plant species are affected by zinc deficiency. Zinc deficiency is common in the major staple cereal crops: rice, wheat and maize. In many countries maize receives the highest proportion of applications of zinc fertilizer and it is the crop species which is most susceptible to zinc deficiency (Alloway,
Maize is an important cereal crop of the world and has great value in livestock and poultry production. (Harris et al., 2007).

For the evaluation of bio-activated Zn maize has been selected because it is an important cereal crop of the world known as king of the crops. With huge potential to feed a growing human population. Currently, it is a staple food in sub-Saharan Africa, some parts of Asia, and Latin America. In south and south-east Asia, maize is mostly used as animal feed. It provides raw material for number of industries. Among those corn starches, corn oil, dextrose, corn syrup, corn flakes, cosmetics, wax and alcohol producing industries are of prime importance. It is also grown for its vegetative portion to feed the dairy and other animals. Largest producers of maize are United States, China, Brazil, Mexico, Argentina, Indonesia, France and South Africa. And maize has short growing season as compared to other cereals (Broudly et al., 2007).

Maize (Zea mays L.) is cross pollinated crop belonging to family Poaceae and tribe Maydeae. Maize grain is a rich source of many important nutrients. Maize flour contains 9.6% moisture contents, 70.4% carbohydrate, 10.7% crude protein, 3-18% oil, 2.2% crude fiber, 1.7% ash and 5.4% ether extracts and several important vitamins and minerals (Bressani et al., 1990). Maize ranks third largest cereal crop after wheat and rice on hectare basis in Pakistan.

In Pakistan, maize occupied one thousand hectares with grain yield of 4,527 thousand tons in 2013-14 (Pakistan Economic Survey, 2013-14) which is very low as compared to other maize growing regions of the world especially in the developed countries.

Keeping in view the above comprehensive facts the present study is planned with the objectives below.

- Isolation, screening, characterization and identification of zinc solubilizing bacteria
- Formulation of bio-activated Zn (BOZ) and assessment of the temporal release of Zn in soil
- Evaluation of bio-activated Zn (BOZ) for improving yield and quality of maize under pot and field conditions
CHAPTER 2

REVIEW OF LITERATURE

Zn is a significant part of enzymes that motivate and raise the rate of metabolic reactions involved in development and growth of crop. Zinc deficiency is a common issue not only in human beings but in plants and animals as well. One-third of world’s population is affected by zinc deficiency. Its deficiency is a global problem for plants and can be found in every part of the world. More than 70% of Pakistani soils are zinc deficient. So, the cereals crops grown on these soils are zinc deficient. PGPR with organic material may also increase the availability of native and applied zinc to the plants. These PGPR alone and also in combination with organic matter and ZnO, promote plant growth by increasing zinc bioavailability in soil along with some other mechanisms. In this chapter, importance of zinc with a special reference to zinc solubilizing been reviewed. Particularly, mechanisms used by PGPR containing zinc solubilizing ability to improve plant growth have been reviewed.

2.1. Zinc as an essential nutrient for plants

Zn is an essential micronutrient for plants was recognized for the first time by Sommer and Lipman (1926). In developed countries the use of essential micronutrients in soil considered as pillars of agriculture. Plant nutrition is an important factor for enhancing over all plant growth and plants products. Zn is applied in minute concentration but small concentrations to allow plants physiological pathways to function properly (Mousavi et al., 2011).

Zn is a vital nutrient for normal growth and development of plants and animals. In plants it is required for optimum fruit size, crop production and yield, it also used in the carbonic anhydrase activity which is part of all photosynthetic tissues, and important for biosynthesis of chlorophyll (Xi-Wen et al., 2011). Generally, Zn has key role in the activation of enzymes, synthesis of protein, recovery reactions, oxidation and metabolism of carbohydrates. By utilization of micronutrients and zinc contained fertilizer, crops quality is improving and with lack of these elements due to decline in photosynthesis process, RNA,
carbohydrates and protein synthesis is reduced ultimately quality of crops and performance will be limited (Efe and Yarpuz, 2013).

Maize is an important cereal crop. Its yield is two times high than other cereal crops (Tollenar and Lee, 2002). On the other hand, the quantities of nutrients are almost same (Benton Jones, 2003). As it is well documented that zinc exerts a great influence in plant physiology for example photosynthesis, chlorophyll synthesis, nitrogen metabolism, protein synthesis, resistance to stresses protection from oxidative stress. (Cakmak, 2008). Maize was recognized by farmers for a long time as a crop of high response to zinc supply. In arid and semi-arid areas zinc application to crops is very important because maize growth is considered highly sensitive to many external and internal stresses, which in turn induce grain yield reduction (Leach and Hameleers, 2001; Subedi and Ma, 2009). The foliar application of zinc is a simple way for making quick correction of plant nutritional status, as reported for wheat (Erenoglu et al., 2002) and maize (Grzebisz et al., 2008). It is very clear that the proper application of zinc to maize is necessary to boost up the growth and yield of maize.

Plants uptake Zn as free divalent cation (Zn$^{2+}$), but it may be absorbed as monovalent cation (ZnOH$^+$) at higher pH (Marschner, 1995). In root Zn taken up through plasma membrane is carried mediatory by secondary active transport. Primary uptake system in plants is by metal carriers of ZIP (Zinc Iron Permeases) family, but channel proteins also exist. However, it is not clear yet to what degree specific membrane channels and specific carriers are involved in Zn passage into root cells (Lee et al. 2010).

Zn plays a role in physiological processes of plant growth and metabolism e.g., enzyme activation, protein synthesis, carbohydrates metabolism, lipids, auxins and nucleic acids production, gene expression and regulation and reproductive development (pollen formation) (Chang et al., 2005). It is also essential for the activity of a number of plant proteins (Broadley et al., 2007) primarily because of its role in their maintenance (Christianson 1991). Fox and Guerinot (1998) stated that Zn is vital for functioning of more than 300 enzymes. It is a structural part of carbonic anhydrase, alcohol dehydrogenase, RNA polymerase and assists as a cofactor for all six enzyme classes (oxidoreductases, transferases, hydrolases, lysases, isomerase and ligases). Zn metallo-enzyme molecules are linked with DNA and RNA synthesis (Broadley et al., 2007).
Zn is directly involved in auxin synthesis in plants (Skoog 1940); so its lack leads to leaf distortion and a restriction of internodes (Irshad et al., 2004). It is also involved in sustaining the structural and functional integrity of biological membranes (Sadeghzadeh and Rengel 2011). As Zn is an integral part of Cu/Zn-superoxide dismutase (SOD), it is involved in detoxifying reactive oxygen species (Cakmak and Marschner, 1998) and avoiding damage to membrane lipids and sulphhydryl groups in Zn-scarce plants (Cakmak, 2000b). It is important to observe the impairment of membranes caused by Zn deficiency which cannot be inverted contrasting that caused by calcium (Ca) deficiency (Welch et al., 1982).

Auxins are known to play a basic role in cell division and elongation (Teale et al., 2006). The most distinct Zn deficiency indications are stunted growth and small leaves (Irshad et al., 2004), which are probably due to variations in auxin metabolism, particularly of IAA (Alloway, 2003). Brown et al. (1993) reported that addition of zinc in calcareous field of plants significantly increased tryptophan concentration which is a precursor for the biosynthesis of IAA in rice grains. Moreover, Zn deficient plants had reduced pollen production, leading to an increased fraction of empty grain positions (Marschner, 1995). Zinc is also a central part of transcription factors which are important for cell proliferation and differentiation (Vallee and Falchuk, 1993). It can be recognized by reduced enzyme activities (antioxidants) and high oxidative stress damage in chloroplasts due to obstruction of energy spillover from PS-II to photosystem-I (PS-I) (Chen et al., 2009). Such destruction to photosynthetic centers, decreased leaf photosynthetic capacity due to a decreased number of PS-II units per unit leaf area, making them susceptible to photo destruction (Chen et al., 2008). A decrease in CO₂ assimilation is mainly due to ROS-induced damage to the photosynthetic apparatus and a decline in Rubisco activity (Sasaki et al., 1998) in Zn-deficient plants. Nevertheless, accumulation of saccharides in leaves (Cakmak, 2000b) due to a decline in CO₂ concentration and stomatal conductance may be a credible reason for decreased photosynthetic rate under Zn scarcity (Marschner, 1995).

Zinc metabolic functions are centered on its great affinity to form N-O- and S-donor ligands. The predominant Zn forms are free ions, storage metallo-proteins, insoluble cell wall structures and low molecular weight complexes. Zn is deactivated throughout complexes with organic ligands or phosphorous complexes at an intracellular level. As zinc is required for the synthesis of tryptophan (Alloway, 2004) and tryptophan is a pioneer of Indole-3-
acetic acid, it also has a dynamic role in the auxin production which is a crucial growth hormone (Brennan, 2005). The contribution was further described in transduction of signals through mitogen-activated protein kinases (Hänisch and Mendel 2009).

The contact of Zn with membrane proteins of sulphydryl groups and phospholipids further adds for the protection of membranes. Being a prosthetic component of enzymes within cells i.e., isomerases, dehydrogenases, transphosphorylases, aldolases, RNA and DNA polymerases (Lo pez-Millan et al., 2005), Zn is involved in the production of energy and synthesis of protein (Hänisch and Mendel, 2009). It is also taking part in synthesis of nucleic acid, lipid and carbohydrate metabolisms (Marschner 1995) and it forms RNA and DNA complexes, convincing their stability (Coleman, 1992).

Zn is involved in photosynthetic apparatus, including enzyme kinetics and proteolytic activities such as the repair of PS-II (Hänisch and Mendel, 2009). Zn is also involved in the opening of stomata, probably as a constituent of CA (Carbonic anhydrase) to maintain appropriate HCO_3^- supply in the guard cells (Sharma et al., 1995), while similarly in these cells Zn regulates the influx of potassium ion (Brennan, 2005).

Zinc involves in the photosynthetic metabolism and in the regulation of the biochemical reactions which integrate the structure of Rubisco (Alloway, 2004). In the thylakoid lamellae (cellular membranes), some ligands e.g., histidine and cysteine bind with zinc with great tendency and more stability than to iron (Berg and Shi, 1996; Brennan 2005). It is evident that zinc is involved in oxidative stress-induced appearance of genes encoding antioxidative enzymes defense, for example, glutathione reductase and H_2O_2 scavenging ascorbate peroxidase (Cakmak, 2000a).

2.2. Zinc as essential nutrient for humans

Zinc deficiency is common in humans, animals and plants. More than 30% world’s population suffers from severe Zn deficiency (Welch, 2002). Zinc plays a basic role in cellular functions of all living organisms and is also improving the immune system of human. The optimal dietary consumption for human adults is 15 mg Zn/day. Zinc has catalytic and also structural component of several body enzymes. Deficiency of Zn may cause by unsatisfactory consumption and inappropriate absorption of Zn in the body. The human body suffers from hair and memory loss, skin complications and weakness in body muscles due to
Zn deficiency. During Pregnancy, insufficient Zn intake also causes stunted brain development of the fetus. Infertility has also been perceived in Zn deficient men. Zn deficiency causes congenital diseases like Acrodermatitis enteropathica (Zimmermann, 2001).

Zn has no store house in human body (Johnson et al., 1993). As it is a basic component of hundreds of proteins which are zinc binding and nucleic acid, zinc is located almost uniformly throughout the human body but it is not measurable; therefore, now-a-days it is a big challenge in the detection and diagnosis of zinc deficiency in human body by estimating zinc concentration in serum and other tissues (Hambridge and Krebs, 2007). It is a common recommendation as an average male needs 11 mg Zn per day while an average female needs 9 mg of Zn daily. During pregnancy and lactation, the female needs 13 to 14 mg of Zn on daily basis. Newborns from 7 months to 3 years need 3 mg, 4 to 8 years need 5 mg and children from 9 to 13 years need 8 mg of Zn/day (Hotz and Brown, 2004). Zn is stored in the rice husks and grains and with the intake of this cereal zinc deficiency in human can be ameliorated. Zn-rich foods are beef, pork, chicken, and breakfast cereals, nuts like roasted peanuts, almonds, walnuts, oats and dairy products like yogurt, cheese and milk (Cakmak, 2002b).

2.3. Zinc as essential nutrient for animals

In animal production the significance of trace minerals have a great concern for producers, veterinarians, feed manufactures and food scientists. Sufficient Zn absorption and uptake is required for many metabolic functions as immunity response to pathogen, reproduction and growth. Mineral including zinc supplementation become complex and costly because modifications in trace mineral range of all avian species and livestock is perilous to get optimal production in current system of animal production. (Wikse, 1992). In immune system functions of Zn are protein synthesis, energy production, antibody production, antioxidant enzyme production, stabilization of membranes against bacterial endotoxins and maintenance of lymphocyte replication (Kidd, 1996). Inadequate intake of Zn results in lowered cellular immunity, disrupted growth of T-dependent tissue and decreased antibody response (Fletcher et al., 1988).
Zn mineral supplementation for cattle boosted recovery rate in infectious bovine rhinotracheitis usually in virus-stressed cattle (Chirase et al., 1991). It is founded that zinc methionine increases antibody titer against bovine herpesvirus-1 (Spears et al., 1991). During lactation Zn Supplementation to dairy cows resulted in fewer mammary gland infections (Spain et al., 1993). Cattle reproductive system may be helpful if copper, manganese or zinc is in the marginal to deficient range. In cattle the common deficiency symptoms are infertility, decreased conception, embryo death, and delayed or suppressed estrus (Corah and Ives, 1991). Insufficient Zn concentration have been related with abnormal estrus, reduced fertility and altered myometrial contractibility with prolonged labor and abortion (Maas, 1987). In vision of the role of all minerals (micronutrients) in growth, zinc function as a component in several enzyme systems related with protein and carbohydrate metabolism.

2.4. Fate of zinc in soil

The zinc applied to the soil is either used by the microorganisms and plants or becomes fixed on the soil collides (Alloway, 2008). When the amount of Zn to plants is not sufficient, then quality of production and crop yields will be badly affected. Therefore certain minimum level of Zn supply is necessary for proper function of crops. Zn deficiency is a ubiquitous problem of plants (Hotz and Brown, 2004). Deficit zinc has been identified in several parts of the world (Cakmak, 2002). About 30% of the world’s soils are zinc deficient (Alloway, 2004). When Zn is applied to the soil, it is either precipitated or adsorbed to the soil matrix. A number of factors including soil texture, pH, soil water content, organic matter and calcareousness of the soil are known to influence solubility and bioavailability of Zn in soil (Imran et al., 2014). In India more than 50% of the agricultural soils are Zn deficient. Any approach to improve the zinc uptake and its transport to grains has important practical consequence. (Kabata-Pendias, 2000). Zn is a nutrient which is required by the plants in small quantity because plants absorb Zn from soil solution, adequate levels of dissolved Zn are needed for the optimal growth of crops (Reed and Martens, 1996).

The availability of Zn in soil is mostly measured by the process of adsorption-desorption and it’s partitioning between the solid phase and solution (Gaudalix and Pardo, 1995; Catlett et al., 2002). Zhao and Selim (2010) found that sorption was less in acidic soils compared to neutral soil. Only 9-11% of sorbed Zn was unconfined in neutral soils over time.
while it was 42-51% in acidic soils. Clayey soils had shown more retention of Zn on their adsorptive sites than sandy soils. Yoo and James (2002) also found a decrease in water soluble Zn and increase in (CEC) cation exchange capacity of soil with an increase in soil pH from 4.0 to 7.0. An increase in pH results in more retention by soil colloids due to increase in cation exchange capacity (adsorptive capacity), chemosorption on CaCO$_3$ and iron oxides. Water content in soil is very important factor, which determines the availability of Zn to plants (Patnaik et al., 2008). Usually, flooding of soil reduces Zn availability most probably due to dissolution of indigenous P (Neue and Lantin, 1994), formation of insoluble compounds with manganese, iron, carbonate and sulfide under strictly anaerobic conditions (Alloway, 2004).

According to Mandal and Hazra (1997), a high concentration of Fe$^{2+}$ under submerged conditions contributed to reduced Zn availability in soils. Adsorption isotherms can be used to determine the equilibrium relationship between the amounts of dissolved and adsorbed species at a given temperature. A sorption isotherm measures the factors of quantity, intensity and capacity which are important for calculating the amount of soil nutrient essential for maximum plant growth. Texture, clay minerals, organic matter, CEC, CaCO$_3$ and the properties which need not to be measured in order to determine soil requirements using sorption technique affect the amount of nutrients required by soil (Solis and Torrent, 1989).

Soil organic matter increases solubility of Zn and reduces fixation, which results in its more uptake by plant roots (Marschner, 1993; Obrador et al., 2003; Cakmak, 2009). According to Hodgson (1963), the production of complexing agent from organic matter is responsible for enhanced Zn solubility and extractability. Katyal and Sharma (1991) founded a significant relation between organic carbon and diethylenetriamine penta acetic acid extractable Zn. Similarly, Sidhu and Sharma (2010) observed an increase in DTPA extractable Zn with soil organic carbon.

A global study to evaluate nutrient status of 30 different countries was conducted by Sillanpaa (1982). They collected a total of 3538 soil and plant samples for nutrient analysis. Pakistan, Turkey, Iraq, Syria, India and Lebanon were the countries where soil Zn status was the lowest. Rafique et al. (2006) showed a nutrient indexing of farmer grown rain-fed wheat in 1.82 Million hectare of Potohar plateau. In more than 80% of the sampled fields the crop
was zinc deficient with good relationship between surface soil ammonium bicarbonate diethylene triamine penta-acetic acid extractable Zn content and plant Zn concentration. Keeping in view causes of poor Zn availability, there is a dire need to review strategies which could be effective in enhancing availability of Zn to plants.

2.5. Factor effecting zinc availability

Zn deficiency is an abundant problem of plants (Hotz and Brown, 2004). It can be found anywhere in world and almost all crops respond positively to Zn application (Welch, 2002). Soils inherit their trace elements, including Zn, primarily from rocks through geochemical and pedochemical weathering processes. In addition to mineralogical composition of the parent material, Zn level in the soil during soil formation is also dependent on climate, type and intensity of weathering and various other predominating factors. (Saeed and Fox, 1977).

After application, Zn is precipitated or adsorbed to the soil components. The factors that influence bioavailability of Zn in soil including soil texture, pH, soil water content, organic matter and calcareousness of the soils (Alloway, 2008). High pH results in more retention by soil colloids due to increase in cation exchange capacity (adsorptive capacity), chemosorption on CaCO3 and iron oxides (Yoo and James, 2002). Zhao and Selim (2010) found that sorption was less in acidic soils as in neutral soil by comparing the sorption and desorption capacity of acidic and neutral soils. Only 9-11% of sorbed Zn was unconfined in neutral soils over time while it was 42-51% in acidic soils. Clayey soils had shown more retention of Zn on their adsorptive sites than sandy soils. A negative link between DTPA extractable Zn and clay content of the soil was found by Sidhu and Sharma in 2010.

Water content of soil is very important factor, which defines the availability of Zn to plants (Patnaik et al., 2008). The decrease in available Zn observed in flooding and submergence because of the changes in pH value and the formation of insoluble Zn compounds. Temporarily, the insoluble Zn compounds formed with Mn and Fe hydroxides from the adsorption on carbonates and breakdown of oxides, specifically magnesium carbonate. In submerged conditions for rice cultivation, Zn is transformed into amorphous sesquioxide precipitates or franklinite; ZnFe2O4 (Sajwan, 1988). Solubility of Zn increases
and reduces fixation by soil organic matter, which results in its more uptake by plant roots (Cakmak, 2009).


2.6. Zn uptake and translocation in plant tissues

Zn uptake is measured by the concentration and composition of the growing media, although it varies among plant species. Zn uptake simply arises as inorganic divalent cation or as organic complexes, inclines to show a direct pattern with its nutrient solution concentration (Kabata-Pendias and Pendias, 2001), and the roots loads the shoots tissues through xylem. Zinc translocation via xylem follows symplast and apoplast to roots (Broadley et al., 2007), but in the phloem higher level of Zn have also been identified, denoting that this metal is translocated through both phloem tissues and xylem (Haslett et al., 2001). Under anaerobic conditions, the concentration of zinc absorption decreases in barley roots, due to declining temperatures and uncoupled metabolism. Zinc metal intake occurs by plant roots through an active transport (Schmidt et al., 1965).

Bowen (1969) evaluated that Zn absorption in the sugarcane leaves is disturbed by the oxidative phosphorylation inhibition, while Bowen et al. (1974) explained that low temperature inhibits zinc absorption in the roots of Pinus radiate. Likewise, as Zn\textsuperscript{2+} uptake does not respond to metabolic inhibitors, it has also been determined that it is metabolically-independent process. (Kochian, 1993).

Separately Zn\textsuperscript{2+} kinetics of uptake, the process regulating its movement in the root and other cell membranes appears to be controlled metabolically. Electrochemical gradient fixated the process by carrier proteins, ion channels or against the electrochemical gradient through electrogenic pumps (Weiss et al., 2004). Hille (2001) described Zn\textsuperscript{2+} transport across membrane is mainly dominated by electrogenic pumps and ion channels, in place of carrier-mediated transport. Under this background, these pore forming proteins ion channels can develop a voltage gradient and maintain its voltage across the plasma membrane of cells,
allowing passage of ions down to their electrochemical gradient. Certain channels might also let the flow of ions based merely on their negative charges or positive charge, while grouping of ion channels order the passage through the pore and can close or open with the help of electrical or chemical temperature and signals. Moreover, Non-Selective Cation Channels have the capability to speed up passive transport, through membranes of plants (Demidchik et al., 2002).

In plant species to evaluate the Zn\textsuperscript{2+} mechanism uptake and translocation, rapid development has been attained by consideration of the molecular mechanisms of metal transport through cell membranes in the last 10-15 years. Many gene families responsible for the transport of heavy metal in plants have been recognized through the application of molecular and genetic techniques (Hall and Williams, 2003). The genes of ZIP1-4 family (Zn regulated IRT/ZRT-like transporter proteins), have been reported to be down regulated or uninfluenced by fertilization of Zn (Burleigh et al., 2003). CDF is a family of plant transporter gene was first time characterized in designated ZAT and Arabidopsis (Van der zaal et al., 1999). MtZIP2 gene encoding plasma membrane localized zinc transporter was replicated from Medicago truncatula (Burleigh et al., 2003). The sole features of MtZIP2 are being regulated in roots by proper fertilization of Zn contrasting the other Zn transporters of plant. Hussain et al. (2004) delivered that HMA4 and HMA2 Arabidopsis transporters from subfamily P-type ATPases also play a key role in homeostasis and transport of Zn in plants.

### 2.7. Exogenous application of zinc

Fertilizer approach can be a swift solution to the problematic soils and considered as a significant balancing technique to the on-going breeding programs. The basic aim of studies related to fertilizers specifically on enhancing zinc concentration in grain (or other edible parts) but it is very strange, on the other hand, a large number of studies are available on the role of soil and foliarly applied zinc fertilizers in correction of Zn deficiency and increasing plant growth and yield (Rengel et al., 1995).

#### 2.7.1. Soil & foliar application of zinc fertilizers

Zn is applied directly to soil as both organic and inorganic compounds. Most commonly zinc sulfate (ZnSO\textsubscript{4}) is applied as inorganic source of Zn because it is highly
soluble and cost effective. Zinc can also be applied to soils in the form of ZnEDTA, ZnO and Zn-oxysulfate. The efficiency of agronomic practices (i.e., level of the crop response per unit used micronutrient) of zinc fertilizers is less with inorganic Zn fertilizers as compared to the ZnEDTA (Martens and Westermann, 1991). But in cereal farming use of ZnEDTA is limited due to its high cost. In Turkey convincing results have been obtained in a field trials, about the importance of zinc fertilizer to enhance Zn concentration in wheat grain through agronomic bio-fortification. Wheat grown in field in Central Anatolia with zinc fertilizer improved Zn concentration in grains and productivity (Yilmaz et al., 1997).

The application of Zn fertilizers improves Zn contents in grains up to four folds depending on the application method. Combined application of soil and foliar application were the most useful method for grain Zn concentration that resulted in an increase of about 3.5-fold Zn concentration in the grain. When a high grain Zn concentration is targeted with high yield of grains, combined soil and foliar application is recommended. On the other hand, by using fertilizers with seeds at sowing combined with Zn foliar application is also an active approach to enhance both grains Zn concentration and grain yield (Table 2.1).

Zn availability increases with decreasing soil pH, because the solubility of minerals reduced and soil colloidal particles (clay minerals, calcium carbonate, organic matter and iron and aluminum oxides) increases Zn uptake. Zn application is necessary for the root development. Zinc utilization by plants increases by decreasing levels of soil phosphorus. Certain metal cations like Fe$^{2+}$ and Cu$^{2+}$ (due to the similar transporters for these metals in the plant roots) inhibit the Zn uptake by plants. Zn fertilizers are used with the help of three types of compounds and these vary significantly in the Zn content, efficiency and price for crops on different types of soils.
Table 2.1. Effect of different Zn application methods on Zn concentration in whole shoots and grain, and the increases in shoot biomass and grain yield by Zn applications

<table>
<thead>
<tr>
<th>Zn application methods(^a)</th>
<th>Zn concentration</th>
<th>Increases in yield by Zn application</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole shoot ((mg \ kg^{-1}))</td>
<td>Grain</td>
</tr>
<tr>
<td>1 – Control</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2 – Soil</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>3 – Seed</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>4 – Foliar</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>5 – Soil+foliar</td>
<td>69</td>
<td>35</td>
</tr>
<tr>
<td>6 – Seed+foliar</td>
<td>73</td>
<td>29</td>
</tr>
</tbody>
</table>
The major zinc sources are inorganic Zn compounds, synthetic Zn-chelates and organic Zn compounds. The inorganic compounds include zinc sulphate (ZnSO₄), zinc carbonate (ZnCO₃), zinc oxide (ZnO), zinc chloride (ZnCl₂) and zinc nitrate (Zn(NO₃)₂). The most common zinc source used around the world is ZnSO₄ and available in both the heptahydrate and crystalline monohydrate form. The synthetic Zn-chelates are complex generally produced by reacting a metallic ion with a chelating agent such as EDTA (Ethylene Diamine Tetra- acetic Acid) and the stability of the chelate-metal complex form regulates the availability of the metal to plants. C- Organic compounds (natural) are those complexes which are manufactured by combining Zn salts with organic products or by-products from paper pulp manufacturing such as lingo sulphonates, phenols and polyflavonoids or with citrates. Usually synthetic chelates like Zn-EDTA are more costly as compared to natural organic complexes, but they are comparatively very effective. The chief fertilizer sources of zinc are shown in Table 2.2 (Alloway, 2008).

The effective source of Zn is ZnSO₄ in improving Zn in grains as compared to ZnO and ZnEDTA (Cakmak, 2008). Better potential of zinc fertilizer for quick improvement of Zn concentrations in grain, mainly when late foliar Zn applied. It is recognized that foliar Zn uptake is encouraged by mixing Zn fertilizer with urea (Mortvedt and Gilkes 1993). Urea fertilizers containing Zn (e.g., zinc coated urea) may characterize a better foliar Nitrogen fertilizer to increase both protein and grain Zn concentrations.

There are different ways for mixing Zn into NPK fertilizers. Zinc can be added to a compound fertilizer either during manufacturing by combining it into granules, or by bulk mixing Zn fertilizers with granular NPK fertilizer or by coating of Zn onto granular compound fertilizer (Mortvedt, 1991). There is little information in the literature regarding the effectiveness of various Zn-supplemented compound fertilizers and the type of the Zn-supplementation manner e.g. coating, incorporation or bulk-blending, on Zn uptake and accumulation in plants.
Table 2.2. The main sources of zinc fertilizers (Alloway, 2008).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Zinc Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inorganic Compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zinc sulphate monohydrate</td>
<td>ZnSO$_4$.H$_2$O</td>
<td>36</td>
</tr>
<tr>
<td>Zinc sulphate heptahydrate</td>
<td>ZnSO$_4$.7H$_2$O</td>
<td>22</td>
</tr>
<tr>
<td>Zinc oxysulphate</td>
<td>xZnSO$_4$.xZnO</td>
<td>20-50</td>
</tr>
<tr>
<td>Basic zinc sulphate</td>
<td>ZnSO$_4$.4Zn(OH)$_2$</td>
<td>55</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>ZnO</td>
<td>50-80</td>
</tr>
<tr>
<td>Zinc carbonate</td>
<td>ZnCO$_3$</td>
<td>50-56</td>
</tr>
<tr>
<td>Zinc nitrate</td>
<td>Zn(NO$_3$)$_2$.3H$_2$O</td>
<td>23</td>
</tr>
<tr>
<td>Zinc phosphate</td>
<td>Zn$_3$(PO$_4$)$_2$</td>
<td>50</td>
</tr>
<tr>
<td>Zinc frits</td>
<td>Fritted glass</td>
<td>10-30</td>
</tr>
<tr>
<td>Ammoniated zinc sulphate solution</td>
<td>Zn(NH$_3$)$_3$SO$_4$</td>
<td>10</td>
</tr>
<tr>
<td><strong>Organic Compounds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disodium zinc EDTA</td>
<td>Na$_2$ZnEDTA</td>
<td>8-14</td>
</tr>
<tr>
<td>Sodium zinc HEDTA</td>
<td>NaZnHEDTA</td>
<td>6-10</td>
</tr>
<tr>
<td>Sodium zinc EDTA</td>
<td>NaZnEDTA</td>
<td>9-13</td>
</tr>
<tr>
<td>Zinc polyflavonoid</td>
<td>-</td>
<td>5-10</td>
</tr>
<tr>
<td>Zinc lignosulfonate</td>
<td>-</td>
<td>-8</td>
</tr>
</tbody>
</table>
2.8. Rhizosphere microflora and zinc bioavailability to plants

Rhizosphere is the zone of soil around roots that is directly swayed by root secretions and is a hot spot of microflora, having manifold increase in microbial population as compared to bulk soil. The rhizosphere microflora may advantage plants through miscellaneous mechanisms e.g., fixation of atmospheric nitrogen, mobilization of nutrients, production of phytohormones, altering native level of phytohormones, improving plant stress tolerance to salinity, toxicity, drought, metal and pesticide load and acts as a biocontrol agent (Khalid et al., 2009).

Though, each and every mechanism has its own implication, mobilization of nutrients by microflora has been considered with pivotal role to enhance nutrient content in plant tissues. There is a good deal of research on mobilization of phosphorus in the rhizosphere by these tiny creatures, but increase in Zn bioavailability in the rhizosphere due to the activities of microbes in rhizosphere has not been well explored yet. Still there are sufficient reports demonstrating substantial potential of these microbes in improving Zn bioavailability in the rhizosphere of plants and Zn content in plant tissues (Subramanian et al., 2009).

Since these microbes play a key role in improving food quality, thus they would be given key importance in future while devising approaches to alleviate Zn malnutrition in humans through food, especially where diverse food is not available to common people and they can also not afford food supplements. Among microbes, both bacteria and fungi have shown terrific ability to improve plant Zn availability in the rhizosphere and also enhance zinc in plant parts (Subramanian et al., 2009).

2.8.1. Mechanisms of zinc bioavailability by zinc solubilizing bacteria

Some organisms use single mechanism while other uses multiple mechanisms to improve Zn in soil and finally improve Zn uptake in plant tissues. Availability of micronutrients in soil is very much sensitive to soil pH. A slight change in soil pH may have a great influence on micronutrient mobility/solubility in soil. It has been reported that availability of Zn decreases 100 times by increase of one unit in pH (Havlin et al., 2005). Thus bioavailable fraction of Zn can be enhanced to a significant level by decreasing the pH of alkaline soil. It has been reported that rhizosphere microflora lower the soil pH to a great extent (Wu et al., 2006) due to secretion of some organic acids and protons extrusion (Fasim
et al., 2002). For example, *Pseudomonas fluorescens* secreted gluconic acid and 2-ketogluconic acid in the culture during solubilization of Zn phosphate. Additionally, concentration of protons was also higher in the culture after incubation period (Di Simine et al., 1998). Similarly, Fasim et al. (2002) observed that solubilization of Zn oxide and phosphate was attended by proton extrusion and production of 2-ketogluconic acid. Martino et al. (2003) recognized that ericoid mycorrhizal fungi secreted organic acid to solubilize Zn from insoluble Zn$_3$(PO$_4$)$_2$ and ZnO. When *Pseudomonas* and *Bacillus* spp. were used to solubilize ZnS, ZnO and ZnCO$_3$ in broth culture then a change in pH was observed (Saravanan et al., 2004).

Subramanian et al. (2009) also indicated that bioavailability of Zn through bioinoculants and acid phophatase activity in arbuscular mycorrhizae inoculated soil would have lower the rhizospheric soil pH and contributed to the release of zinc from mineral fraction. However, degree of reduction in rhizosphere pH vary among microorganisms as Giri et al. (2005) observed a 1.1 unit reduction in pH of rhizosphere soil with mycorrhizal inoculation, while Wu et al. (2006) observed a decrease in pH up to 0.47 unit with bacterial inoculation. Thus, pH goes down due to the release of organic acids and H$^+$, which improves Zn solubilization and uptake by plants.

### 2.8.2. Zn-chelation

Zinc ions have high contact with the soil component due to which its persistency in the soil solution is very low (Alloway, 2009). Due to low persistency/high reactivity of Zn in soil solution, plant available Zn fraction in the soil is poor. Though, bioavailability of Zn could be increased by Zn chelating compounds (Obrador et al., 2003). These compounds are either synthetic or manufactured and released by the plant roots and potential rhizosphere microflora into the rhizosphere to chelate the Zn and increase its bioavailability. The chelates of microflora are metabolites that form complexes with metal cations like Zn$^{2+}$ (Tarkalson et al., 1998) and reduce their reaction with the soil. These Zn chelates consequently move towards the roots and release chelating ligand (Zn$^{2+}$) at the root surface, making them free to chelate another Zn ion from the soil solution. Chelation has been perceived as dominant phenomena to improve bioavailability and uptake by plant roots in some microorganisms. Whiting et al. (2001) concluded that possible mechanism used by bacteria e.g.,
*Microbacterium sapereae, Pseudomonas monteilii, and Enterobacter cancerogenes*, for enhancing water soluble Zn which is bioavailable in soil was the production of Zn chelating metallophores.

In another study, Tariq *et al.* (2007) observed that *Azospirillum lipoferum* (JCM-1270, ER-20), *Pseudomonas* sp. (96-51) and *Agrobacterium* sp. (Ca-18) mobilized Zn and made it bioavailable for longer period of time (when they were applied as a biofertilizer) to rice by producing chelating agent (ethylene diaminetetraacetate (EDTA)). Kucey (1987) reported that inoculation of *Penicillium bilaji* enhanced Zn solubilization and uptake in plant which might happen through chelating mechanism.

### 2.8.3. Changes in root architecture

Zinc is taken up by plants specifically by diffusion because it is immobile in soil (Havlin *et al.*, 2005). Depletion zones are formed around roots due to poor native bioavailable Zn and low exogenous supply. For improving Zn uptake it should be in close vicinity of roots. This can be gained either by more Zn application or improving root growth and surface area so that roots can take nutrients away from the depletion zone. Mycorrhizal fungus is well known for its effect on root architecture. Over long distances, mycorrhizal plants uptake Zn by crossing the depletion zone. Arbuscular mycorrhizae can obtain Zn from a distance of 40 mm from the root surface (Burkert and Robson, 1994). Subramanian *et al.* (2009) found that mycorrhizal fungus increased root length, spread and volume of roots as compared to the plants with absence of fungal inoculation and this increased the Zn concentration significantly by up to 4% in the grain. Similarly, Tariq *et al.* (2007) observed an extensive increase with bacterial inoculation in root weight, length and volume and Zn uptake in straw and grain of rice.

### 2.8.4. Fungal inoculants

AM fungus is considered highly effective among the fungal inoculants for improving the availability and absorption of immobile nutrients by higher plants (Liu *et al.*, 2000). AM fungi are recognized in improving the availability of phosphorus to plant roots. According to other reports, mycorrhizal symbiosis is also very effective in improving availability of Zn to plants (Subramanian *et al.*, 2009). By comparing of both types of soils, a substantial increase
in soil bioavailable content of Zn has been stated in fungal inoculated soils compared to uninoculated soils. This bioavailable Zn is taken up by the plant root and stores in root, or translocated to other plant parts. So the concentration of Zn in plant tissues is dependent on its availability in soil directly. There are a number of reports about the application of bioinoculants directly increasing the Zn uptake (Subramanian et al., 2009), which might have followed through increase in bioavailable Zn in soil.

Similarly, Subramanian et al. (2009) observed that inoculation of *Glomus intraradices* caused an increase of 43% overall in bioavailable Zn in soil after 75 days as compared to uninoculated soils on different Zn levels. When available Zn increases it resulted in more acquirement of Zn and its splitting to maize grain. Chen et al. (2003) found 11% higher Zn in root of red clover plants inoculated with *G. mosseae* while Wu et al. (2011) found 450, 225 and 200% more Zn accumulation in roots of peach plants inoculated with *G. mosseae*, *G. versiforme*, and *Paraglomus occultum*, as compared to uninoculated plants. By observing response of AM fungus inoculation, it is very promising in terms of Zn accumulation in shoot and leaves. With comparison of +AM and -AM plants, Giri et al. (2005) found about 15 times more Zn in the shoots of +AM compared to -AM plants. Chen et al. (2003) also concluded extensive increase in shoot Zn concentration by fungal inoculation. Similarly Wu et al. (2011) found that mycorrhizal infection also improved concentration of Zn in the leaves of AM plants compared to uninoculated plants. However when Zn is applied at higher rate to soil, response of AM fungi on Zn acquisition is comparatively low.

Chen et al. (2003) found more Zn in shoots of red clover plants having mycorrhizal symbiosis with Zn application at the rate of 0 and 50 mg kg\(^{-1}\) soil, but when Zn was applied at the rate of 100 and 300 mg kg\(^{-1}\), Zn concentration was observed even less in inoculated plants. This is fact; either no Zn is applied to soil or applied at very lower rate in most of the developing countries. So under these conditions, AM fungi inoculations are very powerful for enhancing the Zn content in plant tissues. Free living fungi are also capable to convert insoluble Zn compounds into soluble compounds and improve Zn concentration in plant tissues e.g., fungi belonging to genera *Aspergillus* and *Penicillium* (Sayer et al., 1995) as AM fungi. Kucey (1988) resulted a significant increase in Zn uptake by wheat plants with inoculation with *Penicillium bilaji*. It increased from 161 (uninoculated control) to 232
μg/pot under greenhouse conditions while from 9.61 to 10.7 mg/plot in field study with fungal bioinoculant. Fungal inoculants could be effectively used to increase Zn availability to plants is clearly demonstrated by these studies.

### 2.8.5. Bacterial inoculants

Bacterial bioinoculants are also helpful in increasing solubilization/availability of Zn in soil and its further uptake by plants to improve plant Zn content. It has been reported that several bacterial species are able to solubilize insoluble Zn compounds in liquid medium (Saravanan et al., 2007) and in soil (Tariq et al., 2007).

Saravanan et al. (2004) observed that *Pseudomonas* and *Bacillus* can solubilize various Zn compounds like ZnS, ZnO and ZnCO$_3$ to a greater extent in liquid medium in an *in vitro* study. Similarly, Saravanan et al. (2007) reported that zinc solubilizing prospective of *Glomus diazotrophicus* PA15 in another study. It is resulted by inoculation with *G. diazotrophicus* PA15 a 41, 15.7 and 60 times increase in soluble Zn content in case of ZnO, ZnCO$_3$ and Zn$_3$(PO$_4$)$_2$, respectively after 48 h of incubation compared to uninoculated control. Fasim et al. (2002) observed a high potential of *Pseudomonas aeruginosa* to solubilize ZnO in liquid medium similarly. Tariq et al. (2007) observed almost 5.6 time higher bioavailable Zn in inoculated soil compared to uninoculated soil. Whiting et al. (2001) have also recorded through bacterial inoculation about 0.45 fold increase in bioavailable Zn in rhizosphere soil. It has also been widely reported that bacterial inoculation increases plant Zn content (Biari et al., 2008). A 2-fold more Zn concentration in the shoot of *T. caerulescens* compared to control while uptake was increased up to 4-fold observed by Whiting et al. (2001). Eleiwa et al. (2012) reported that inoculation of *Azotobacter* and *Azospirillum* was useful in controlling Zn deficiency in wheat as up to 18% increase in Zn uptake in response to inoculation was measured compared to uninoculated control under no Zn application. Likewise, inoculation of corn with *Azotobacter* and *Azospirillum* caused a significant increase Zn content in grain (Biari et al., 2008) and also observed up to 107, 85, 95 and 107% increase in Zn content in seed with *Azospirillum* sp. strain 21, *Azospirillum brasiliense* DSM2286, *Azotobacter* sp strain 5, *Azotobacter chroococcum* DSM2286 compared to uninoculated control. Mishra et al. (2012) observed consortium of *Pseudomonas* spp. and *Rhizobium* leguminosarum-pr-1 enhanced the shoot Zn content. While, Tariq et al.
(2007) observed 133% increases in Zn concentration in grain with inoculation by conducting experiment on rice. The bacterial application also relieved the deficiency symptoms of Zn in plant. Sadaghiani et al. (2008) also found a substantial increase in Zn acquisition in wheat and barley with Bacillus M-13 and Pseudomonas aeruginosa 7NSK2. Therefore, use of such inoculants could be valuable to increase solubilization of Zn in soil and its subsequent availability to plants.

2.8.6. Role of ZSB with combination of zinc and other organic amendment

Organic amendments also increase Zn bioavailability by increasing \( C_{mic} \) (microbial biomass carbon), which not only increases the rate of decomposition of organic matter (source of Zn), but also increases the bioavailability of complex Zn by decreasing the soil pH and by chelating agents release. Improvement in \( C_{mic} \) and microbial activity in soil with the addition of organic amendments ensures better soil quality and also has a beneficial impact on soil fertility by mineralization process, these organic amendments also ensure the availability of those macro and micronutrients (including Zn), which are generally ignored by the farmers. It is crucial to increase bioavailability of Zn to plants by solubilizing fixed Zn and/or by reducing fixation of the applied Zn fertilizers (Imran et al., 2014). This can be achieved either by using organic amendments or potential Zn solubilizing bioinoculants alone or in combination (consortium).

2.9. Bio-activation

It is well documented that bacteria usually Bacillus sp. and Pseudomonas sp. have the ability to produce organic acids which decrease pH. This increases micronutrients (e. g. Zn) availability to plants (Saravanan et al., 2004). It is also found that PGPR (Plant growth promoting rhizobacteria) possibly produce siderophores (Saravanan et al., 2011), gluconate, or the derivatives of gluconic acids, e.g., 2- ketogluconic acid (Fasim et al., 2002), 5-ketogluconic acid (Saravanan et al., 2007), and various other organic acids (Tariq et al., 2007) for the solubilization of zinc. Solubilization of insoluble zinc was accompanied by an increase in the \( H^+ \) concentration in the medium, due to the production of 2-ketogluconic acid and other organic acids (Fasim et al., 2002). All these mechanisms help to enhance zinc availability, uptake and its concentration in the crops. If we use these bacteria with insoluble
source of zinc, they have the ability to solubilize the zinc by using the above mentioned mechanisms. The coating of zinc solubilizing bacteria on ZnO is called bio-activation of ZnO.

**Conclusion**

Plant growth promoting rhizobacteria have their own importance, but nutrients mobilization from zinc solubilizing bacteria has been considered the most critical function to improve zinc bioavailability in soil for the plants. There is a good deal of research on zinc mobilization but combination of zinc solubilizing bacteria with organic amendment to solubilize the insoluble source of zinc (ZnO) has not been well studied yet. Keeping in view above review an experiment was design to test the efficacy of bio-activated Zn for improving yield and quality of maize.
CHAPTER 3

MATERIALS AND METHODS

A series of laboratory, wire-house and field experiments were conducted for isolation, identification and characterization of zinc solubilizing bacteria. Efficient bacterial isolates were tested for different plant growth promoting activities. Finally, bio-activated zinc (BOZ) was formulated for improving yield and quality of maize under pot and field conditions. Summary of different studies conducted in this project are given.

3.1. Studies conducted

The research work given in this thesis is divided into following studies.

3.1.1. Study # 1
- Isolation of zinc solubilizing bacteria
- Evaluation of zinc solubilizing potential of isolated bacteria

3.1.2. Study # 2
- Screening of bacteria for plant growth promoting activity in growth room study
- Identification and characterization of selected isolate

3.1.3. Study # 3
- Formulation of bio-activated zinc (BOZ)
- Assessment of the temporal release of Zn from Bio-activated Zn in soil (Incubation Study)

3.1.4. Study # 4
- Evaluation of different formulations of bio-activated Zn vs ZnSO₄ on growth, physiology, yield and quality of maize (Wire House Experiment)

3.1.5. Study # 5
- Evaluation of different formulation of bio-activated Zn vs ZnSO₄ on growth, physiology, yield and quality of maize (Field trial I)

3.1.6. Study # 6
- Confirmatory field evaluation of different formulation of bio-activated Zn vs ZnSO₄ on growth, physiology, yield and quality of maize (Field trial II)
3.2. Study # 1

3.2.1. Isolation of Zinc Solubilizing Bacteria

Zinc solubilizing bacteria were isolated from the rhizosphere of the maize (Research area of Institute of Soil & Environmental Sciences, UAF) by using dilution plate technique on nutrient agar medium. Isolates were purified by repeated streaking on Bunt and Rovira medium (Bunt & Rovira, 1955). Bacterial colonies with prolific growth were selected, purified and preserved in glycerol stock at -40 ºC.

3.2.2. Zinc solubilizing potential of isolated bacteria

An incubation study was conducted to evaluate the zinc solubilizing potential of isolated bacteria with ZnO. The solubilization potential was assessed both qualitatively and quantitatively under in vitro conditions (Saravanan et al., 2003).

3.2.2.1. Qualitative assay

To assess zinc solubilization ability of selected strains, bacteria were subjected to grow on Bunt & Rovira medium containing 0.1% insoluble zinc compound (ZnO) as described by (Bunt & Rovira, 1955). Agar medium consists of glucose 10.0 g; ammonium sulphate 1.0 g; potassium chloride 0.2 g; dipotassium hydrogen phosphate 0.1 g; magnesium sulphate 0.2 g; distilled water 1000 ml at pH 7.0 was autoclaved at 121 ºC at 15 psi for 30 min. Experiments were done in triplicate. After sterilization and plating, freshly grown bacterial cultures were spot inoculated in triplicates on the media using a sterile loop full of bacterial culture. The spotted plates were incubated at 28 ± 1 ºC for 7 days in dark to observe clear halo formation around the colonies. The halo diameters of colonies were measured. Zinc solubilization area (cm²) was calculated according to Saravanan et al., 2003.

\[ \text{Area} = \pi r^2 \]

3.2.2.2. Quantitative assay

Basal medium (Bunt & Rovira, 1955) was prepared, after autoclaved at 121ºC and kept in incubator at optimal temperature. Broth medium consisted of glucose 10.0 g; ammonium sulphate 1.0 g; potassium chloride 0.2 g; dipotassium hydrogen phosphate 0.1 g;
magnesium sulphate 0.2 g; distilled water 1000 ml and pH 7.0. Inoculum of each bacterial isolate was prepared by growing them in nutrient broth. Flasks were incubated at 28 ± 1 °C for 15 days in shaking incubator. The samples were collected after 15th day. After incubation, pH in the culture medium was recorded. The aliquot of the medium was centrifuged (7000 rpm, 15 minutes) and filtered (0.22 µm). The culture supernatant was directly fed to atomic absorption spectrophotometer for determination of soluble zinc content. The amount of zinc solubilized was obtained by comparing the soluble zinc of the inoculated sample from the corresponding un-inoculated control and expressed as ml L⁻¹ of Zn culture (Saravanan et al., 2003).

Total 52 rhizobacterial colonies were selected and from them only 14 were positive for zinc solubilization (Qualitative). On the basis of holozone diameter, solubilizing area, pH reduction and zinc solubilizing ability, 10 isolates were selected for further screening of bacteria for plant growth promotion activity.

3.3. Study # 2

3.3.1. Screening of bacteria for plant growth promotion activities in growth room

The experiment was conducted in the growth room under axenic conditions to screen the bacteria for plant growth promotion activities. Under controlled conditions ten different isolates of zinc solubilizing bacteria were used to check the effect of bacteria on the maize seedling. Different zinc solubilizing strains were inoculated in Bunt and Rovira broth medium (Bunt & Rovira, 1955). Growth and physiological parameters were studied. On the basis of growth (Shoot length, root length, fresh and dry biomass of root and shoot), and physiological parameters (photosynthesis rate, transpiration rate, stomatal conductance and chlorophyll contents) one effective growth promoting bacterial isolate was selected for further identification and characterization.

3.3.2. Identification of the selected bacterial isolate

The selected bacterial strain was identified through amplification with polymerase chain reaction (PCR), sequencing and bioinformatics analysis of its 16S rRNA gene sequence. For this purpose, crude DNA of the selected isolate AZ6 was extracted from the
cell culture using proteinase K treatment (Cheneby et al., 2004). The 16S rRNA sequence was amplified from 2.5 µL of crude DNA template using the universal primers 27f and 1492R as previously described by Hussain et al., (2011). The PCR reaction was carried out using 2.5 µL crude DNA as a template in total 25 µL reaction mixture according to the following program: 1 cycle of 4 min at 94 °C; 39 cycles of 1 min at 94 °C, 1 min at 55 °C, 1.5 min at 72 °C and a final extension step at 72 °C for 5 min. The size of the amplified 16S rRNA was confirmed by separating on 1% agarose gel along with GeneRuler 1kb DNA (Fermentas, country name). The 16S rRNA PCR product was purified using a PCR Purification Kit (Favorgen, Taiwan) and sequenced by Macrogen (Seoul, Korea). 16S rRNA of AZ6 was compared with the known nucleotide sequences using BlastN accessed at http://www.ncbi.nlm.nih.gov/BLAST. A phylogenetic tree was constructed by carrying out multiple alignments using ClustalX (Thompson et al., 1997) and processing the data using NJ Plot for neighbor joining method (Perriere and Gouy, 1996). The partial sequence was deposited in the GenBank database under the accession AZ6 KT221633.

3.4. Characterization of selected strain

Selected zinc solubilizing strain (Bacillus sp. AZ6) was characterized for various traits such as IAA (Indole-3-acitic acid) production activity (Sarwar et al., 1992), ACC-deaminase activity (Honma and Shimomura, 1978; Penrose and Glick, 2003), Siderophores production activity (Schwn and Neilands 1987) and production of organic acids (Butsat et al., 2009).

3.4.1. ACC-deaminase activity

ACC-deaminase activity was measured according to a modification of the method of Honma and Shimomura (1978) and Penrose and Glick (2003) which measure the amount of α-ketobutyrate produced when the enzyme ACC-deaminase cleaves ACC. The number of nmol of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1.5 µmol. A stock solution of 1 mM α-ketobutyrate was prepared in 0.1 M Tris-HCl (pH 8.5) and stored at 4 °C. Just prior to use, the stock solution was diluted with the same buffer to make a 10 µM solution from which a standard concentration curve was developed. In each
series of standard containing 2 mL of known concentration of \(\alpha\)-ketobutyrate, 3 mL of 2,4-dinitrophenylhydrazine reagent (0.2\% 2,4-dinitrophenylhydrazine in 2 M HCl) was added and the contents were vortexed and incubated at 30 \(^\circ\)C for 30 minutes, during which time the \(\alpha\)-ketobutyrate was derivatized as phenylhydrazone. The color of the phenylhydrazone was developed by the addition of 20 mL 2 M NaOH to each standard; after mixing the absorbance of the mixture was measured at 540 nm. ACC-deaminase activity was induced in bacterial strains as described by Glick \textit{et al.} (1995). ACC deaminase activity was measured in bacterial extracts prepared by the following manner. Bacterial cell pellets prepared as described above were suspended in 10 mL of 0.1 M Tris-HCl (pH 7.6) and transferred to 15 mL centrifuge tube. The contents of the tube were centrifuged at 13500 g for 5 minutes and the supernatant was removed. The pellet were suspended in 6 mL 0.1 M Tris-HCl (pH 8.5). Three milliliters of toluene was added to the cell suspension and vortexed at the highest setting for 30 seconds. The tolenized cell suspension was immediately assayed for ACC deaminase activity. All sample measurements were carried out in duplicate. Two milliliters of the tolenized cells were placed in a fresh 15 mL centrifuge tube; 0.2 mL of 0.5 M ACC was added to the suspension, briefly vortexed, and then incubated at 30\(^\circ\)C for 15 min. Following the addition of 10 mL of 0.56 M HCl, the mixture was vortexed and centrifuge for 5 minutes at 13500 g at room temperature. Ten milliliters of the supernatant was vortexed together with 8 mL of 0.56 M HCl. Thereupon 3 mL of the 2,4-dinitrophenylhydrazine reagent was added to the glass tube, the contents were vortexed and then incubated at 30\(^\circ\)C for 30 min. Following the addition and mixing of 20 mL of 2 N NaOH, the absorbance of the mixture was measured at 540 nm. Two series were run for the absorbance assay. In the first series, reagents included ACC, bacterial cells and assay reagents with their control containing ACC and assay reagents. Second series included bacterial cell and assay reagents with its control containing assay reagents only. The assay was carried out on digital spectrophotometer, so the control value was subtracted automatically from the treatment values. Values of the first series were subtracted from the values of the second series for respective bacterial inoculation, to estimate the amount of \(\alpha\)-ketobutyrate in nmol from the standard curve. Value of ACC-deaminase activity of strain was further estimated on the basis of per gram biomass of bacterial cell.
3.4.2. Auxin production as Indoe-3-acetic acid (IAA) production

*In vitro* assay was carried out to determine auxin production (IAA equivalents) by the selected isolate as indole acetic acid both in the presence and absence of L-tryptophan by following the procedure as described by Sarwar *et al.*, 1992. For this 25 mL of Luria Bertani (LB) media was taken in 100 mL Erlenmeyer flasks, autoclaved and cooled. L-Tryptophan was filtered by passing through 0.2 µm membrane filter and was added at a concentration of 100 µg mL⁻¹ to the LB media. The contents of the flask were inoculated by adding 1 mL of bacterial culture (population density of 10⁷-10⁸ CFU mL⁻¹). The flasks were plugged and incubated at 28 ± 1 °C for 48 hours. After incubation, the contents were filtered through Whatman No. 2. Un-inoculated control with LB broth and L-TRP alone was also prepared for comparison. While measuring IAA-equivalents, 3 mL filtrate was taken and mixed with 2 mL Salkowski’s reagent (2.0 mL of 0.5 M FeCl₃ + 98 mL of 35% HClO₄). After color development, intensity of the color was measured at 535 nm by using spectrophotometer. Standard calibration curve was prepared by measuring the intensity of the color of pure IAA standards.

3.4.3. Siderophore production

Siderophore production was qualitatively assessed by plate assay described by Schwn and Neilands (1987). The bacterial strain AZ6 was inoculated to plates containing Chrom azurol (CAS) agar and incubated at 28±2 C for 4-5 days. A change in blue to orange colour depicted production of siderophores.

3.4.4. Bacterial metabolite (Organic acid profiles, detected by HPLC)

Selected isolate of zinc solubilizing bacterium *Bacillus sp.* AZ6 was inoculated in test tubes containing 15 mL of LB broth medium and incubated at 28 ±1 °C for 72 hours. Three biological replications were maintained for this experiment. After incubation, each sample was centrifuged at 10000 rpm at 4°C and the supernatant was collected. Metabolites were extracted thrice by vigorous shaking with Methanol (HPLC grade) in 1:1 ratio using separating funnel. Organic acids (cinamic, Ferulic, caffeic, chlorggenic, syrirgic and gallic acids) were determined by HPLC (Shimadzu, Japan) with LC 10AT, UV-visible and SPD 10
AV, after running the samples along with standards for organic compounds (Butsat et al., 2009).

3.5. Study # 3

3.5.1. Preparation of bio-activated zinc

The organic material (Grinded Orange Peel) was first dried in oven at 80 °C to make it free of microbes then it was bioaugmented with the inoculum. The inoculum was prepared on LB medium in the ratio of 90:10 (W/V) and incubated for 72 hours at 28±2 °C in an incubator. This resulted to development of maximum (10^7 to 10^8 CFU g⁻¹) population of beneficial bacterial strain. Zinc oxide was crushed to 300-400 mesh size and mixed with an appropriate amount and source of powder of organic material in different ratios of Zn: O.M (9:1, 8:2, 7:3 and 6:4). The organic material had already been bioaugmented with efficient zinc solubilizing bacterial strain (Bacillus sp. AZ6) and mixture was amended with inoculated LB broth medium 10% @ 100 ml kg⁻¹. This mixture was again incubated for 3 days at 28±2 °C to achieve maximum chelation of Zn with organic complexes along with sustainability of much population of beneficial bacterial strain. In all the formulations bacterial population of 10^7 to 10^8 CFU g⁻¹ were maintained. Four different formulations of bio-activated zinc were prepared (BOZ1, BOZ2, BOZ3 and BOZ4). Detail of recipe of bio-activated zinc formulations are given in Table 3.1.

3.5.2. Evaluation of the temporal release of Zn from bio-activated Zn in soil (Incubation Study)

The study was conducted in the wire house. The main purpose of this study was to investigate the rate of Zn release with respect to time (temporal zinc release) and effect of bio-activation process on Zn solubilization. Soil for pot experiment was taken from surface layer of the research area of Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad. Soil was air dried, ground, and after passing through a 2 mm sieve it was mixed thoroughly and the pots (5.5 kg pot size) were filled with 5 kg soil.
Experiment was comparison of 8 treatments with 3 replications and laid down by following completely randomized design. The concentration of Zn was 4.9 kg ha\(^{-1}\) in all the zinc-containing treatments but the sources and formulation were different.

The treatment plan was as follows:
T1: No Zinc (Control)
T2: Zn from ZnO (Unavailable form)
T3: Zn from ZnSO\(_4\) (available form)
T4: Zn from Bio-activated Zn (ZnO + OM, 9:1) +ZSB (BOZ 1)
T5: Zn from Bio-activated Zn (ZnO + OM, 8:2) +ZSB (BOZ 2)
T6: Zn from Bio-activated Zn (ZnO + OM, 7:3) +ZSB (BOZ 3)
T7: Zn from Bio-activated Zn (ZnO + OM, 6:4) +ZSB (BOZ 4)
T8: Zinc solubilizing bacteria (ZSB) Only inoculation

Different treatments (ZnO, ZnSO\(_4\), zinc bio-activated formulations and ZSB) were applied before sowing. Pots were irrigated with tap water to maintain optimum moisture (35% field capacity). Physicochemical properties of soil were determined before conducting the experiment (Table 3.2).

Zn concentration from soil was analyzed 6 times after every 12\(^{th}\) day by ABDTPA extraction (Soltanpour and Schwab, 1977).

3.6. Pre-sowing soil analyses

Before the filling of pots, a soil sample was analyzed for its various physico-chemical properties including particle size analysis (Gee and Bauder, 1986), soil pH, organic matter (Nelson and Sommer, 1982), electrical conductivity (EC), saturated percentage, nitrogen and phosphorus, extractable potassium and available zinc. The results of this analysis are presented in the Table 3.2.

3.6.1. Soil textural class

For determining soil textural class, 50 g of each soil was taken and 40 mL of 1\% sodium hexametaphosphate (NaPO\(_3\))\(_6\) solution and 250 mL of distilled water were added and
kept overnight. Soil was stirred with a mechanical stirrer for 10 minutes, transferred to a one liter graduated cylinder and the volume was made up to the mark. After mixing the suspension reading was recorded with Bouyoucos hydrometer (Moodie et al., 1959). Soil textural class was designated after using the International Textural Triangle.

3.6.2. Saturation percentage (SP)

Saturated paste was prepared and a portion of paste was transferred to a tarred china dish and weighed. Weighed soil paste was placed in an oven and dried to a constant weight at 105°C and weighed again. Saturation percentage was calculated by using following formula (Method 27a, U.S. Salinity Lab. Staff, 1954).

\[
SP = \frac{\text{Mass of wet soil} - \text{Mass of dry soil}}{\text{Mass of oven dry soil}} \times 100
\]

3.6.3. pH of saturated soil paste (pHs)

The pH of saturated soil paste was determined after preparing saturated soil paste. About 250 g soil was saturated with distilled water. The paste was allowed to stand for one hour and pH was recorded (Method 21a, U.S. Salinity Lab. Staff, 1954) by using pH meter (pH 6-38W Microprocessor pH/mV/Temperature meter).

3.6.4. Electrical conductivity (ECe)

For determining ECe, extract of each soil paste was obtained by using vacuum pump. Electrical conductivity was measured by using digital Jenway conductivity meter (Jenway 4510 Conductivity meter) as described in Method 3a and 4b of U.S. Salinity Lab. Staff (1954).

3.6.5. Organic matter

Soil organic matter contents were determined according to the method described by Moodie et al. (1959). According to this, one g of soil sample was mixed thoroughly with 10 mL 1N potassium dichromate solution and 20 mL concentrated sulphuric acid. Then, 150 mL of distilled water and 25 mL of 0.5 N ferrous sulphate solution were added and the excess was titrated against 0.1N potassium permanganate to pink end point.
3.6.6. **Total nitrogen**

Nitrogen was determined by Ginning and Hibbard’s method (Jackson, 1962) of sulphuric acid digestion and distillation was made with macro Kjeldhal’s apparatus (VELP Scientifica UDK 126 D).

3.6.7. **Available phosphorus**

Five grams of soil was extracted with 0.5 M NaHCO₃ solution adjusted to pH. Five mL of clear filtrate was taken in 100 mL volumetric flask and five mL color developing reagent (ascorbic acid) was added. Volume was made up to the mark. Reading was recorded on spectrophotometer (T 60 PG instrument) using 880 nm wavelength with the help of standard curve (Watanabe and Olsen, 1965).

3.6.8. **Extractable potassium**

Extraction was done with ammonium acetate (1 N of pH 7.0) and potassium was determined by using Flame photometer (Sherwood 410) following the Method 11a, Salinity Lab. Staff, 1954).

3.6.9. **Soil zinc determination**

Soil zinc was determined by Ammonium Bicarbonate-DTPA extraction method developed by Soltanpour and Schwab (1977), and later modified by Soltanpour and Workman (1979a). For this 10 g air dried soil (2-mm) was taken into a 125 mL conical flask. Extracting solution was added 20 mL and shaken on a reciprocal shaker for 15 minutes at 180 cycles/minute with flasks kept open. The extracting solution was 1 M in the ammonium bicarbonate (NH₄HCO₃), and 0.005 M DTPA adjusted to pH 7.6. The extracts were then filtered through Whatman No. 42 filter paper. Zinc was determined by atomic absorption spectrophotometer (A ANALYST 100). The standard solutions of the metal were made in the extracting solution.

3.7. **STUDY # 4 (Pot experiment)**

The experiment was conducted in the wire house of Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad. The main purpose of the study was the
evaluation of different formulation of bio-activated Zn vs ZnSO₄ on growth, physiology, yield and quality of maize. Soil for pot experiment was taken from surface layer of the research area of Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad. Soil was air dried, ground, and after passing through a 2 mm sieve it was mixed thoroughly and the pots was filled with 25 kg soil. Maize hybrid variety (Syngenta NT662 taken from Syngenta dealer at Faisalabad) was used in the experiment. Soil used in pot was analyzed for physical and chemical characteristics as described in Section 3.6 (Table 3.2).

Experiment comprised of 8 treatments with 3 replications and laid down by following completely randomized design. The concentration of Zn was 4.9 kg ha⁻¹ in all the zinc containing treatments but the sources and formulation were different.

The treatments were:

T1: No Zinc (Control)

T2: Zn from ZnO (Unavailable form)

T3: Zn from ZnSO₄ (available form)

T4: Zn from Bio-activated Zn (ZnO + OM, 9:1) +ZSB (BOZ 1)

T5: Zn from Bio-activated Zn (ZnO + OM, 8:2) +ZSB (BOZ 2)

T6: Zn from Bio-activated Zn (ZnO + OM, 7:3) +ZSB (BOZ 3)

T7: Zn from Bio-activated Zn (ZnO + OM, 6:4) +ZSB (BOZ 4)

T8: Zinc solubilizing bacteria (ZSB) Only inoculation

Different treatments (ZnO, ZnSO₄, zinc bio-activated formulations and ZSB) were applied before sowing. In treatments 8 where only zinc solubilizing was applied, seeds of maize treated with inoculated LB media by using carriers (Orange peel). Different treatments (ZnO, ZnSO₄ and zinc bio-activated formulations) were applied before sowing.

Recommended dose of NPK (175 kg ha⁻¹, 160 kg ha⁻¹ and 125 kg ha⁻¹) was applied using Urea, Diammonium phosphate, sulphate of potash and as sources of N, P and K respectively. All the fertilizers were applied at the time of sowing except nitrogen (Urea), which was applied in two splits. Soil in each pot was remixed to homogenize it with respect to NPK. Pots were irrigated with tap water to maintain optimum moisture for plant growth. The plants were irrigated as and when required.
3.7.1. Pre-sowing soil analyses
Same as given in section 3.6.9

3.7.2. Data collection:
Data of following parameters were recorded by adopting standard measures. These parameters were recorded during growth stages and at the time of harvesting of crop and soil parameters were collected before and after crop harvest. Following growth, physiological, yield, chemical and quality parameters were taken.

3.7.2.1. Growth parameters
At maturity (after 4 months), plants were harvested and data about growth parameters were recorded. Plant height, root length, shoots length and cob length was recorded with the help of measuring rod from top to bottom. Fresh shoot and root biomass were weighted on digital electrical balance (Setra bl-4100S). Fresh shoots and roots were firstly sun dried (30-35 ºC) and then placed in the oven (65 ºC) till constant weight. Then the dry root biomass and shoot biomass was recorded on digital electrical balance. Cob diameter and plant girth were measured with the help of vernier caliper.

3.7.2.2. Physiological parameters
After 55 days sowing, the physiological parameters i.e. photosynthetic rate (A), transpiration rate (E), and stomatal conductance (gs) in all treatments was measured through CIRAS-3 (Potable photosynthesis measuring system). These parameters were taken in the morning.

3.7.2.3. Carbonic anhydrase activity (CA)
Carbonic anhydrase activity CA activity was determined by the method of Dwivedi and Randhawa (1974). The leaf samples were cut into small pieces (1 cm²) at a temperature below 25°C. After mixing them, 200 mg leaf pieces were weighed and suspended in 0.2 M cysteine hydrochloride solution. The samples were incubated at 40°C for 20 min. The pieces were blotted and transferred to the test tubes containing phosphate buffer (pH 6.8), followed by the addition of 0.2 M alkaline bicarbonate solution and 0.002% bromothymol blue
indicator. The test tubes were incubated at 50°C for 20 min. After the addition of 0.2 mL of methyl red indicator, the reaction mixture was titrated against 0.05 N HCl. The results were expressed as: μmol (CO₂) kg⁻¹ s⁻¹.

3.7.2.4. Leaf chlorophyll content (SPAD Value)

For the determination of leaf chlorophyll contents, 3 leaves from each plant were selected and with the help of chlorophyll meter (Konica Minolta sensing, INC, made in Japan) chlorophyll contents were measured after its calibration and average was calculated.

3.7.2.5. Electrolyte leakage

It was used to assess membrane permeability, as described by Lutts et al., 1995. Samples were washed three times with DDW to remove the surface contamination. The leaf discs were prepared by cutting the young leaves and were placed in a closed vial containing 10 mL of DDW and incubated on a rotatory shaker for 24 h; subsequently, the electrical conductivity of the solution (EC1) was determined (EC meter Jenway 4510). Samples were then autoclaved at 120°C for 1 hour and 20 min and then electrical conductivity (EC2) was measured after cooling the solution at room temperature. The electrolyte leakage was calculated as

\[ \text{Electrolyte leakage (\%)} = \frac{\text{EC}_1}{\text{EC}_2} \times 100 \]

3.7.2.6. Yield parameters

At maturity, plants were harvested and data about yield parameters were recorded. Grains weight, 100 grain weight and stover yield per cob were recorded. The parameters like number of grains per cob, no of grains per line, no of lines per cob were also calculated.

3.7.2.7. Quality parameters

The quality parameters of maize grains i.e. crude protein, oil contents, crude fiber ash content, moisture percentage and dry matter were analyzed using following methods.
3.7.2.7.1. Crude protein (CP)

Nitrogen content in maize grains samples was determined by Kjeltech Apparatus (VELP Scientifica UDK 126 D). The protein percentage was measured by multiplying nitrogen with conversion factor 6.25 (Shih et al., 1999).

3.7.2.7.2. Oil contents

The Soxhelt apparatus was used for the determination of oil in each sample according to AACC (2000) method No. 30-25. For the purpose, 5g of flour was extracted with petroleum ether at a condensation rate of 2-3 drops/sec. for 8hrs. After distilling excess ether the residue of extraction flask was dried at 100°C for 30 min. until a constant weight. Oil content was calculated by the formula given below.

\[
\% \text{ Oil} = \frac{\text{Weight of ether extract}}{\text{Weight of flour sample}} \times 100
\]

3.7.2.7.3. Crude fiber (CF)

The maize grain samples were subjected for determination of crude fiber content by following the procedure mentioned in AACC (2000) Method No. 32-10. The crude fiber was estimated in 2g fat and moisture free sample and boiled for 30 minutes in the presence of 1.25% H\textsubscript{2}SO\textsubscript{4}, and then filtered and washed. Then these samples were again boiled in 1.25% NaOH for 30minutes, and then filtered and washed. The resultant residue was dried at 130°C for 2 hrs and weighed. The dried residue was ignited at 600°C + 15°C, cooled and reweighed. The crude fiber was calculated according to following expression.

\[
\% \text{ Fiber} = \frac{\text{Loss in weight on ignition - blank}}{\text{Weight of sample}} \times 100
\]

3.7.2.7.4. Ash content

Each flour sample was tested for ash content by following the procedure outlined in AACC (2000) method No. 08-01. The whole maize grain sample were taken in pre- weighed crucibles and charred on bunson burner before incinerating in the muffle furnace where a temperature of 550°C was maintained till the sample converted to grayish white residue.
\[
\text{% Ash} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100
\]

3.7.2.7.5. Dry matter

The moisture of sample is lost by volatilization caused by heat. The amount of material left after the removal of water is the dry matter (AOAC, 1990). For dry matter determination, first aluminum containers were oven dried and weighed by electric balance. 10 gram of plant sample was weighed in each container and placed in oven at 105°C till constant weight. Dry matter percentage was calculated by following formula:

\[
\text{Dry matter} \% = \frac{\text{Weight of oven dry sample}}{\text{Weight of sample before drying}} \times 100
\]

3.7.2.7.6. Moisture percentage

Maize grains were evaluated for moisture content by using an air forced draft oven (Memmet Germany) at a temperature of 105± 5°C by following the procedure described in AACC (2000) method No. 44-15A.

\[
\text{Moisture} \% = \frac{\text{Weight of sample} - \text{Oven dry weight of sample}}{\text{Weight of sample}} \times 100
\]

3.7.2.8. Analysis of zinc in different plant parts (shoot, root and grains) by wet digestion

Total recovery of zinc was not possible by dry ashing procedure. Therefore the plant material was wet digested with HNO\textsubscript{3}-HClO\textsubscript{4}. The reagents i.e., nitric acid, perchloric acid (HNO\textsubscript{3}-HClO\textsubscript{4}) a 2:1 ratio, one liter of conc. nitric acid was added to 500 mL of conc. perchloric acid. Air dried ground material (1g) was placed in digestion flask. 10 mL of concentrated HNO\textsubscript{3} was added in it and let it stand to overnight. Next day samples were heated on hot plate carefully until the production of red NO\textsubscript{2} fumes has ceased. Allow the flasks to cool down and then add a small amount (2-4 mL) of 70% HClO\textsubscript{4}. The samples were heated again and allow evaporating to a small volume. When the vapors were condensed, transfer the flask to a 50 mL flask and volume was made with distilled water. Each batch of
samples to digestion contained at least one reagent blank (no plant material). Samples were then filtered and used for determination of zinc by atomic absorption spectrometry.

Calibrated standards of zinc were prepared from the commercially available ZnSO$_4$.7H$_2$O. Stock solution was made by dissolving ZnSO$_4$.7H$_2$O 4.398 g/L. Substock of 100 ppm was prepared by taking 10 mL stock solution in 100 mL flask. Volume was made with distilled water. Working standards of 0.5, 1, 1.5, 2, 2.5, and 3 ppm were prepared by taking sub stock.

After digestion shoot, roots and grain samples of maize were then filtered and used for determination of zinc by atomic absorption spectrophotometer (A ANALYST 100).

### 3.7.2.9. Zinc concentration in soil after harvesting the crop

Same as given in section 3.6.9

### 3.7.2.10. Phytates

For phytate determination, 60 mg finely ground samples were extracted with 10 mL of 0.2 N HCl at room temperature for 2 h under continuous shaking. Phytate in the extract was determined by an indirect method that uses absorption of pink color developed by un-reacted Fe(III) with 2,2′-bi-pyridine (Haug and Lantzsch, 1983). Briefly, 0.5 mL extract was mixed with 1 mL 0.4 mM ammonium ferric sulphate solution (dissolved in 0.2 N HCl) in a capped glass tube and heated in a boiling water bath for 30 min. After cooling in ice cold water for 15 min, the samples were allowed to adjust at room temperature. After adjusting, 2 mL of 2,2′-bi-pyridine solution (5 g dissolved in 500 mL water with 1% v/v thioglycollic acid) was added to the mixture and the absorbance was measured at 519 nm with a spectrophotometer. Phytate in the extract was calculated from a calibration curve established using standard phytate solutions made with sodium phytate. All samples for phytate determinations were prepared and analyzed in triplicates.

### 3.7.2.11. Phytate to Zn ratio:

Concentrations of both Zn and phytate in maize grains were used to calculate [phytate]: [Zn] ratio.
3.8. Study # 5&6 (Field trial I & II)

Two field experiments were conducted in the research area of the Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad. Filed trials were conducted in different seasons 1st one was in March and the other one in July. The main purpose of the study 5 (Field trial I) was the field evaluation of different formulation of bio-activated Zn vs ZnSO₄ on growth, yield and quality of maize. The study 6 (Field trial II) was the confirmatory field evaluation of different formulation of bio-activated Zn vs ZnSO₄ on growth, yield and quality of maize. And the main purpose of field trial II was to check the effect of bio-activated zinc formulation in seasonal variation (In Pakistan, there are two seasons of maize sowing - in March & in July).

**For treatment** (Same as given in section 3.7)

Different treatments (ZnO, ZnSO₄, zinc bio-activated formulations and ZSB) were applied before sowing. In treatments 8 where only zinc solubilizing was applied, seeds of maize treated with inoculated LB media by using carriers (Orange peel). Different treatments (ZnO, ZnSO4 and zinc bio-activated formulations) were applied before sowing.

Recommended N, P and K were applied in each treatment. Recommended dose of NPK (175 kg ha⁻¹, 160 kg ha⁻¹ and 125 kg ha⁻¹) was applied using Urea, DAP and SOP as sources of N, P and K respectively. All the fertilizers were applied at the time of sowing except Nitrogen (Urea), which was applied in two splits. Field was irrigated with canal water to maintain optimum moisture for plant growth. The plants were irrigated as and when required.

3.8.1. Pre-sowing soil analyses

Same as given in section 3.6.9.

3.8.2. Data collection:

Data of following parameters were recorded by adopting standard measures. These parameters were recorded during growth stages and at the time of harvesting of crop and soil
parameters were collected. Following growth, physiological, yield, chemical and quality parameters were taken.

3.8.3.1. Growth parameters

At maturity, plants were harvested and data about growth parameters were recorded. Plant height, shoots length and cob length was recorded with the help of measuring rod from top to bottom. Fresh shoot biomass was weighted on digital electrical balance. Fresh shoots was firstly sun dried and then placed in the oven till constant weight. Then the shoot biomass was recorded on digital electrical balance. Cob diameter and plant girth were measured with the help of Verner caliper.

3.8.3.2. Physiological parameters

After 55 days of sowing, the physiological parameters i.e. photosynthetic rate (A), transpiration rate (E) stomatal conductance (gs) in all treatments was measured through CIRAS-3 (Potable photosynthesis system). These parameters were taken in the morning.

3.8.3.3. Carbonic anhydrase activity (CA)

Same as given in section 3.7.2.3.

3.8.3.4. Leaf chlorophyll content (SPAD Value)

Same as given in section 3.7.2.4.

3.8.3.5. Electrolyte leakage

Same as given in section 3.7.2.5.

3.8.3.6. Yield parameters:

At maturity, plants were harvested and data about yield parameters were recorded. Grain yield kg ha\(^{-1}\), 1000 grain weight and stover yield kg ha\(^{-1}\) and per cob were recorded. The parameters like no of grains per cob, no of grains per line, no of lines per cob were also calculated.
3.8.3.7. Quality parameters
Same as given in section 3.7.2.7.

3.8.3.8. Analysis of zinc in different plant parts (shoot, root and grains) by wet digestion
Same as given in section 3.7.2.8.

3.8.3.9. Phytates
Same as given in section 3.7.2.10.

3.8.3.10. Phytate to Zn ratio:
Same as given in section 3.7.2.11.

3.9. Statistical analysis:
The Analysis of data was done using analysis of variance technique (ANOVA) with completely randomized design (CRD) for controlled conditions (wire house experiments) and randomized complete block design (RCBD) for field experiments (Steel and Torrie, 1997). For this purpose computer software Statistix 8.1 was used and means were compared by least significant difference (LSD) test.
<table>
<thead>
<tr>
<th>Zinc Formulations</th>
<th>ZnO + Organic material + ZSB</th>
<th>Zn Concentration in BOZ formulation</th>
<th>Organic matter (Grinded Orange peel)</th>
<th>Recommended application of BOZ formulations</th>
<th>Contain total Zn</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-activated zinc formulation 1 (BOZ 1)</td>
<td>90 : 10 (ZSB)</td>
<td>72%</td>
<td>10%</td>
<td>6.8 kg ha⁻¹</td>
<td>4.91 kg</td>
<td>5.1</td>
</tr>
<tr>
<td>Bio-activated zinc formulation 2 (BOZ 2)</td>
<td>80 : 20 (ZSB)</td>
<td>64%</td>
<td>20%</td>
<td>7.65 kg ha⁻¹</td>
<td>4.91 kg</td>
<td>5.1</td>
</tr>
<tr>
<td>Bio-activated zinc formulation 3 (BOZ 3)</td>
<td>70 : 30 (ZSB)</td>
<td>56%</td>
<td>30%</td>
<td>8.74 kg ha⁻¹</td>
<td>4.91 kg</td>
<td>4.9</td>
</tr>
<tr>
<td>Bio-activated zinc formulation 4 (BOZ 4)</td>
<td>60 : 40 (ZSB)</td>
<td>48%</td>
<td>40%</td>
<td>10.20 kg ha⁻¹</td>
<td>4.91 kg</td>
<td>4.8</td>
</tr>
</tbody>
</table>
Table 3.2: Physicochemical characteristics of the soil used for release trial

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Units</th>
<th>Observations (Incubation Trial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>%</td>
<td>53.1</td>
</tr>
<tr>
<td>Silt</td>
<td>%</td>
<td>27.5</td>
</tr>
<tr>
<td>Clay</td>
<td>%</td>
<td>19.4</td>
</tr>
<tr>
<td>Textural class</td>
<td>--</td>
<td>Sandy clay loam</td>
</tr>
<tr>
<td>Saturation percentage</td>
<td>%</td>
<td>33.0</td>
</tr>
<tr>
<td>pH</td>
<td>--</td>
<td>7.7</td>
</tr>
<tr>
<td>EC</td>
<td>dS m⁻¹</td>
<td>1.44</td>
</tr>
<tr>
<td>O.M</td>
<td>%</td>
<td>0.73</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>%</td>
<td>0.05</td>
</tr>
<tr>
<td>Available phosphorus</td>
<td>mg kg⁻¹</td>
<td>5.35</td>
</tr>
<tr>
<td>Extractable potassium</td>
<td>mg kg⁻¹</td>
<td>84</td>
</tr>
<tr>
<td>Available zinc</td>
<td>mg kg⁻¹</td>
<td>0.57</td>
</tr>
</tbody>
</table>
Table 3.3: Physicochemical characteristics of the soil used for Pot trial

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Units</th>
<th>Observations (Pot Trial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>%</td>
<td>51.9</td>
</tr>
<tr>
<td>Silt</td>
<td>%</td>
<td>28.6</td>
</tr>
<tr>
<td>Clay</td>
<td>%</td>
<td>19.5</td>
</tr>
<tr>
<td>Textural class</td>
<td>--</td>
<td>Sandy Clay Loam</td>
</tr>
<tr>
<td>Saturation percentage</td>
<td>%</td>
<td>33.25</td>
</tr>
<tr>
<td>pH</td>
<td>--</td>
<td>7.8</td>
</tr>
<tr>
<td>EC</td>
<td>dS m⁻¹</td>
<td>1.52</td>
</tr>
<tr>
<td>O.M</td>
<td>%</td>
<td>0.74</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>%</td>
<td>0.043</td>
</tr>
<tr>
<td>Available phosphorous</td>
<td>mg kg⁻¹</td>
<td>5.60</td>
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<tr>
<td>Extractable potassium</td>
<td>mg kg⁻¹</td>
<td>92</td>
</tr>
<tr>
<td>Available zinc</td>
<td>mg kg⁻¹</td>
<td>0.59</td>
</tr>
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</table>
Table 3.4: Physicochemical characteristics of the soil used for the field trial I & field trial II

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Units</th>
<th>Observations (Field Trial I)</th>
<th>Observations (Field Trial II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>%</td>
<td>51.2</td>
<td>53.1</td>
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<tr>
<td>Silt</td>
<td>%</td>
<td>29.6</td>
<td>27.5</td>
</tr>
<tr>
<td>Clay</td>
<td>%</td>
<td>19.2</td>
<td>19.4</td>
</tr>
<tr>
<td>Textural class</td>
<td>--</td>
<td>Sandy clay loam</td>
<td>Sandy clay loam</td>
</tr>
<tr>
<td>Saturation percentage</td>
<td>%</td>
<td>32.0</td>
<td>33.0</td>
</tr>
<tr>
<td>pH</td>
<td>--</td>
<td>7.9</td>
<td>7.7</td>
</tr>
<tr>
<td>EC</td>
<td>dS m(^{-1})</td>
<td>1.41</td>
<td>1.43</td>
</tr>
<tr>
<td>O.M</td>
<td>%</td>
<td>0.68</td>
<td>0.71</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>%</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Available phosphorous</td>
<td>mg kg(^{-1})</td>
<td>6.79</td>
<td>5.30</td>
</tr>
<tr>
<td>Extractable potassium</td>
<td>mg kg(^{-1})</td>
<td>84</td>
<td>89</td>
</tr>
<tr>
<td>Available zinc</td>
<td>mg kg(^{-1})</td>
<td>0.61</td>
<td>0.58</td>
</tr>
</tbody>
</table>
CHAPTER 4

RESULTS

Zinc supply in adequate amount is considered indispensable for proper growth and development of plants. There are several reasons for low Zn availability in soil such as Zn deficient parent material, high soil pH, high calcareousness, high salt content and waterlogging conditions. Because Zn is very low in soils and cereals crops grown on such soils are also Zn deficient. Among the nutrients which are deficient in human, Zn ranks 5th. However, human major source of daily calorie intake are cereals grains (wheat, maize and Rice) low in essential minerals. It is crucial to increase bioavailability of Zn in cereals especially maize. It is crucial to increase bioavailability of Zn in maize. ZnSO\textsubscript{4} is most commonly used Zn source. The problem with this, only 4-8\% of total applied zinc is available to plants; all other applied zinc is fixed in soil.

The present study was undertaken to explore the zinc solubilizing bacteria in combination with insoluble source of zinc (ZnO) and organic material may increase the availability of applied source and native Zn in soil. The objective of the study was to formulate & evaluate bio-activated zinc (BOZ) for improving yield and quality of maize. Several strains of zinc solubilizing bacteria were isolated from rhizosphere of maize grown in different areas. The strains capable to solubilize ZnO were further screened for their plant growth promoting activity under axenic conditions. The selected strain was later identified as Bacillus sp. AZ6 and characterized for its plant growth promoting attributes. The selected strain was used with the organic material to bio-activate the insoluble zinc with different formulations. Efficient formulations were evaluated for temporal release of zinc. A pot experiment was conducted for the evaluation of different formulations of bio-activated Zn vs ZnSO\textsubscript{4} on growth, physiology, yield and quality of maize. Then the results of pot trial were confirmed under field conditions by conducting experiments in two maize growing seasons (Field trial I in March, Field trial II in July). The results of all the studies are discussed below.
4.1. Study 1: Zinc solubilizing potential of isolated bacteria

Zinc solubilizing bacteria were isolated from the rhizosphere of the maize by using dilution plate technique on nutrient agar medium and later enrichment culture tin Bunt & Revira media. Total 52 rhizobacterial isolates were selected and from them only 14 were positive for zinc solubilization. Zinc solubilizing potential of bacterial isolates with insoluble source of zinc (ZnO) was assessed both qualitatively and quantitatively under in vitro conditions. The results of zinc solubilization potential of isolated bacteria are given below.

4.1.1. Qualitatively indicator of zinc

In plate assay, the strains produced a clear solubilization halo on Bunt and Revira medium amended with insoluble zinc sources are given in Table 4.1. Most of the bacterial isolates that solubilized zinc performed better than the rest of the isolates. Among the bacterial isolates maximum holozone diameter was observed in AZ6 (3.13 cm). The strains AZ1, AZ3, AZ4, and AZ5 showed minimum zinc solubilization while AZ2, AZ6, AZ7, AZ9, and AZ13 showed holozone diameter ranging from 1.73 to 2.33 cm.

Data revealed (Table 4.1) that the maximum colony diameter was observed in bacterial isolates AZ2, AZ6, AZ7, AZ9 and AZ13. Among the bacterial isolates, maximum colony diameter was observed in AZ6 (0.97 cm) as compared to all bacterial isolates. The bacterial isolates AZ1, AZ3, AZ4, and AZ5 showed minimum colony diameter in plates.

Solubilizing area of different zinc solubiling bacterial isolates were presented in Table 4.1. Most of the bacterial strain solubilizes zinc but AZ6, AZ7, AZ9 and AZ13 AZ2, (7.69, 4.26, 3.71, 2.91 and 2.35 cm² respectively) perform better than all other strains. Among the bacterial isolates, maximum solubilization area was observed in AZ6 (7.69 cm²). The bacterial isolates, AZ1, AZ3, AZ4, and AZ5 were showed low potential.

4.1.2. Quantitative assay:

4.1.2.1. Amount of solubilized zinc (µg zinc/mL)

Zinc solubilizing on liquid Bunt and Revira medium supplemented with zinc oxide at 0.1% zinc concentration are presented in Fig 4.1. Most of the bacterial isolates solubilized zinc but AZ6, AZ13, AZ9, AZ7 and AZ2 (14, 9, 9, 8 and 4 µg zinc/mL respectively) perform better than all other bacterial isolates and compared to control (2 µg
Among the bacterial isolates, AZ6 showed maximum zinc solubilization that was 14 µg zinc/mL. The bacterial isolates, AZ1, AZ3, AZ4, and AZ5 showed minimum zinc solubilizing compared to other isolates. Compared to control all bacteria solubilized zinc significantly.

4.1.2.2. pH of culture medium

There was a significant decrease in pH of the medium inoculated with zinc solubilizing bacterial isolates as compared to un-inoculated control. Data regarding pH decrease is represented in Fig. 4.2. Most of the bacterial strains reduce pH of the broth medium and solubilized insoluble zinc, but maximum pH reduction was observed with AZ6. The strains AZ1, AZ3, AZ4, and AZ5 showed minimum decrease in pH. It caused decrease in pH of broth media containing zinc oxide 4.9 as compare to control.
### Table 4.1. Zinc solubilizing potential of isolated bacteria (Qualitatively)

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>After 7 days</th>
<th>Parameters</th>
<th>Colony diameter (cm)</th>
<th>Area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Holozone diameter (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ1</td>
<td>1.27 k</td>
<td>0.28 i</td>
<td>1.27 k</td>
<td></td>
</tr>
<tr>
<td>AZ2</td>
<td>1.73 f</td>
<td>0.39 f</td>
<td>2.35 f</td>
<td></td>
</tr>
<tr>
<td>AZ3</td>
<td>1.37 j</td>
<td>0.23 k</td>
<td>1.47 j</td>
<td></td>
</tr>
<tr>
<td>AZ4</td>
<td>1.43 i</td>
<td>0.26 j</td>
<td>1.61 i</td>
<td></td>
</tr>
<tr>
<td>AZ5</td>
<td>1.30 k</td>
<td>0.32 h</td>
<td>1.33 k</td>
<td></td>
</tr>
<tr>
<td>AZ6</td>
<td>3.13 a</td>
<td>0.97 a</td>
<td>7.69 a</td>
<td></td>
</tr>
<tr>
<td>AZ7</td>
<td>2.33 b</td>
<td>0.46 c</td>
<td>4.26 b</td>
<td></td>
</tr>
<tr>
<td>AZ8</td>
<td>1.60 h</td>
<td>0.35 g</td>
<td>2.02 h</td>
<td></td>
</tr>
<tr>
<td>AZ9</td>
<td>2.17 c</td>
<td>0.53 b</td>
<td>3.71 c</td>
<td></td>
</tr>
<tr>
<td>AZ10</td>
<td>1.73 f</td>
<td>0.34 g</td>
<td>2.36 f</td>
<td></td>
</tr>
<tr>
<td>AZ11</td>
<td>1.90 d</td>
<td>0.42 de</td>
<td>2.84 d</td>
<td></td>
</tr>
<tr>
<td>AZ12</td>
<td>1.87 e</td>
<td>0.41 e</td>
<td>2.75 e</td>
<td></td>
</tr>
<tr>
<td>AZ13</td>
<td>1.93 d</td>
<td>0.42 d</td>
<td>2.91 d</td>
<td></td>
</tr>
<tr>
<td>AZ14</td>
<td>1.67 g</td>
<td>0.38 f</td>
<td>2.18 g</td>
<td></td>
</tr>
</tbody>
</table>

*Means sharing the same letter do not differ significantly (P< 0.05)*
Means sharing the same letter do not differ significantly ($P < 0.05$)

Fig. 4.1. Zinc solubilizing potential of isolated bacteria (Quantitative)
Means sharing the same letter do not differ significantly ($P<0.05$)

Fig. 4.2. Zinc solubilizing potential of isolated bacteria (Quantitative)
4.2. Study 2: Effective zinc solubilizing bacteria for improving growth of maize seedlings (Growth room study)

Ten effective zinc solubilizing bacterial isolates were selected on the basis of zinc solubilization potential from study # 1. The selected isolates were further screened for their growth promoting activity under axenic conditions. The results imply that the zinc solubilizing isolates significantly improved the growth of maize seedlings. The results are given below.

4.2.1. Growth parameters

Data (Table 4.2) showed positive effect of most of the isolates of zinc solubilizing bacteria on shoot length of maize seedling as compared to un-inoculated control. It was observed that maximum shoot length was produced by AZ6, AZ13, AZ7 and AZ2. Bacterial isolates AZ6 increased the shoot length 59% more as compared un-inoculated control.

Root lengths of maize seedling were also improved by most of the zinc solubilizing bacteria. It was observed that maximum root length was produced by AZ6 (12.13 cm) and minimum was observed un-inoculated control (7.70 cm).

Maximum shoot fresh biomass was produced by AZ6 (2.39 g plant⁻¹) followed by AZ11 (2.20 g plant⁻¹), AZ10 (2.14 g plant⁻¹) and AZ9 (2.06 g plant⁻¹) while in un-inoculated shoot fresh biomass was up to 7.70 g plant⁻¹.

Root fresh biomass of maize seedling was also increased by the zinc solubilizing bacterial isolates. It was observed that maximum root fresh biomass was produced by AZ6 (1.81 g plant⁻¹) and minimum was observed in un-inoculated control (0.43 g plant⁻¹). The other isolated produced dry root biomass in the range of 0.50 to 1.54 g plant⁻¹.

Zinc solubilizing bacterial isolates significantly improved shoots dry biomass of maize seedling. It was observed that maximum shoot dry biomass was produced by AZ6 (0.55 g plant⁻¹) minimum was observed in un-inoculated control (0.29 g plant⁻¹).

Bacterial isolates showed positive impact on root dry biomass of maize seedling. It was observed that maximum root dry biomass was produced by AZ6 (0.57 g plant⁻¹) and minimum was observed in un-inoculated control (0.12 g plant⁻¹). The other isolated produced dry root biomass in the range of 0.15 to 0.55 g plant⁻¹.

It was evident from the all the growth promoting parameters that all the bacterial isolates enhanced growth of maize seedling as compared to un-inoculated control. It was
also observed that AZ6 was best growth promoting strain compared from to all other strains.

4.2.1. Physiological parameters

Data (Table 4.3) showed positive effect of most of the isolates of zinc solubilizing bacteria on photosynthetic rate of maize as compared to un-inoculated control. It was observed that maximum photosynthetic rate was observed in AZ6 (6.14 μmol CO$_2$ m$^{-2}$ s$^{-1}$) and minimum was in un-inoculated control (3.23 μmol CO$_2$ m$^{-2}$ s$^{-1}$). Bacterial isolates AZ6 increased the photosynthetic rate 90% more as compared un-inoculated control.

Transpiration rate of maize plant were also increased by most of the zinc solubilizing bacterial isolates. It was observed that maximum transpiration rate was produced by AZ6 (2.32 mmol H$_2$O m$^{-2}$ s$^{-1}$) and minimum was observed un-inoculated control (1.11 mmol H$_2$O m$^{-2}$ s$^{-1}$).

Bacterial isolates showed positive impact on stomatal conductance of maize plants. It was observed that maximum stomatal conductance was produced by AZ6 (135 mmol m$^{-2}$ s$^{-1}$) and minimum was observed in un-inoculated control (106 mmol m$^{-2}$ s$^{-1}$). The other isolated showed stomatal conductance in the range of 112 to 130 mmol m$^{-2}$ s$^{-1}$.

Zinc solubilizing bacterial isolates significantly improved chlorophyll contents of maize plant. It was observed that maximum chlorophyll contents were in AZ6.

Overall it was observed that all the bacterial isolates improved physiology of maize plant as compared to un-inoculated control but AZ6 was best growth promoting strain compared from to all other strains.
Table 4.2. Effect of zinc solubilizing bacteria on growth of maize seedlings

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Shoot length</th>
<th>Root length</th>
<th>Shoot fresh biomass</th>
<th>Root fresh biomass</th>
<th>Shoot dry biomass</th>
<th>Root dry biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units</td>
<td>cm</td>
<td>cm</td>
<td>g/plant</td>
<td>g/ plant</td>
<td>g/ plant</td>
<td>g/ plant</td>
</tr>
<tr>
<td>Control</td>
<td>20.33 i</td>
<td>7.70 i</td>
<td>1.707 i</td>
<td>0.43 h</td>
<td>0.29 f</td>
<td>0.12 h</td>
</tr>
<tr>
<td>AZ2</td>
<td>29.00 cd</td>
<td>10.20 de</td>
<td>1.940 e</td>
<td>0.76 f</td>
<td>0.30 f</td>
<td>0.23 f</td>
</tr>
<tr>
<td>AZ6</td>
<td>32.33 a</td>
<td>12.13 a</td>
<td>2.393 a</td>
<td>1.81 a</td>
<td>0.55 a</td>
<td>0.57 ab</td>
</tr>
<tr>
<td>AZ7</td>
<td>29.33 bc</td>
<td>10.43 cd</td>
<td>1.880 g</td>
<td>0.50 g</td>
<td>0.41 d</td>
<td>0.15 g</td>
</tr>
<tr>
<td>AZ8</td>
<td>26.53 f</td>
<td>8.70 h</td>
<td>1.777 h</td>
<td>0.80 e</td>
<td>0.26 g</td>
<td>0.25 e</td>
</tr>
<tr>
<td>AZ9</td>
<td>28.50 d</td>
<td>9.90 e</td>
<td>2.060 d</td>
<td>1.57 b</td>
<td>0.41 d</td>
<td>0.53 b</td>
</tr>
<tr>
<td>AZ10</td>
<td>27.16 e</td>
<td>9.50 f</td>
<td>2.143 c</td>
<td>1.03 d</td>
<td>0.45 c</td>
<td>0.36 c</td>
</tr>
<tr>
<td>AZ11</td>
<td>28.70 d</td>
<td>10.53 c</td>
<td>2.220 b</td>
<td>0.80 e</td>
<td>0.51 b</td>
<td>0.24 ef</td>
</tr>
<tr>
<td>AZ12</td>
<td>25.50 g</td>
<td>8.43 h</td>
<td>2.013 e</td>
<td>1.54 b</td>
<td>0.36 e</td>
<td>0.55 b</td>
</tr>
<tr>
<td>AZ13</td>
<td>29.76 b</td>
<td>11.33 b</td>
<td>2.037 de</td>
<td>1.09 c</td>
<td>0.40 d</td>
<td>0.36 c</td>
</tr>
<tr>
<td>AZ14</td>
<td>24.66 h</td>
<td>9.13 g</td>
<td>1.967 f</td>
<td>1.01 d</td>
<td>0.34 e</td>
<td>0.32 d</td>
</tr>
</tbody>
</table>

Means sharing the same letter do not differ significantly (P< 0.05)
Table 4.3. Effect of zinc solubilizing bacteria on physiology of maize seedlings (Growth room study)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Photosynthetic rate (A)</th>
<th>Transpiration rate (E)</th>
<th>Stomatal conductance (gs)</th>
<th>Chlorophyll Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units</td>
<td>µmol CO₂ m⁻² s⁻¹</td>
<td>mmol H₂O m⁻² s⁻¹</td>
<td>mmol m⁻² s⁻¹</td>
<td>Spade value</td>
</tr>
<tr>
<td>Control</td>
<td>3.23 g</td>
<td>1.11 i</td>
<td>106.67 i</td>
<td>13.10 j</td>
</tr>
<tr>
<td>AZ2</td>
<td>4.23 f</td>
<td>1.41 g</td>
<td>130.33 b</td>
<td>31.36 b</td>
</tr>
<tr>
<td>AZ6</td>
<td>6.14 a</td>
<td>2.32 ab</td>
<td>135.00 a</td>
<td>35.73 a</td>
</tr>
<tr>
<td>AZ7</td>
<td>5.43 b</td>
<td>1.91 e</td>
<td>126.00 c</td>
<td>27.50 d</td>
</tr>
<tr>
<td>AZ8</td>
<td>4.65 cd</td>
<td>1.20 h</td>
<td>115.67 g</td>
<td>14.63 i</td>
</tr>
<tr>
<td>AZ9</td>
<td>4.40 e</td>
<td>2.03 d</td>
<td>122.67 de</td>
<td>20.23 f</td>
</tr>
<tr>
<td>AZ10</td>
<td>4.26 f</td>
<td>1.53 f</td>
<td>118.00 fg</td>
<td>15.90 gh</td>
</tr>
<tr>
<td>AZ11</td>
<td>4.61 d</td>
<td>2.10 c</td>
<td>125.33 cd</td>
<td>22.56 e</td>
</tr>
<tr>
<td>AZ12</td>
<td>4.20 f</td>
<td>1.18 h</td>
<td>112.33 h</td>
<td>15.43 hi</td>
</tr>
<tr>
<td>AZ13</td>
<td>4.74 c</td>
<td>2.19 b</td>
<td>127.33 c</td>
<td>29.30 c</td>
</tr>
<tr>
<td>AZ14</td>
<td>4.30 ef</td>
<td>1.53 f</td>
<td>120.67 ef</td>
<td>16.76 g</td>
</tr>
</tbody>
</table>

*Means sharing the same letter do not differ significantly (P< 0.05)*
Picture 4.1: Effect of zinc solubilizing isolates on the growth of maize seedlings
4.3. Identification and characterization of selected strain

4.3.1. Identification of selected strain

The 16S rRNA gene (1354 bp) amplified from the strain AZ6 was sequenced; the sequence was deposited in the GenBank database under the accession no. AZ6 KT221633. The Blast analysis of the 16S rRNA amplicon indicated its maximum similarity with the bacterial strains belonging to genus Bacillus. In-silico analysis of the 16S rRNA of the bacterial strain AZ6 was carried out by constructing the phylogenetic tree following the neighbor joining method. The bacterial strain AZ6 was observed to be phylogenetically positioned in the cluster comprising the bacterial strains belonging to the genus Bacillus. Following the phylogenetic relationship of the strain AZ6 with several Bacillus sp. (Fig. 4.1), this bacterial isolate was named as Bacillus sp. AZ6. The GeneBank accession numbers of the strains used for in-silico analysis are given in brackets, whereas, Bootstrap values greater than 800% are marked as black circles.

Identified strain Bacillus sp. AZ6 KT221633 potential to solubilize insoluble source of zinc (ZnO) is shown in picture 4.2.
Fig. 4.3. Neighbor-joining phylogenetic analysis resulting from the multiple alignment of 16S rRNA gene sequence of Bacillus sp. with those of other bacterial strains found in GeneBank database.
4.3.2. Characterization

Selected zinc solubilizing strain (*Bacillus sp. AZ6*) was characterized for various characters such as IAA (Indole-3-acitic acid) production activity, ACC-deaminase activity, siderophore production activity and production of organic acids. The results are given below.

4.3.2.1. ACC Deaminase activity (α-ketobutyrate nmol g\(^{-1}\) biomass hr\(^{-1}\))

ACC Deaminase activity (Table 4.4) of the selected zinc solubilizing bacterial (*Bacillus sp. AZ6*) was 211.34 α-ketobutyrate nmol g\(^{-1}\) biomass hr\(^{-1}\).

4.3.2.2. Auxin production (µg/mL)

Data regarding auxin biosynthesis (Table 4.4) by *Bacillus sp. AZ6* indicated auxin production (as IAA equivalents) both in the presence and absence of L-tryptophan. In the absence of L-tryptophan it was 12.03 µg/mL auxin. In the presence of L-auxin production was 35.30 µg/mL.

4.3.2.3. Siderophore production

The production of low molecular weight, iron chelating siderophores by Zn mobilizing bacterial strain was detected on blue agar. The solubilizing bacterial strain expressed the capability to chelate Fe but with various strength. The orange holozone were produced by the strain. A change in blue to orange color depicted production of siderophores (Table 4.4).

4.3.2.4. Analysis of bacterial metabolites by HPLC

The bacterial metabolites as extracted by methanol was analyzed by HPLC (Shimadzu, Japan) with LC 10AT, UV-visible and SPD 10 AV). The results showed that the chromatograms of bacterial metabolites exhibited different patterns (Fig. 4.5) and many peaks were detected. Based on comparison with the standards, some of the compounds in the metabolites could be identified. The values detected in chromatogram are explained in Fig 4.4. Cinamic acid (9.8 mg/L), Ferulic acid (8.1 mg/L), Caffeic acid (5.9 mg/L), Chlorggenic acid (5.8 mg/L), Syrirgic acid (3.8 mg/L), Gallic acid (3.8 mg/L) were present in the metabolites of *Bacillus sp. AZ6*. 
Table 4.4. Growth promoting characteristics of *Bacillus sp. AZ6*

<table>
<thead>
<tr>
<th>Character</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC Deaminase activity</td>
<td>211.34 $\alpha$-ketobutyrate nmol g$^{-1}$ biomass hr$^{-1}$</td>
</tr>
<tr>
<td>Auxin production</td>
<td>12.03 $\mu$g/mL (Without L-Tryptophan)</td>
</tr>
<tr>
<td></td>
<td>35.30 $\mu$g/mL (With L-Tryptophan)</td>
</tr>
<tr>
<td>Siderophore production</td>
<td>+++</td>
</tr>
</tbody>
</table>
**Shimadzu, Japan LC 10AT, UV-visible and SPD 10 AV**

**Fig. 4.4:** HPLC standardization peaks for organic release

**Fig. 4.5.** Bacterial metabolite profiles, detected by HPLC
Picture 4.2: Holozone and colony diameter of *Bacillus sp. AZ6*
4.5. Study 3: Impact of different formulations of bio-activated zinc (BOZ) vs ZnSO₄ on temporal release of zinc in soil (Incubation Study)

4.5.1. Temporal release of zinc in soil

The main purpose of this study was to investigate the rate of Zn release with respect to time (temporal zinc release) and effect of bio-activation on Zn solubilization.

Comparative effectiveness of various sources of zinc such as ZnO, ZnSO₄ and different formulations of bio-activated zinc (BOZ) on the temporal release of zinc is evident from Fig. 4.6. Data shows that different bio-activated zinc formulations significantly improved the zinc availability during the incubation trial over control and all the other combinations. During incubation, zinc release was increased as incubation time increased in all the formulation of Zn in the form of chemical, biological and bio-activated combinations and became maximum at 60th day of incubation in the all the combination under investigation. However, decrease in zinc release pattern was noted after 60th day of incubation. Different chemical, biological and bio-activated combinations of Zn released different amount of Zn during the incubation trial. The maximum zinc release was noted with the application of BOZ4 combination. It released the highest zinc during all the sampling times compared to all other combinations. It was followed by BOZ3, BOZ2, ZnSO₄, BOZ1, ZSB, ZnO and control. The statistical analysis showed a significant interactive effect of different combinations of Zn and time of incubation. Accordingly, the maximum release was noted with the application of BOZ4 at 60th day (1.65 mg kg⁻¹) of incubation and the minimum was noted in case of control at 24th days (0.60 mg kg⁻¹) of incubation. It was also noted that with the application of BOZ 4 formulation zinc bioavailability increased up to 23% as compared to ZnSO₄ at 60th days of incubation.
Fig. 4.6: Impact of different formulations of bio-activated zinc (BOZ) vs ZnSO$_4$ on temporal release of zinc in soil
4.6. Study # 4:
Evaluation of different formulations of bio-activated Zn vs ZnSO₄ on growth, physiology, yield and quality of maize (Wire House Experiment)

The pot experiment was conducted in the wire house. The main purpose of the study was the evaluation of different formulations of bio-activated Zn vs ZnSO₄ on growth, physiology, yield and quality of maize. The results of the above study are given.

4.6.1. Plant height

Comparative effectiveness of various sources of zinc such as ZnO (unavailable form), ZnSO₄ (available form) and different formulations of bio-activated zinc (BOZ) on plant height is evident from Fig. 4.7(a). All the plants received basal doses of NPK. Statistical analysis of data revealed that various treatments had significant effect on plant height as compared to control. Application of ZnO alone did not have any significance influence on plant height whereas ZnSO₄ promoted plant height (147 cm) significantly. Plant height was increased by most of the bio-activated formulations and maximum plant height was recorded in BOZ 4 (168 cm) and BOZ3 (161 cm). Inoculation with zinc solubilizing bacteria (143 cm) also promoted plant height as compared to uninoculated control.

4.6.2. Root length

The impact of various sources of zinc i.e ZnO, ZnSO₄ and different formulations of bio-activated zinc (BOZ) on root length is indicated in Fig. 4.7(b). The results showed that BOZ4 and BOZ3 promoted root length as compared to available form of zinc (ZnSO₄). BOZ4 increased root length by 20% as compared to available form of zinc (ZnSO₄) and 35% as compared to unavailable form of zinc (ZnO). Inoculation with zinc solubilizing bacteria also promoted root length by 14% as compared to uninoculated control. BOZ1 and BOZ2 have similar effect as ZnSO₄.
Means sharing the same letter do not differ significantly ($P < 0.05$)

Fig. 4.7: Effect of bio-activated zinc vs ZnSO$_4$ on (a) plant height & (b) root length of maize in pot trial

Treatments Description:

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO$_4$ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
4.6.3. Fresh shoot biomass (g pot\(^{-1}\))

The effectiveness of applied treatments on fresh shoot biomass is evident from Fig. 4.8(a). All the treatments are statistically significant as compared to control except unavailable form of zinc (ZnO). The comparison among treatment revealed that formulation BOZ4 successfully increased fresh shoot biomass (262 g pot\(^{-1}\)) as compared to ZnSO\(_4\) (245.33 g pot\(^{-1}\)). Inoculations of zinc solubilizing bacteria promoted fresh shoot biomass (237.67 g pot\(^{-1}\)) as compared to inoculated control (220 g pot\(^{-1}\)).

4.6.4. Dry shoot biomass (g pot\(^{-1}\))

The results related to dry shoot biomass are presented in Fig. 4.8 (b). With the application of various sources of zinc i.e ZnO, ZnSO\(_4\) and different formulations of bio-activated zinc (BOZ) showed better improvement in dry shoot biomass as compared to control (no zinc). Among bio-activated zinc formulations, BOZ4 showed maximum shoot dry biomass, 26% higher as compared to ZnSO\(_4\). BOZ1 and BOZ2 have similar effect as ZnSO\(_4\). The measured shoot dry biomass was promoted with the application of ZnO (122 g pot\(^{-1}\)), ZnSO\(_4\) (132 g pot\(^{-1}\)) and zinc solubilizing bacteria (123 g pot\(^{-1}\)) as compared to control (112 g pot\(^{-1}\)).

4.6.5. Fresh root biomass (g pot\(^{-1}\))

The effectiveness of applied treatments on fresh root biomass is given in Fig.4.9 (a). The results indicated that fresh root biomass was promoted by the application of all the zinc sources as compared to control. Among bio-activated zinc formulations, BOZ4 improved fresh root biomass by 31% more as compared to ZnSO\(_4\) application. The measured fresh root biomass was 18% more with inoculation of zinc solubilizing bacteria as compared to uninoculated control. BOZ 2 & BOZ 1 showed fresh root biomass at par with ZnSO\(_4\).

4.6.6. Dry root biomass (g pot\(^{-1}\))

The results regarding root dry weight are indicated in Fig. 4.9 (b). The results showed that recorded root dry weight was higher in all the treatments where any source of zinc was applied. All the bio-activated zinc formulation improved dry root biomass as compared to control (no zinc). Among formulations of bio-activated zinc, BOZ4 followed by BOZ3 and
BOZ2 showed maximum root dry weight as compared to ZnSO4. Inoculation of ZSB only improved dry root biomass 26% more as compared to un-inoculated control.

4.6.7. Cob length

The evidence of results regarding cob length is given in Fig. 4.10(a). All the treatments showed significantly higher cob length as compared to control. The BOZ4 showed 19% higher cob length as compared to ZnSO4. The ascending order of treatments for cob length is control < ZnO < ZSB < ZnSO4 ≤ BOZ1 ≤ BOZ2 < BOZ3 < BOZ4.

4.6.8. Cob diameter

The results regarding cob diameter are indicated in Fig. 4.10 (b). The results revealed that cob diameter was promoted by the application of all the zinc sources as compared to control. Among bio-activated zinc formulations, BOZ 4 improved cob diameter 16% more as compared to ZnSO4 and 31% more as compared to ZnO. Inoculation of zinc solubilizing bacteria (10.17 cm) improved cob diameter non-significantly as compared to uninoculated control (9.17 cm).
Means sharing the same letter do not differ significantly ($P < 0.05$).

Fig. 4.8: Effect of bio-activated zinc vs commercial ZnSO$_4$ on (a) fresh shoot biomass & (b) dry shoot biomass of maize in pot trial.

**Treatments Description:**

**T1:** Control (No zinc)

**T2:** ZnO (unavailable form)

**T3:** ZnSO$_4$ (available form)

**T4:** Bio-activated Zn formulation 1 (BOZ 1)

**T5:** Bio-activated Zn formulation 2 (BOZ 2)

**T6:** Bio-activated Zn formulation 3 (BOZ 3)

**T7:** Bio-activated Zn formulation 4 (BOZ 4)

**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly (P< 0.05)

Fig.4.9: Effect of bio-activated zinc vs commercial ZnSO₄ on (a) fresh root biomass & (b) dry root biomass of maize in pot trial

Treatments Description:

T1: Control (No zinc)                      T5: Bio-activated Zn formulation 2 (BOZ 2)
T2: ZnO (unavailable form)                T6: Bio-activated Zn formulation 3 (BOZ 3)
T3: ZnSO₄ (available form)                T7: Bio-activated Zn formulation 4 (BOZ 4)
T4: Bio-activated Zn formulation 1 (BOZ 1) T8: Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly ($P < 0.05$)

**Fig. 4.10:** Effect of bio-activated zinc vs commercial ZnSO$_4$ on (a) cob length & (b) cob diameter of maize in pot trial

**Treatments Description:**

- **T1:** Control (No zinc)  
- **T2:** ZnO (unavailable form)  
- **T3:** ZnSO$_4$ (available form)  
- **T4:** Bio-activated Zn formulation 1 (BOZ 1)  
- **T5:** Bio-activated Zn formulation 2 (BOZ 2)  
- **T6:** Bio-activated Zn formulation 3 (BOZ 3)  
- **T7:** Bio-activated Zn formulation 4 (BOZ 4)  
- **T8:** Zinc solubilizing bacteria (ZSB), only inoculation
4.6.9. Plant girth

The effectiveness of bio-activated zinc formulation vs ZnSO\textsubscript{4} on plant girth is given in Fig. 4.11 (a). Statistical analysis revealed that all the treatments promoted plant girth significantly as compared to control. With the application of BOZ4 34% more plant girth was obtained as compared to ZnSO\textsubscript{4} and 69% more as compared to ZnO. BOZ 2 and BOZ1 had almost similar result as that of ZnSO\textsubscript{4}. Inoculation with zinc solubilizing bacteria also promoted plant girth significantly as compared to control (no zinc).

4.6.10. Carbonic anhydrase activity

The results regarding carbonic anhydrase activity are illustrated in Fig. 4.11 (b). Treatment comparison revealed that various zinc sources (ZnO, ZnSO\textsubscript{4} & bio-activated zinc formulations) improved the CA activity of prescribed enzyme as compared to control (no zinc). Among the bio-activated zinc formulation, BOZ 4 showed CA enzyme activity to be 38% more as compared to ZnSO\textsubscript{4} and 54% more as compared ZnO. All other bio-activated zinc formulations also showed better CA activity as compared to ZnSO\textsubscript{4}. Inoculation with zinc solubilizing bacteria also promoted CA activity significantly as compared to control (no zinc).

4.6.11. Photosynthetic rate

Comparative effectiveness of various sources of zinc such as ZnO (unavailable form), ZnSO\textsubscript{4} (available form) and different formulations of bio-activated zinc (BOZ) on photosynthetic rate is evident from Fig. 4.12 (a). Statistical analysis of data revealed that various treatments had significantly effects on photosynthetic rate as compared to control. Photosynthetic rate was increased by the most of the bio-activated formulations and maximum photosynthetic rate was recorded in BOZ 4 (23.23 μmol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}) and BOZ3 (19 μmol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}). Among bio-activated zinc formulations, BOZ4 gave statistically maximum photosynthetic rate, 40% higher than ZnSO\textsubscript{4}. Inoculation with zinc solubilizing bacteria (15 μmol CO\textsubscript{2} m\textsuperscript{-2} s\textsuperscript{-1}) also promoted photosynthetic rate as compared to uninoculated control.
4.6.12. Transpiration rate

The Fig. 4.12 (b) showed the results of transpiration rate. Comparison among treatments revealed that all treatments showed higher transpiration rate as compared to control. Among the bio-activated zinc formulations, BOZ4 improved transpiration rate by 39% more as compared to ZnSO₄. Inoculation with zinc solubilizing bacteria (6.58 mmol H₂O m⁻² s⁻¹) showed higher transpiration rate as compared to inoculated control but the effect of ZSB was non-significant. BOZ 1 showed lower transpiration rate as compared to ZnSO₄.

4.6.13. Stomatal conductance

The results regarding stomatal conductance are indicated in the Fig. 4.13 (a). Results revealed that all the applied zinc sources (ZnO, ZnSO₄, bio-activated zinc formulations) showed higher stomatal conductance as compared to control. Among the bio-activated zinc formulations, BOZ4 showed 14% more stomatal conductance as compared to ZnSO₄. The slight increase in stomatal conductance was observed with ZSB (197 mmol m⁻² s⁻¹) as compared to control (185.3 mmol m⁻² s⁻¹). BOZ 2 and BOZ were statistically at par as compared to ZnSO₄.


Efficacy of bio-activated zinc vs ZnSO₄ on chlorophyll contents of maize plants are indicated in Fig.4.13 (b). Treatment comparison revealed that various sources of zinc i.e., ZnO, ZnSO₄ and bio-activated zinc formulations showed higher chlorophyll contents as compared to control. Among all treatments, BOZ4 formulation followed by BOZ 3 and BOZ2 increased chlorophyll contents more as compared to ZnSO₄ treatment. BOZ4 showed 22% more chlorophyll contents as compared to unavailable source of zinc (ZnSO₄). The increase in chlorophyll contents was also observed with the application of ZnO (36.6 spade value), and inoculation with zinc solubilizing bacteria (38.17 spade value) as compared to control (30.18 spade value).
Means sharing the same letter do not differ significantly ($P < 0.05$)

Fig.4.11: Effect of bio-activated zinc vs commercial ZnSO$_4$ on (a) plant girth & (b) carbonic anhydrase activity of maize in pot trial

**Treatments Description:**

**T1:** Control (No zinc)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)

**T2:** ZnO (unavailable form)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)

**T3:** ZnSO$_4$ (available form)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)

**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly (P< 0.05)

Fig.4.12: Effect of bio-activated zinc vs commercial ZnSO₄ on (a) photosynthetic rate & (b) transpiration rate of maize in pot trial

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO₄ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly (P< 0.05)

Fig.4.13: Effect of bio-activated zinc vs commercial ZnSO₄ on (a) stomatal conductance & b) chlorophyll contents of maize in pot trial

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO₄ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
4.6.15. Electrolyte leakage (%)

The effect of various sources of applied zinc on electrolyte leakage is presented in Fig. 4.14 (a). The results regarding treatment comparison revealed that most of the treatments had significantly decreased electrolyte leakage as compared to control (no zinc). Among the bio-activated zinc formulations, BOZ4 & BOZ 3 showed 36 and 32% decrease in electrolyte leakage as compared to available form of zinc (ZnSO₄). Statistically ZnO had no effect on the electrolyte leakage while inoculation with zinc solubilizing bacteria showed 6% decreased in electrolyte leakage as compared to control (no zinc).

4.6.16. Grain yield (g pot⁻¹)

Comparative effectiveness of various sources of zinc such as ZnO (unavailable form), ZnSO₄ (available form) and different formulations of bio-activated zinc (BOZ) on grain yield is evident from Fig. 4.14 (b). Statistical analysis of data revealed that various treatments had significantly effects on grain yield compared to control. The results showed that recorded grain yield (g pot⁻¹) was higher in bio-activated zinc formulation, i.e, BOZ2 < BOZ3 < BOZ4. Among the bio-activated zinc formulations BOZ4 improved grain yield by 11% as compared to ZnSO₄ and 14% more as compared to ZnO. Inoculation with zinc solubilizing bacteria increased grain yield (g pot⁻¹) 6% as compared to control (no zinc).

4.6.17. 100 grain weight (g)

The data regarding 100 grain weight in presented in in Fig. 4.15 (a). The results revealed that bio-activated zinc formulations and ZnSO₄ significantly increased 100 grain weight. Application of BOZ4 formulation increased 5% more 100 grain weight as compared to ZnSO₄. The treatment comparison showed that among bio-activated zinc formulations, BOZ4 and BOZ3 showed same results regarding 100 grain weight while BOZ1 and ZnSO₄ had same effect in the increment of grain weight. Inoculation with zinc solubilizing bacteria showed 4% more 100 grain weight as compared to control (no zinc).

4.6.18. Stover yield (g pot⁻¹)

The effectiveness of treatments regarding stover yield is indicated in Fig. 4.15 (b). The results showed that all the treatments significantly increased stover yield as compared to
control (no zinc). The observed increment in stover yield with BOZ4 was 20% over ZnSO$_4$. ZnO alone improved stover yield but the increment was statistically non-significant with respect to control. Inoculation with zinc solubilizing bacteria showed slightly higher stover yield as compared to control (no zinc).

4.6.19. Number of grains per cob

The result for number of grains per cob is given in Fig. 4.16 (a). The treatments comparison revealed that bio-activated zinc formulations (BOZ2, BOZ3 & BOZ4) showed higher number of grains per cob as compared to ZnSO$_4$. Among these formulations, BOZ 4 improved number of grains by 5% per cob as compared to available form of zinc (ZnSO$_4$). Statistical analysis showed that sole application of ZnO and inoculation with zinc solubilizing bacteria had no effect significant on number of grains per cob over control.

4.6.20. Number of grains per line

The data regarding number of grains per line is presented in graphical form in Fig. 4.16 (b). The results revealed that all the zinc sources i.e., bio-activated zinc formulations, ZnSO$_4$ and ZnO successfully increased number of grains per line. Among the bio-activated zinc formulations BOZ2 < BOZ3 < BOZ4 showed higher no. of grains per line as compared to ZnSO$_4$. BOZ4 showed 7% increased number of grains per line as compared to ZnSO$_4$. Sole application of ZnO and inoculation with zinc solubilizing bacteria had no significant effect in the increment of number of grain as compared to control (no zinc).
Means sharing the same letter do not differ significantly (P< 0.05)

Fig.4.14: Effect of bio-activated zinc vs commercial ZnSO₄ on (a) electrolyte leakage & (b) grain yield/pot of maize in pot trial

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO₄ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly \( P < 0.05 \)

Fig.4.15: Effect of bio-activated zinc vs commercial ZnSO\(_4\) on (a) 100 grain weight & (b) stover yield/pot of maize in pot trial

**Treatments Description:**

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO\(_4\) (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
**Means sharing the same letter do not differ significantly (P< 0.05)**

**Fig.4.16:** Effect of bio-activated zinc vs commercial ZnSO$_4$ on (a) number of grain per cob & (b) number of grain per line of maize in pot trial

**Treatments Description:**

**T1:** Control (No zinc)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)

**T2:** ZnO (unavailable form)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)

**T3:** ZnSO$_4$ (available form)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)

**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
4.6.21. Number of line per cob

The results regarding on number of line per cob are indicated in Fig. 4.17 (a). The results showed that higher number of line per cob was observed with the application of formulations of bio-activated zinc. Statistical analysis showed that application of ZnO and ZSB had not significantly increase number of line per cob as compared to control (no zinc). All bio activated Zn formulations had increased number of line per cob as BOZ1 < BOZ2 < BOZ3 < BOZ4. BOZ4 showed 23% more number of line per cob as compared to ZnSO4.

4.6.22. Crude protein

The effect of different sources of zinc (ZnO, ZnSO4 and bio-activated zinc formulations) on crude protein is given in Fig. 4.17 (b). The crude protein is found maximum with formulations of bio-activated zinc i.e. BOZ4 (13.23 % crude protein) and minimum with control (9.61% crude protein). Statistical analysis revealed that ZnO and ZSB had no significant effect on crude protein while all other treatments significantly improved crude protein as compared to control. Among bio-activated zinc formulations, the higher crude protein was found with BOZ4 followed by BOZ3, BOZ2 and BOZ1. Bio-activated zinc formulation (BOZ4) improved crude protein 16% more as compared to control (no zinc).

4.6.23. Oil contents

The results regarding oil contents are indicated in Fig. 4.18 (a). The maximum oil contents were found with BOZ4 (5.49% oil contents) and minimum with control (3.35 % oil contents). Statistical analysis revealed that application of ZnSO4 (4.49% oil contents) and inoculation with zic solubilizing bacteria (4.06% oil contents) also showed slightly higher in oil contents while sole application of ZnO had no significant effect as compared to control. Among formulations of bio-activated zinc, the sequence of increment was, BOZ4 followed by BOZ3, BOZ2 and BOZ1. Statistically BOZ1& BOZ2 had same effect as ZnSO4. BOZ4 showed 22% more oil contents as compared to ZnSO4.

4.6.24. Crude fiber

The effectiveness of treatments on crude fiber is given in Fig. 4.18 (b). The crude fiber is found maximum with formulations of bio-activated zinc i.e. BOZ4 (2.94% crude
fiber) and minimum with control (1.74% crude fiber). BOZ4 improved crude fiber 33% more as compared to ZnSO$_4$. Statistical analysis revealed that ZnO had no significant effect on fiber while all other treatments significantly improved crude fiber as compared to control (no zinc). Inoculation with zinc solubilizing bacteria also improved fiber contents as compared to control (no zinc).

4.6.25. Ash contents

The data regarding ash contents are given in Fig. 4.19 (a). The ash contents were found maximum with formulations of bio-activated zinc i.e. BOZ4 (1.6%) and minimum with control (1.14%). Sole application of ZnO and inoculation with zinc solubilizing bacteria had not increased ash contents significantly. While all other treatments had significant effect on ash contents as compared to control. Among bio-activated zinc formulations, the higher ash contents were found with BOZ4 and BOZ3 while BOZ2 and BOZ1 had same effect as compared to ZnSO$_4$. Application of BOZ4 improved 18% more ash contents as compared to ZnSO$_4$.

4.6.26. Grain dry matter

The impact of various sources of zinc i.e ZnO, ZnSO$_4$ and different formulations of bio-activated zinc (BOZ) on grain dry matter is indicated in Fig. 4.19 (b). All the treatments showed higher grain dry matter as compared to control. Among the bio-activated zinc formulations, BOZ4 increased grain dry matter 2.5% more as compared to available form of zinc (ZnSO$_4$) and 3% more as compared to unavailable form of zinc (ZnO). Inoculation with zinc solubilizing bacteria also promoted grain dry matter 2% more as compared to uninoculated control. BOZ1, BOZ2 and BOZ3 had statistically similar effect as ZnSO$_4$. 

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**Means sharing the same letter do not differ significantly (P< 0.05)**

Fig.4.19: Effect of bio-activated zinc vs commercial ZnSO₄ on (a) no of line per cob & (b) crude protein of maize in pot trial

**Treatments Description:**

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO₄ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Mean sharing the same letter do not differ significantly ($P<0.05$)

Fig. 4.18: Effect of bio-activated zinc vs commercial ZnSO$_4$ on (a) oil contents & b) crude fiber of maize in pot trial

Treatments Description:

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO$_4$ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Mean sharing the same letter do not differ significantly (P< 0.05)

Fig. 4.19: Effect of bio-activated zinc vs commercial ZnSO₄ on (a) Ash contents & (b) dry matter of maize in pot trial

Treatments Description:

**T1:** Control (No zinc)

**T2:** ZnO (unavailable form)

**T3:** ZnSO₄ (available form)

**T4:** Bio-activated Zn formulation 1 (BOZ 1)

**T5:** Bio-activated Zn formulation 2 (BOZ 2)

**T6:** Bio-activated Zn formulation 3 (BOZ 3)

**T7:** Bio-activated Zn formulation 4 (BOZ 4)

**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
4.6.27. Moisture percentage

The effectiveness of bio-activated zinc formulation vs ZnSO₄ on moisture percentage are indicated in Fig. 4.20 (a). Treatment comparison revealed that all the treatments showed significantly decreased trend for moisture contents in grain significantly as compared to control (no zinc). Among bio-activated zinc formulations i.e. BOZ4 exhibited decreased moisture contents in grains 22% as compared to ZnSO₄. The sole application of ZnO and inoculation with zinc solubilizing bacteria also showed decrease in moisture percentage of grains.

4.6.28. Zinc in shoot

The results regarding Zn concentration in shoot is given in Fig. 4.20 (b). All treatments had significantly increased the concentration of zinc in shoot. The increment in Zn concentration in shoot with BOZ4 was 10% over ZnSO₄. Sole application of ZnO and ZSB slightly increased the zinc concentration in shoots.

4.6.29. Zinc in root

The comparison among the different sources of zinc such as ZnO, ZnSO₄ and bio-activated zinc formulations in root is given in Fig. 4.21(a). The concentration of Zn in root significantly increased with all treatments over control. Maximum concentration of Zn in root was found with BOZ4 that was 29% more as compared to available form of zinc (ZnSO₄). While BOZ3, BOZ2 and BOZ1 had showed statistically same improvement as compared to ZnSO₄. Statistical analysis revealed that application of ZnO and ZSB had not increased Zn concentration in root as compared to control.

4.6.30. Zinc in grains

Comparative effectiveness of various sources of zinc such as ZnO (unavailable form), ZnSO₄ (available form) and different formulations of bio-activated zinc (BOZ) on grain zinc concentration is evident from Fig. 4.21 (b). The concentration of Zn in grain had significantly increased with all treatments over control except with ZnO and ZSB. Maximum concentration of Zn in grain was found with BOZ4 (56.33 µg g⁻¹) and minimum with control (40.66 µg g⁻¹). The increment in Zn concentration in grain with BOZ4 was 27% over ZnSO₄.
Statistical analysis revealed that application of ZnO and ZSB had not significantly increased Zn concentration in grain as compared to control (no zinc).

4.6.31. Phytates

The effect of different sources of zinc (ZnO, ZnSO₄ and bio-activated zinc formulations) on phytates in grains is given in Fig. 4.22 (a). Statistical analysis revealed that sole application of insoluble form of zinc (ZnO) and inoculation of ZSB had no significant effect on phytates while all other treatments significantly decrease phytates as compared to control. Among bio-activated zinc formulations, the lower phytates was found with BOZ4 followed by BOZ3, BOZ2 and BOZ1. Bio-activated zinc formulation (BOZ4) decrease 48% phytates as compared to ZnSO₄.

4.6.32. Phytate to zinc ratio

The effect of treatments on phytate to Zn ratio is given in Fig. 4.22(b). All the sources of zinc decreased phytate to zinc ratio as compared to control (no zinc). All bio activated Zn formulations (BOZ1, BOZ2, BOZ3 BOZ4) showed lower phyatat to zinc ratio as compared ZnSO₄. Among the formulations, BOZ4 decreased 59% phytate to zinc ratio as compared to ZnSO₄.

4.6.33. Zinc in soil

The results of impact of bio-activated zinc vs ZnSO₄ on soil zinc availability after the harvesting of maize is given in Fig. 4.23 (a). The concentration of Zn in soil was increased with all treatments over control except with ZnO. Maximum concentration of Zn in soil was found with bio-activated zinc formulations i.e. BOZ4 (0.71 mg kg⁻¹) and minimum with control (0.51 mg kg⁻¹). All other bio-activated zinc formulations also increased zinc availability in soil. The increment in Zn concentration in soil with BOZ4 application was 8% more over ZnSO₄. Inoculation with zinc solubilizing bacteria slightly improved zinc availability in soil after harvesting as compared to control. Statistical analysis revealed that application of ZnO had not increased Zn concentration in soil as compared to control.
Means sharing the same letter do not differ significantly \((P < 0.05)\)

**Fig.:4.20 Effect of bio-activated zinc vs commercial ZnSO\(_4\) on (a) moisture percentage & (b) zinc in shoot of maize in pot trial**

**Treatments Description:**

**T1:** Control (No zinc) \hspace{1cm} **T5:** Bio-activated Zn formulation 2 (BOZ 2)

**T2:** ZnO (unavailable form) \hspace{1cm} **T6:** Bio-activated Zn formulation 3 (BOZ 3)

**T3:** ZnSO\(_4\) (available form) \hspace{1cm} **T7:** Bio-activated Zn formulation 4 (BOZ 4)

**T4:** Bio-activated Zn formulation 1 (BOZ 1) \hspace{1cm} **T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Mean sharing the same letter do not differ significantly (P< 0.05)

Fig.4.21: Effect of bio-activated zinc vs commercial ZnSO₄ on (a) zinc in roots & b) zinc in grain of maize in pot trial

Treatments Description:

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO₄ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Mean sharing the same letter do not differ significantly (P< 0.05)

Fig. 4.22: Effect of bio-activated zinc vs commercial ZnSO₄ on (a) phytate & (b) phytate to zinc ratio of maize in pot trial

**Treatments Description:**

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO₄ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly ($P < 0.05$)

Fig. 4.23. Effect of bio-activated zinc vs commercial ZnSO$_4$ on (a) zinc in soil after harvesting maize in pot trial

**Treatments Description:**

**T1:** Control (No zinc) 

**T2:** ZnO (unavailable form) 

**T3:** ZnSO$_4$ (available form) 

**T4:** Bio-activated Zn formulation 1 (BOZ 1) 

**T5:** Bio-activated Zn formulation 2 (BOZ 2) 

**T6:** Bio-activated Zn formulation 3 (BOZ 3) 

**T7:** Bio-activated Zn formulation 4 (BOZ 4) 

**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
4.7. Study # 5& 6:

Evaluation of different formulations of bio-activated Zn vs ZnSO₄ on growth, physiology, yield and quality of maize (Field Experiments)

Two field trials were conducted in the Farm area of the Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad. Both filed trials were conducted in different seasons one was in March (Field trial I) and the other in July (Field trial II). The results of the above studies are given below.

4.7.1. Plant height

Comparative effectiveness of different formulations of bio-activated zinc (BOZ) vs ZnSO₄ on plant height in field trial I&II is evident from Fig. 4.24 (a). All the plants received basal doses of NPK. In both the field trials statistical analysis of the data revealed that various treatments had significant effects on plant height compared to control.

In field trial I application of ZnO alone did not have any significant influence on plant height whereas ZnSO₄ promoted plant height (179 cm) significantly. Plant height was increased by most of the bio-activated zinc formulations and maximum plant height was recorded in BOZ 4 (219 cm) and BOZ3 (211cm) as compared to ZnSO₄ (183 cm). BOZ4 improved plant height 19% more as compared to ZnSO₄. Inoculation with zinc solubilizing bacteria (162 cm) also promoted plant height as compared to uninoculated control.

In field trial II, the results revealed that most of the treatments promoted plant height. BOZ4 improved plant height 21% more as compared to ZnSO₄. ZSB also promoted plant height while ZnO had no effect as compared to control. Among the bio-activated zinc formulations, BOZ4 and BOZ3 showed maximum plant height as compared to ZnSO₄ while BOZ1 has same effect as ZnSO₄. In both the field trials BOZ 4 & BOZ 3 formulations improved plant height significantly as compared to ZnSO₄. Overall, both the trials showed similar results.

4.7.2. Fresh shoot biomass (kg ha⁻¹)

The effectiveness of applied zinc sources on shoot fresh biomass of field trial I & II are evident from Fig. 4.24 (b). Results of field trial I revealed that all treatments are statistically significant as compared to control except ZnO. The comparison among
treatments revealed that BOZ4 has successfully increased shoot fresh biomass 8% more as compared to ZnSO₄ respectively. Inoculations of zinc solubilizing bacteria promoted shoot fresh biomass as compared to control while insoluble source ZnO alone had no effect. The results of field trial II related to shoot fresh biomass revealed that the most of treatments statistically improved the shoot fresh biomass as compared to control. BOZ4 increased shoot fresh biomass 9% more as compared to ZnSO₄. Overall it was observed that in field trial I & II, most of the bio-activated zinc formulation significantly improved fresh shoot biomass.

4.7.3. Dry shoots biomass (kg ha⁻¹)

The effectiveness of applied treatments of field trial I & II on dry shoot biomass is given in Fig. 4.25 (a). The results of field trial I indicated that dry shoot biomass was promoted in all the applied zinc sources as compared to control. Among bio-activated zinc formulations, BOZ4 improved dry shoots biomass 19% more as compared to ZnSO₄. Statistical analysis of the data revealed that application of ZnO and zinc solubilizing bacteria had promoted dry shoot biomass but that was statistically non-significant. BOZ1 has less dry shoots biomass as compared to compared to ZnSO₄. Inoculation with zinc solubilizing bacteria slightly improved dry shoot biomass while ZnO had no effect as compared to control. Among bio-activated zinc formulations, BOZ4, followed by BOZ3 and BOZ2 showed maximum dry shoot biomass as compared to ZnSO₄ while BOZ1 had same effect as that of ZnSO₄. In field trial I & II, overall, it was observed that most of the bio-activated zinc formulations had positive impact to increase the dry shoot biomass as compared to the treatment where ZnSO₄ was applied.

4.7.4. Cob length

The evidence of results regarding cob length of field trial I & II are given in Fig. 4.25 (b). In both the field trials all the treatments showed significantly higher cob length as compared to control. Among the applied treatments, cob length was maximum with bio-activated zinc formulations i.e. BOZ4 (Field trial I: 25 cm, Field trial II: 25.5 cm). All the BOZ formulation improved cob length as compared to ZnSO₄. The descending order of treatments for improved cob length in both the field trials is ZnO < ZSB < ZnSO₄ < BOZ1 < BOZ3 < BOZ2 < BOZ4 < control.
Means sharing the same letter do not differ significantly (P < 0.05)

Fig. 4.24: Effect of bio-activated zinc vs ZnSO₄ on (a) Plant height & (b) fresh shoot biomass of maize in Field trial I & II

Treatments Description:

**T1:** Control (No zinc)

**T2:** ZnO (unavailable form)

**T3:** ZnSO₄ (available form)

**T4:** Bio-activated Zn formulation 1 (BOZ 1)

**T5:** Bio-activated Zn formulation 2 (BOZ 2)

**T6:** Bio-activated Zn formulation 3 (BOZ 3)

**T7:** Bio-activated Zn formulation 4 (BOZ 4)

**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly (P< 0.05)

Fig. 4.25: Effect of bio-activated zinc vs ZnSO₄ on (a) dry shoot biomass & (b) cob length of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO₄ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
4.7.5. Cob diameter

The results of field trial I & II regarding cob diameter are indicated in Fig. 4.26 (a). Treatment comparison of bio-activated zinc formulations vs ZnSO$_4$ revealed that various treatments improved cob diameter as compared to control. Among all the bio-activated zinc formulations, the maximum cob diameter was recorded with BOZ4 and BOZ 3 as compared to ZnSO$_4$. The BOZ 4 increased the cob diameter 6.2% as compared to ZnSO$_4$ in field trial I, and 6% in field trial II.

4.7.6. Plant girth

For field trial I & II effectiveness of various sources of zinc on plant girth are given in Fig. 4.26 (b). Statistical analysis revealed that all the treatments promoted plant girth significantly as compared to control except ZnO. The higher plant girth was observed with the application of BOZ4 (Field trial I: 13 cm, Field trial II: 12 cm). With BOZ 4 formulation the increase in plant girth was 49% more in field trial I, and 47% more in field trial II as compared to ZnSO$_4$. The plant girth was also increased with the application of ZnSO$_4$ and ZSB while ZnO had no significant effect on plant girth as compared to control.

4.7.7. Carbonic anhydrase activity

The results regarding carbonic anhydrase activity of field trial I & II are illustrated in Fig. 4.27 (a). Treatment comparison revealed that various sources of zinc (ZnO, ZnSO$_4$ and bio-activated zinc formulations) improved the activity of CA enzyme activity as compared to control.

In field trial I, carbonic anhydrase activity was higher with BOZ4 followed by BOZ 3 and BOZ2 (365, 350 and 338 μmol (CO$_2$) kg$^{-1}$ s$^{-1}$ respectively) as compared to ZnSO$_4$. The increase in enzyme activity was also observed with the application of ZnSO$_4$ but the increase was less than all BOZ formulations. Inoculation with zinc solubilizing bacteria also promoted carbonic anhydrase activity as compared to uninoculated control. In field trial II, the results revealed that the carbonic anhydrase activity was promoted mostly by the application of bio-activated zinc as compared to ZnSO$_4$. In field trial I, BOZ4 increased the CA activity 19% more and in field trial II 18% more as compared to ZnSO$_4$. Furthermore, it was evident from
the results of both field trials that bio-activated zinc formulations performed better than the commercial ZnSO₄.

4.7.8. Photosynthetic rate

The results related to photosynthetic rate of field trial I & II are presented in Fig. 4.27(b). In field trial I, most of the treatments increased photosynthetic rate as compared to control. The maximum photosynthetic rate was observed with BOZ4. Among the bio-activated zinc formulations, BOZ4 provided statistically maximum photosynthetic rate i.e., 12% more as compared to ZnSO₄. Photosynthetic rate was increased with the application of ZnO (5%), ZnSO₄ (27%) and ZSB (12%) more as compared to control (no zinc).

The results of field trial II revealed that photosynthetic rate is found maximum with bio-activated zinc i.e. BOZ4. Statistical analysis revealed that ZnO had minor effect on photosynthetic rate while all other treatments significantly improved photosynthetic rate as compared to control. Among bio-activated zinc formulations, the higher photosynthetic rate was found with BOZ4 followed by BOZ3, BOZ2 and BOZ1. Application of ZnSO₄ and ZSB also improved photosynthetic rate as compared to control. Overall, it was observed that bio-activated formulations enhanced photosynthetic rate of plants in both the field trials.
Means sharing the same letter do not differ significantly (P < 0.05)

Fig. 4.26: Effect of bio-activated zinc vs ZnSO₄ on (a) cob diameter & (b) plant girth of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)
T2: ZnO (unavailable form)
T3: ZnSO₄ (available form)
T4: Bio-activated Zn formulation 1 (BOZ 1)
T5: Bio-activated Zn formulation 2 (BOZ 2)
T6: Bio-activated Zn formulation 3 (BOZ 3)
T7: Bio-activated Zn formulation 4 (BOZ 4)
T8: Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly (P < 0.05)

Fig. 4.27: Effect of bio-activated zinc vs ZnSO₄ on (a) carbonic anhydrase activity & (b) photosynthetic rate of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO₄ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
4.7.9. Transpiration rate

The Fig. 4.28(a) showed the results of transpiration rate of maize crop in field trial I & II. Comparison among treatments revealed that all the treatments exhibited higher transpiration rate as compared to control. The performance of BOZ4, BOZ3 & BOZ2 was statistically superior as compared to ZnSO4 in field trial I and almost similar results were found in field trial II. BOZ4 increased transpiration rate by 8% more in field trial I, and 37% in field trial II as compared to ZnSO4. Inoculation with zinc solubilizing bacteria showed higher transpiration rate as compared to control but the effect of ZSB was significant in field trial I and non-significant in field trial II. The ZnO showed minor improvement in transpiration rate as compared to control in both the field trial.

4.7.10. Stomatal conductance

Different formulations of bio-activated zinc showed statistically significant effect on the stomatal conductance of maize plant (Fig. 4.28 b). The BOZ4 increased stomatal conductance by 12% & 11% more as compared to ZnSO4 in field trial I and field trial II, respectively. A slight increase in stomatal conductance was observed with the inoculation of zinc solubilizing bacteria as compared to control in both the trials.

4.7.11. Chlorophyll contents

The results regarding chlorophyll contents of field trial I & II are indicated in Fig. 4.29 (a). Treatment comparison of both field trials revealed that various sources of zinc significantly improved chlorophyll contents of maize plants as compared to control except ZnO. Among all the treatments, bio-activated zinc formulations i.e., BOZ4 followed by BOZ3 increased chlorophyll contents more as compared to ZnSO4. Inoculation with zinc solubilizing bacteria improved chlorophyll contents in field trial I and II.

4.7.12. Electrolyte leakage

The efficacy of bio-activated zinc vs ZnSO4 for decreasing electrolyte leakage (%) under field conditions (Field trial I, & II) is evident from Fig. 4.29(b). In field trial I, it is obvious that all the treatments have competitive edge over the control in decreasing electrolyte leakage. The minimum electrolyte leakage was observed in the BOZ 4 which
decreased 42% more electrolyte leakage as compared to ZnSO₄. Further comparison among treatments revealed that BOZ3 and BOZ 2 also decreased electrolyte leakage as compared to ZnSO₄. Inoculation with zinc solubilizing bacteria also decreased electrolyte leakage as compared to the uninoculated control.

In the field trial II, decrease in electrolyte leakage showed the same trend as in the field trial I. BOZ4 decreased electrolyte leakage 37% more as compared to ZnSO₄.
Means sharing the same letter do not differ significantly ($P < 0.05$)

**Fig. 4.28:** Effect of bio-activated zinc vs ZnSO$_4$ on (a) transpiration rate & (b) stomatal conductance of maize in Field trial I & II

**Treatments Description:**

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO$_4$ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly (P< 0.05)

Fig. 4.29: Effect of bio-activated zinc vs ZnSO₄ on (a) chlorophyll contents & (b) electrolyte leakage of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO₄ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
4.7.13. Grain yield (kg ha\(^{-1}\))

Comparative effectiveness of different formulations of bio-activated zinc (BOZ) vs ZnSO\(_4\) on grain yield (kg ha\(^{-1}\)) in field trial I&II is evident from Fig. 4.30 (a). The results of field trial I showed that recorded grain yield was higher in most of the bio-activated zinc formulations i.e. BOZ4 and BOZ 3 increased grain yield by 11% and 5% more as compared to ZnSO\(_4\). Application of ZnSO\(_4\) also showed 12% increase in grain yield treatment while ZnO had no significant effect as compared to control. Inoculation with zinc solubilizing bacteria also promoted 12% more grain yield as compared to uninoculated control.

The results of field trial II regarding treatment comparison revealed that most of the treatments had significantly increased grain yield over control. BOZ4 and BOZ 3 increased grain yield 8% & 13% respectively as compared to ZnSO\(_4\). Application of ZnSO\(_4\) also showed 17% increase in grain yield while ZnO had no significant effect as compared to control. Inoculation with zinc solubilizing bacteria also increased the grain yield of maize. Overall it was observed that in both the field trials, BOZ3 & BOZ4 increased grain yield 20-30 % over control and 10-15% over ZnSO\(_4\).

4.7.14. Grain yield (g cob\(^{-1}\))

The data of field trial I & II regarding grain yield (g cob\(^{-1}\)) are given in Fig. 4.30 (b). The grain yield cob\(^{-1}\) was found maximum with bio-activated zinc formulations i.e. BOZ4 and BOZ3 showed maximum grain yield cob\(^{-1}\) as compared to ZnSO\(_4\) in both the field trials. Statistical analysis revealed that sole application of ZnO had not increased grain yield cob\(^{-1}\) while all other treatments significantly increased grain yield cob\(^{-1}\) as compared to control. Among bio-activated zinc, the higher grain yield cob\(^{-1}\) was found with BOZ4 (Field trial I: 5% more, Field trial II: 4% more) when compared with available form of zinc (ZnSO\(_4\)). Zinc solubilizing strain also improved the grain yield cob\(^{-1}\) as compared to control but it was less than ZnSO\(_4\).

4.7.15. 1000 grain weight

Comparative efficacy of different formulations of bio-activated zinc vs ZnSO\(_4\) of field trial I & II on 1000 grain weight is presented in Fig.4.31 (a). The results revealed that bio-activated zinc formulations and ZnSO\(_4\) successfully increased 1000 grain weight as
compared to control, and the increment was maximum with BOZ4 (Field trial I: 314 g, Field trial II: 315 g). Zinc solubilizing strain also improved the 1000 grain weight as compared to control but it was less than ZnSO₄. The treatment comparison showed that among bio-activated zinc, BOZ4 and BOZ3 showed statistically significant improvement in 1000 grain weight over ZnSO₄, while BOZ2, BOZ1 and ZnSO₄ had same effect in the increment of grain weight in both the field trials.

4.7.16. Stover yield (kg ha⁻¹)

The effectiveness of different sources of zinc (ZnO, ZnSO₄ and bio-activated zinc formulations) regarding stover yield (kg ha⁻¹) of field trial I & II are indicated in Fig. 4.31 (b). The results of both the field trials showed that all the treatments improved stover yield as compared to control. Among bio-activated zinc formulations i.e., BOZ4 improved stover yield by 14% in field trial I & 5% more in field trial II as compared to ZnSO₄. Zinc solubilizing bacteria also had positive impact on stover yield of maize as compared to control. Application of ZnO had no significant effect on stover yield as compared to control. Similar trend was found in field trial II.
Means sharing the same letter do not differ significantly (P< 0.05)

Fig. 4.30: Effect of bio-activated zinc vs ZnSO₄ on (a) grain yield & (b) grain yield g/cob of maize in Field trial I & II

Treatments Description:

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO₄ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly (P < 0.05)

Fig. 4.31: Effect of bio-activated zinc vs ZnSO₄ on (a) 1000 grain weight & (b) stover yield of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO₄ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
4.7.17. Stover yield (g cob\(^{-1}\))

The Fig. 4.32 (a) depicts the results of stover yield (g cob\(^{-1}\)) of maize crop for field trial I & II. Comparison among the different sources of applied zinc (ZnO, ZnSO\(_4\), bio-activated zinc formulations) revealed that all treatments showed higher stover yield (g cob\(^{-1}\)) as compared to control but statistically most of the treatments are significant. The performance of BOZ4 and BOZ3 was statistically superior as compared to ZnSO\(_4\) in field trial I and almost similar results were found in field trial II. BOZ4 improved stover yield 16\% more in field trial I and 9\% more in field trial II as compared to ZnSO\(_4\). While inoculation with zinc solubilizing bacteria improved stover yield (g cob\(^{-1}\)) as compared to control in both the field trials. The ZnO gave non-significant results in case of stover yield (g cob\(^{-1}\)) as compared to control.

4.7.18. Number of grains per cob

The result of (Field trial I & II) number of grains per cob is given in Fig. 4.32 (b). In field trial I, the treatment comparison revealed that all the applied zinc sources (ZnO, ZnSO\(_4\), bio-activated zinc formulations) improved number of grains per cob as compared to control (no zinc). Among bio-activated zinc formulations, BOZ4 improved number of grains per cob followed by BOZ3. 17\% increase in number of grains per cob for BOZ4 in field trial I and 24\% more in field trial II as compared to available form of zinc (ZnSO\(_4\)). Statistical analysis showed that in field trial II, ZnO had no significant effect on number of grains per cob while in field trial I, slight increment over control. Almost similar trend was found in field trial II in most of the treatments.

4.7.19. Number of grains per line

The data regarding (Field trial I & II) number of grains per line is presented in Fig. 4.33(a). In field trial I, the results revealed that bio-activated zinc formulations, ZnSO\(_4\) and ZSB successfully increased number of grains per line and the increment was maximum with BOZ4 (645). BOZ4 improved number of grain per line by 5\% in field trial I and 8\% more in field trial II as compared to ZnSO\(_4\). The treatment comparison revealed that statistically all bio-activated zinc formulations showed same results except BOZ4 regarding number of grains per line while ZnO had no significant effect in the increment of number of grain as
compared to control (no zinc). Inoculation with zinc solubilizing bacteria increased number of grains per cob but the increase was statistically non-significant. Almost similar results were found in field trial II.

### 4.7.20. Number of line per cob

Comparative efficacy of various sources of zinc such as ZnO, ZnSO₄ and different formulations of bio-activated zinc (BOZ) in field condition (Field trial I & II) on number of line per cob are indicated in Fig. 4.33(b). The results of field trial I showed that higher number of line per cob was observed with the application of all the applied zinc sources as compared to control. Statistical analysis showed that application of ZnO had not showed significantly increased in the number of line per cob as compared to control. All bio-activated zinc formulations treatments had increased number of line per cob following the same trend. BOZ4, BOZ3 and BOZ2 increased number of lines per cob as compared to ZnSO₄. Almost similar trend of increase in number of line per cob was found with the application of different bio-activated zinc formulations vs ZnSO₄ in field trial II.
Means sharing the same letter do not differ significantly ($P<0.05$)

Fig. 4.32: Effect of bio-activated zinc vs ZnSO$_4$ on (a) stover yield g/cob & (b) No. of grains per cob of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO$_4$ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly ($P < 0.05$)

Fig. 4.33: Effect of bio-activated zinc vs ZnSO$_4$ on (a) No. of grains per line & (b) No. of line in cob of maize in Field trial I & II

Treatments Description:

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO$_4$ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
4.7.21. Crude protein

Comparative efficacy of different formulations of bio-activated zinc (BOZ) vs ZnSO\textsubscript{4} on crude protein in field trial I&II is evident from Fig. 4.34 (a). In both the field trials statistical analysis of data revealed that various bio-activated zinc formulations treatments had significantly effects on crude protein as compared to ZnSO\textsubscript{4}.

In field trial I, application of ZnO alone did not had any significant influence on crude protein as compared to control. Crude protein was increased in most of the bio-activated zinc formulations and maximum crude protein was recorded in BOZ 4 (13% crude protein) and BOZ3 (12% crude protein). BOZ 4 improved crude protein by 17% more as compared to ZnSO\textsubscript{4}. Inoculated with zinc solubilizing bacteria (10% crude protein) also promoted crude protein as compared to uninoculated control.

In field trial II the results showed that most of the treatments promoted crude protein (%). Whereas 12% increment in crude protein for BOZ4. ZSB also promoted crude protein while ZnO had statistically no effect as compared to control. Among the bio-activated zinc formulations, BOZ4 and BOZ3 showed maximum crude protein (%) as compared to ZnSO\textsubscript{4} while BOZ1 had same effect as ZnSO\textsubscript{4}.

In both the field trials, BOZ 4, BOZ 3 and BOZ2 formulations improved crude protein (%) significantly as compared to ZnSO\textsubscript{4}. Overall, it was observed that both the trials showed similar results.

4.7.22. Oil contents

The impact of different formulations of bio-activated zinc and different sources of zinc on oil content are indicated in Fig. 4.34(b). In field trial I, the maximum oil contents was found with BOZ4 (4.8% oil contents). Statistical analysis revealed ZSB also showed slightly higher oil content (4.4% oil contents) while ZnO had no significant effect as compared to control. Among all the zinctated treatments the sequence of increment was, BOZ4 followed by BOZ3, BOZ2, BOZ1, ZnSO\textsubscript{4}, ZSB and ZnO. Statistically BOZ1 had same effect as ZnSO\textsubscript{4}. BOZ4 showed 25% more oil contents as compared to insoluble source of zinc (ZnSO\textsubscript{4}).

In field trial II, similar trend was found in the increment of oil content by the bio-activated zinc formulations.
4.7.23. Crude fiber

The evaluation of different formulations of bio-activated zinc vs ZnSO₄ in field condition (Field trial I & II) on crude fiber are given in Fig. 4.35 (a). The crude fiber is found maximum with bio-activated zinc formulations i.e. BOZ4 (Field trial I: 3.02 %, Field trial II: 2.86%). BOZ4 improved crude fiber by 28% in field trial I and 25% more in field trial II as compared to ZnSO₄. BOZ1 showed similar results as ZnSO₄. Statistical analysis revealed that ZnO (Field trial I: 1.78%, Field trial II: 1.83%) had no effect on crude fiber while all other treatments significantly improved crude fiber as compared to control. Inoculation with zinc solubilizing bacteria also improved crude fiber (Field trial I: 1.94%, Field trial II: 1.97%) as compared to control. The improvement through zinc solubilizing strain as compared to control was statistically non-significant.

4.7.24. Ash contents

Comparative efficacy of various sources of zinc such as ZnO, ZnSO₄ and different formulations of bio-activated zinc (BOZ) in field condition (Field trial I & Field trial II) on ash content (%) of maize grains are evident from Fig. 4.35 (b). Statistically analysis of data revealed that various treatments had significantly affected on ash content (%) as compared to control. Application of ZnO alone did not had any influence on ash content (%) whereas ZnSO₄ promoted ash content (%) (Field trial I: 1.41%, Field trial II: 1.140%) significantly. Ash content (%) was increased by the most of the bio-activated formulations and maximum ash content (%) was recorded in BOZ 4 (Field trial I: 1.50%, Field trial II: 1.56%) and BOZ3 (Field trial I: 1.48%, Field trial II: 1.51%). BOZ4 improved ash contents by 6% in field trial I and 11% more in field trial II as compared to ZnSO₄. Inoculation with zinc solubilizing bacteria also promoted ash contents (Field trial I: 1.30%, Field trial II: 1.39%) as compared to uninoculated control.
Means sharing the same letter do not differ significantly (P< 0.05)

Fig. 4.34: Effect of bio-activated zinc vs ZnSO₄ on (a) crude protein & (b) oil content of maize in Field trial I & II

Treatments Description:

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO₄ (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  
**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Mean sharing the same letter do not differ significantly (P< 0.05)

Fig. 4.35: Effect of bio-activated zinc vs ZnSO₄ on (a) crude fiber & (b) ash content of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)   T5: Bio-activated Zn formulation 2 (BOZ 2)
T2: ZnO (unavailable form)   T6: Bio-activated Zn formulation 3 (BOZ 3)
T3: ZnSO₄ (available form)   T7: Bio-activated Zn formulation 4 (BOZ 4)
T4: Bio-activated Zn formulation 1 (BOZ 1)   T8: Zinc solubilizing bacteria (ZSB), only inoculation
4.7.25. Dry matter

The results of (Field trial I & II) for dry matter (%) of maize grain are given in Fig. 4.36 (a). In field trial I, the treatment comparison revealed that bio-activated zinc formulations showed 2% more dry matter as compared to ZnSO₄. Among the bio-activated zinc formulations, BOZ4 showed maximum dry matter (%) followed by BOZ3 and BOZ2. Statistical analysis showed that ZnO had no significant effect on dry matter (%) while inoculation with zinc solubilizing bacteria significantly improved dry matter over control. Almost similar trend was found in field trial II.

4.7.26. Moisture percentage

The results of field trial I & II regarding moisture percentage of maize grain are indicated in Fig. 4.36(b). Treatment comparison of bio-activated zinc formulations vs ZnSO₄ revealed that various treatments decreased moisture percentage as compared to control. Among all the bio-activated zinc formulations i.e. the minimum moisture percentages were recorded with BOZ4 and BOZ3 as compared to control. The BOZ4 decreased moisture percent by 20% in field trial I & 21% in field trial II as compared to ZnSO₄. Inoculation with zinc solubilizing bacteria slightly decreased the moisture percentage as compared to control (no zinc).

4.7.27. Zinc in shoot

The results of field experiment (Field trial I & II) regarding Zn concentration in shoot is given in Fig. 4.37(a). In field experiment I, all treatments had significantly increased the concentration of zinc in shoot as compared to control except for ZnO. Maximum concentration of Zn in shoot was found with BOZ4 (22.09 µg g⁻¹). The increment in Zn concentration in shoot with BOZ4 was 11% over ZnSO₄.

The results related to zinc concentration in shoot of field trial II showed that the concentration of Zn in shoots successfully increased with all treatments over control except with ZnO. Maximum concentration of Zn in shoot was found with BOZ4 (22.87 µg g⁻¹). The increment in Zn concentration in shoot with BOZ4 was 15% over ZnSO₄. Statistical analysis revealed that inoculation with zinc solubilizing bacteria had slightly increased Zn concentration in root as compared to control.
4.7.28. Zinc in grains

The results related to zinc concentration in grain is given in Fig. 4.37 (b). All the applied zinc sources showed maximum improvement for zinc in grains as compared to control. The zinc in grains was found maximum with bio-activated zinc formulations i.e. BOZ4 (Field trial I: 56 µg g⁻¹, Field trial II: 58 µg g⁻¹). BOZ4 improved grain zinc by 27% in field trial I and 18% more in field trial II as compared to ZnSO₄. Statistical analysis revealed that ZnO (Field trial I: 42 µg g⁻¹, Field trial II: 41 µg g⁻¹) had no significant effect on zinc in grains while all other treatments significantly improve zinc in grains as compared to control. Inoculation with zinc solubilizing bacteria also improved zinc in grains (Field trial I: 43 µg g⁻¹, Field trial II: 44 µg g⁻¹) as compared to control (no zinc).
Means sharing the same letter do not differ significantly \((P < 0.05)\)

Fig. 4.36: Effect of bio-activated zinc vs ZnSO\(_4\) on (a) dry matter & (b) moisture percentage of maize in Field trial I & II

Treatments Description:

**T1:** Control (No zinc)  
**T2:** ZnO (unavailable form)  
**T3:** ZnSO\(_4\) (available form)  
**T4:** Bio-activated Zn formulation 1 (BOZ 1)  

**T5:** Bio-activated Zn formulation 2 (BOZ 2)  
**T6:** Bio-activated Zn formulation 3 (BOZ 3)  
**T7:** Bio-activated Zn formulation 4 (BOZ 4)  
**T8:** Zinc solubilizing bacteria (ZSB), only inoculation
Means sharing the same letter do not differ significantly (P< 0.05)

Fig. 4.37: Effect of bio-activated zinc vs ZnSO₄ on (a) zinc in shoots & (b) zinc in grains of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)
T2: ZnO (unavailable form)
T3: ZnSO₄ (available form)
T4: Bio-activated Zn formulation 1 (BOZ 1)
T5: Bio-activated Zn formulation 2 (BOZ 2)
T6: Bio-activated Zn formulation 3 (BOZ 3)
T7: Bio-activated Zn formulation 4 (BOZ 4)
T8: Zinc solubilizing bacteria (ZSB), only inoculation
4.7.29. Phytates

The data regarding phytate concentration of field trials (Field trial I, & II) are given in Fig. 4.38 (a). In field trial I, It is obvious that all the treatments have competitive edge over the control treatment in decreasing phytates ($\mu$g g$^{-1}$). BOZ 4 decreased phytates 52% more as compared to insoluble form of zinc (ZnSO$_4$). Further comparison among treatments revealed that BOZ4, BOZ3 and BOZ 2 decreasing phytates as compared to ZnSO$_4$. Inoculation with zinc solubilizing bacteria decreased phytates as compared to uninoculated control.

However in the field trial II, phytates showed the same trend as in the field trial II. BOZ4 and BOZ3 had minimum phytates as compared to ZnSO$_4$. Overall, it was observed that most of bio-activated zinc formulations showed significant decrease in phytates.

4.7.30. Phytate to Zinc ratio

The effect of treatments on phytate to Zn ratio of Field trial I & Field trial II are given in Fig. 4.38 (b). All the sources of zinc decreased phytate to zinc ratio as compared to control (no zinc). Among the bio-activated zinc formulations, phytate to zinc ratio with BOZ4 decreased by 62% in field trial I and 60% in field trial II as compared to ZnSO$_4$. All other bio-activated Zn formulations showed lower ratio as compared to ZnSO$_4$. Alone application of ZnO and inoculation with zinc solubilizing bacteria slightly decreased the phytate to zinc ratio as compared to control. Application of ZnSO$_4$ also decreases phytate to Zn ratio as compared to control. Effect of bio-activated zinc formulations was almost similar in both the field trials.
Means sharing the same letter do not differ significantly ($P<0.05$)

Fig. 4.38: Effect of bio-activated zinc vs ZnSO$_4$ on (a) phytate (b) phytate to zinc ratio of maize in Field trial I & II

Treatments Description:

T1: Control (No zinc)  
T2: ZnO (unavailable form)  
T3: ZnSO$_4$ (available form)  
T4: Bio-activated Zn formulation 1 (BOZ 1)  
T5: Bio-activated Zn formulation 2 (BOZ 2)  
T6: Bio-activated Zn formulation 3 (BOZ 3)  
T7: Bio-activated Zn formulation 4 (BOZ 4)  
T8: Zinc solubilizing bacteria (ZSB), only inoculation
CHAPTER 5

DISCUSSION

Widespread soil zinc (Zn) deficiency is one of the important factors responsible for yield reduction in different crops (Fageria et al., 2002) and it is also positively correlated with widespread human zinc deficiency (Alloway, 2009). Adequate amount of zinc is considered essential for growth and development of cereal crops. Different strategies are adopted to improve zinc deficiency for human, such as Zn supplementation, food fortification and food diversification. Most of them are not practically possible and are rather uneconomical in developing countries like Pakistan (Bouis et al., 2011). Among these strategies, the combined use of organic material enriched with cheap zinc source ZnO and zinc solubilizing bacteria could be a novel approach.

Keeping in view the above scenario, present study was conducted to evaluate the efficacy of bio-activated zinc for improving yield and quality of maize. In the present study, a number of zinc solubilizing bacteria were isolated from rhizosphere of maize crop from different areas. The bacterial isolates were screened having capability to solubilize ZnO. The bacterial isolates capable to solubilize ZnO were further screened for their plant growth promoting activity under axenic conditions. These bacterial isolates were characterized for their plant growth promoting attributes and were identified by using 16S rRNA sequence. Most effective growth promoting and zinc solubilizing bacterial isolate was used with the organic material to bio-activate the insoluble zinc (ZnO) with different formulations. Efficient formulations were evaluated for temporal release of zinc in soil. Then the pot experiment was conducted to evaluate and compare the different formulations of bio-activated zinc vs ZnSO₄ (comparatively more available in soil but costly source of zinc) on maize crop. Then, the results of pot trial were confirmed under field conditions by conducting experiments in two maize seasons (Field trial I in March, Field trial II in July).

In present study 52 bacterial strains were isolated from rhizosphere of maize. In plate assay, only 14 bacterial isolates showed good potential of zinc solubilizing bacteria from insoluble source zinc (ZnO). As the plate assay has some limitations, it is not considered as relatively authenticated procedure to access the solubilization and mineralization ability of bacteria. Therefore, the bacteria showing good potential of zinc solubilizing on agar plate
were further tested in a broth assay supplemented with ZnO. Similar, studies have been conducted by Sarvanan \textit{et al.}, 2003 in which \textit{Bacillus} sp. and \textit{Pseudomonas} sp. were screened on zinc oxide (ZnO), i.e., Zinc solubilizing bacterial isolate to solubilize zinc oxide. (Di Simine \textit{et al.}, 1998; Saravanan \textit{et al.}, 2007; Sharma \textit{et al.}, 2014).

The bacterial isolates capable to solubilize zinc oxide (ZnO) were further screened for their plant growth promoting activity of maize seedling under axenic conditions. In present study different zinc solubilizing bacterial isolates enhanced zinc availability to plants. Results showed that zinc solubilizing bacterial isolates significantly improved the growth and physiological parameters of maize seedling in a growth room experiment. Bacterial isolates increased significantly growth and physiological parameters of maize plant as compared to un-inoculated control. These finding are agreement with the previous reports that the increase in growth of plant by increasing biological nitrogen fixation and increased the nutrient availability to plants (Son \textit{et al.}, 2006; Muhammadi, 2011) and might be due to the reason that zinc solubilizing bacteria increased the solubilization of nutrients, produced different plant growth promoters (direct) and siderophores (indirect) that suppress the pathogens (Kloepper \textit{et al.}, 1989; Arshad and Frankenberger, 1998). ZSBs strains increased the root length, root hair and surface area, due to which the availability of nutrients increased (Biswas \textit{et al.}, 2000; Adesemoye \textit{et al.}, 2008). According to results, the AZ6 strain resulted in the maximum improvement in growth and physiological parameters of maize seedling. Maximum growth and physiological parameters of maize seedling as compared to all other bacterial isolates is due to AZ6 have more growth promoting attributes compared to other strains.

The selected strain was later identified as \textit{Bacillus} sp. AZ6. Previously it has been reported by Sarvanan \textit{et al.}, 2003 that \textit{Bacillus} sp. has ability to solubilize zinc oxide. The \textit{Bacillus} sp. AZ6 was characterized for their plant growth promoting attributes like auxin production, ACC-deaminase activity, siderophores production and organic acid production. Our bacterial strain produced IAA, which could be useful while interactions with the plants as plants exudates have tryptophan that may help the IAA production potential of the bacteria. These results agreement with earlier reports in which IAA producing rhizobacteria were isolated and had beneficial impact on plant growth promotion (Ali \textit{et al.}, 2013). In our study \textit{Bacillus} sp. AZ6 increased ACC-deaminase activity. ACC-deaminase activity of
PGPR helps plants to withstand stress (biotic and abiotic) by lowering the level of the stress hormone ethylene through the activity of enzyme ACC-deaminase, which hydrolyses ACC into a-ketobutarate and ammonia instead of ethylene (Arshad et al., 2007). In our study the AZ6 strain expressed siderophore production. Siderophores are recognized for making iron (Fe) available to the plant and the production of the siderophores by the microorganisms can bind iron with high affinity, making the iron unavailable for the other microorganisms, and thereby limiting their growth. This strategy may certainly be involved in the biological control of plant disease. These results are similar with the previous results of (Gull and Hafeez 2009; Naureen et al., 2009).

The organic acids produces by Bacillus sp. AZ6 were cinamic acid, ferulic acid, caffeic acid, chlorggenic acid, syrirgic acid, gallic acid. These acids solubilize the insoluble source of zinc by lowering pH. As the production of organic acids can also directly facilitate the mobilization of zinc by reducing sorption of Zn by altering the surface charge characteristics of soil colloids (Jones, 1998).

After evaluation of these selected strains, four different zinc formulations were prepared. It was prepared by the combination of zinc solubilizing strains, ZnO and organic material (Grinded citrus peel). In our study combined use of organic material with zinc solubilizing bacteria and zinc source improved zinc availability. Previously it has been reported that different types of organic matter can enhance the microbial population. Therefore, exogenous application of some potential zinc solubilizing bacteria increase zinc content in the rhizosphere and ultimately in the plants (Imran et al., 2014). The Bacillus sp. AZ6 strain used in the bio-activation of zinc had the ability to produce organic acids, these organic acids solubilize the insoluble source of zinc (ZnO) by lowering the pH. It had been confirmed earlier that production of organic acids can directly facilitate the availability of zinc (Jones, 1998). Organic material used in the bio-activation of zinc may increase available fraction of zinc in soil for plant uptake. Moreover, the application of organic amendments improves biological properties of soil (Tejada et al., 2006). The mechanisms of acquisition of zinc by rhizobacterial strain from insoluble zinc (ZnO) compounds might be a consequence of proton extrusion and production of organic acids of microbial origin possibly in a non-specific way leading to solubilization of zinc and therefore influenceing the bioavailability of
zinc. This type of solubilization of zinc compound carried out through production of organic acids had been reported before (Agusto da Costa and Duta, 2001).

The organic material used in the bio-activated zinc formulations acted as carrier material for the zinc solubilizing bacteria and for the chelation of zinc. Carrier material is one of the important and major portions of carrier based biofertilizers responsible for the delivery of optimum amount of microbes in active condition to the field (Smith, 1992). A good carrier material improves the performance of biofertilizer by providing better niches, easy handling, and long term storage (Mishra and Dadhich, 2010). Carrier material provides the suitable micro-environment to increase the survival of applied bacteria (Roy et al., 2010). Due to the carrier material bacteria survive for long time and make complexes with ZnO and help the slow release of the zinc. This is confirmed from the previous studies that the zinc solubilizing strain solubilized the insoluble source of zinc. As microbial activity in soil is associated with organic matter decomposition, release of chelating agents and organic ligands improve Zn availability by forming soluble complexes with inorganic Zn.

These bio-activated zinc formulations were evaluated for temporal release of zinc in soil in a wire house experiment. It was noted in the experiment that with the application of bio-activated zinc formulations zinc availability increased significantly as compared to control, in all sampling time (maximum at 60th day) of incubation. Zinc translocation efficiency was significantly higher with the application of bio-activated zinc (BOZ4) as compared to available (ZnSO₄), unavailable source of zinc (ZnO) and inoculated with zinc solubilizing bacteria. As the micronutrient zinc is required in 1.24 mg kg⁻¹ of soil concentration (Srivastava & Gangwar, 1990) but the DTP-Zn concentration of the native soils was found 0.3-0.5 mg kg⁻¹ which is very low. With the application of BOZ 4, the increase in zinc concentration at the 60th day of incubation was observed. This is due to zinc solubilizing bacteria which solubilized the insoluble source of zinc (ZnO) and also native zinc from soil, which enhance the availability of zinc in soil with time. BOZ4 formulation contains more organic matter compared to other formulations, so the microbes survive longer time due to more available carbon source to the microbes. Organic amendments improve soil microbial biomass carbon (Flie and Mader, 2000) and the zinc content in soil (Saviozzi et al., 1999). This is confirmed from the previous studies that in some microorganisms, chelation has been observed as dominant phenomena to improve availability. Application of
unavailable source of zinc (ZnO) alone did not have any influence in increasing available zinc in soil, because indigenous bacteria did not have potential to solubilize the unavailable zinc.

Bio-activated zinc formulations significantly improved the growth of maize under pot and field conditions over available sources of zinc (ZnSO₄), unavailable source of zinc (ZnO) and inoculated zinc solubilizing bacteria. Bio-activated zinc formulation especially BOZ4 significantly increased growth parameters (plant height, root length, fresh shoot biomass, fresh root biomass, dry shoot biomass, dry root biomass etc.) of maize in pot and field conditions. Thus increase in growth parameters of maize endorsed the fact that zinc is an essential transition metal, required by plants for their optimum growth and development (McCall et al., 2000). It is involved in several physiological processes of plant growth and metabolism, including enzymes activation, carbohydrates metabolism, lipids, nucleic acids, auxins, gene expression & regulation and reproductive development (Chang et al., 2005) plant resistance, photosynthesis, protein synthesis, pollen formation, structural and catalytic activities, biomembranes stability, DNA replication, energy transfer reactions in plants (Broadley et al., 2012). Bio-activated zinc formulations contain zinc solubilizing bacteria, organic material and ZnO. Zinc solubilizing bacteria used organic material as a carbon source and solubilize insoluble source of zinc (ZnO). These bio-activated formulations are associated with their ability to ensure high bio-availability of free zinc to the plants either slow release of zinc, mineralization or solubilization of bound zinc with other compounds in the form of carbonates, bicarbonates, and hydroxyl carbonates. This is confirmed from the studies conducted by Sarvanan et al., 2003 in which Bacillus sp. solubilized the zinc oxide. These finding are also in an agreement with previous reports (Di Simine et al., 1998; Saravanan et al., 2007; Sharma et al., 2014). This is also confirmed with an increase in soil enzyme activities representing rhizobacterial climax and a turn down in the rhizosphere pH upon bacterial inoculation as reported (Neumann and Romheld, 2002; Oburger et al., 2009). The considerable increase in the maize growth by bio-activated zinc formulations may be due to drought tolerance feature of plant growth promoting rhizobacteria as reported earlier (Marulanda et al., 2007). Various direct and indirect mechanism of PGPR has been explored behind the stress tolerance and plant growth promotion (Glick 1995). The zinc solubilizing bacteria had ability to produce auxin, therefore it might be the reason that better root growth
could be due to auxin biosynthesis (Khalid et al., 2004; Kamilova et al., 2006). The presence of tryptophan-like compounds a precursor of auxin biosynthesis in the root exudates stimulate IAA production due to rhizobacteria and enhance root growth (Khalid et al., 2006; Kamilova et al., 2006). The IAA produced by plant growth promoting rhizobacteria had been reported by many researchers (Zhao et al., 2011; Rashid et al., 2012). Moreover, better plant growth might also be due to better availability of essential nutrients to the plants as these rhizobacteria were good zinc mobilizers as well as auxin producers. Many scientists reported that zinc solubilizer also produce auxin that could improve overall plant nutrition (Hussain et al., 2013a and b). The increase in available zinc by bio-activated zinc formulation might be due to the chelation of zinc with zinc solubilizing bacteria in the incubation period at the time of formulating the product. This is confirmed from the previous studies that in some microorganisms, chelation has been observed as dominant phenomena to improve bioavailability and uptake by plant roots. For instance, Whiting et al. (2001) suggested that possible mechanism used by bacteria (Microbacterium saperdae, Pseudomonas monteilii, and Enterobacter cancerogenes) for increasing water soluble Zn (bioavailable) in soil was the production of Zn chelating metallophores. In another report, Tariq et al. (2007) found that Azospirillum lipoferum (JCM-1270, ER- 20), Pseudomonas sp. (96-51) and Agrobacterium sp. (Ca- 18) mobilized Zn and made it bioavailable for longer period of time when they were applied as a biofertilizer to rice by producing chelating agent like ethylene diaminetetraacetate (EDTA).

BOZ 4 results were also better than other BOZ formulations, because BOZ 4 contain more organic matter in the formulation than the BOZ3, BOZ 2 and BOZ 1. These results are an agreement with the previous reports that organic matter is considered a very crucial factor in nutrient mobility. Various organic amendments such as compost, farmyard manure (FYM), poultry manure, olive husk etc., are applied to soil to improve soil health, fertility and crop yields. The organic material can improve the availability of Zn by releasing Zn with time. These properties may increase soluble/available fraction of Zn in soil for plant uptake. Moreover, application of organic amendments also improves biological properties of soil (Tejada et al., 2006). Application of ZnO alone did not have any significant effect of growth of maize. Zinc oxide (ZnO) is insoluble source of zinc, the indigenous microorganism solubilizes this source but the concentration of available zinc is too small which could not
increase growth of maize. Inoculation with zinc solubilizing bacteria enhances growth of maize under pot and field conditions at par compared to the control but less than ZnSO₄ and BOZ formulations. More growth as compared to control is due to the zinc solubilizing bacteria. Similar increases in dry matter accumulation, zinc acquisition through the inoculation of PGPR have been reported by Mader et al. (2010); Rana et al. (2012); He et al. (2010); Zhao et al. (2011) and Minaxi et al., (2012).

Plant physiology depends on diverse metabolic processes involving a large number of enzymes which, in turn, also depend on other elements such as cofactors or coenzymes to be activated and catalyzed. One of the many enzymes involved in physiological processes is carbonic anhydrase (CA). The gaseous exchange attributes like photosynthetic rate, transpiration, stomatal conductance, chlorophyll contents and carbonic anhydrase were also improved due to application of bio-activated formulations. The more increased was observed in case of BOZ 4 formulation as compared to available, unavailable sources of zinc and inoculation alone. The increase in the physiological parameters of the maize might be due to the increase in zinc concentration in plants that increase the carbonic anhydrase activity. Carbonic anhydrase catalyzes the rapid conversion of carbon dioxide plus water into a proton and the bicarbonate ion (HCO₃⁻) that can be found in prokaryotes and higher organisms; it is represented by four different families. Carbonic anhydrase is a metalloenzyme that requires Zn as a cofactor and is involved in diverse biological processes including pH regulation, CO₂ transfer, ionic exchange, respiration, CO₂ photosynthetic fixation, and stomatal closure. Therefore, relevant aspects about CA morphology, oligomerization, and structural differences in the active site. On the other hand, we consider the general characteristics of Zn, its geometry, reactions, and physiology. We then consider the CA catalysis mechanism that is carried out by the metal ion and where Zn acts as a cofactor. (Escudero-Almanza et al., 2012). Several authors have also reported that CA activity can be specifically inhibited by Zn deficiency in some plants without any significant reduction in the rate of photosynthesis (Edwards and Mohamed, 1973; Randall and Bouma, 1973; Ohki, 1976, 1978). As the carbonic anhydrase activity increase the photosynthetic rate, transpiration rate, stomatal conductance and chlorophyll contents of the maize plant increased in pot and filed condition. This improvement in physiological attributes might be due better root growth which improves over all plant growth due better acquisition of water and nutrients form soil.
As, these zinc solubilizing rhizobacteria had ability to produce auxin, therefore it might be the reason that better root growth could be due to auxin biosynthesis (Khalid et al., 2004; Kamilova et al., 2006). BOZ 4 increased more physiological parameters as compared to other BOZ formulations even contain same amount of zinc, is due to the more organic matter present in the BOZ formulations. These bio-activated formulations are associated with their ability to ensure high bio-availability of free zinc to the plants either slow release of zinc (Sarvanan et al., 2003). Due to zinc chelation with organic matter (Kucy, 1987).

Zinc has role in membrane permeability, with the application of BOZ the electrolyte leakage decreased as compared to ZnSO$_4$, but the maximum decreases were noted in case of BOZ4 formulation. This increase was agreement is due to, in plants, zinc is considered to play a critical physiological role in the structure and function of biomembranes. In plants, this has been demonstrated indirectly. Welch et al. (1995) used root exudates as an indicator of root plasma membrane integrity and found greater leakage of the 32P phosphorus isotope out of roots of zinc-deficient wheat than from zinc-sufficient roots.

Yield (yield per pot, 100 grain weight, yield kg/ha in field, 1000 grain weight) parameters of maize was significantly increased with the application bio-activation zinc (BOZ) formulations as compared to available source of zinc (ZnSO$_4$), unavailable source of zinc (ZnO) and inoculated zinc solubilizing bacteria in most of the cases. From the bio-activated zinc formulations BOZ 4 performed better than other BOZ formulations. As the bio-activated zinc formulation contain organic material along with the bacteria and ZnO. The increase in yield of maize under pot and field conditions is might be due to zinc solubilizing bacteria solubilize by solubilizing of ZnO using organic matter as carbon source and increased the zinc bioavailability in the rhizosphere of maize roots which ultimately increased the yield of maize through different direct and indirect mechanism. The results of our study are similar with the previous results that the inoculation of *Penicillium bilaji* increased Zn solubilization and uptake in plant to greater extent which might occur through chelating mechanism (Kucy, 1987). Similar work has been done previously on rice inoculated with zinc solubilizers (Tariq et al., 2007). The increase in zinc concentration and yield of maize could be attributed to the zinc mobilization potential of ZSB from maize rhizosphere. The considerable increase in the maize yield by bio-activated zinc formulations is due to drought tolerance feature of plant growth promoting rhizobacteria as reported earlier.
Various direct and indirect mechanism of PGPR have been explored behind the stress tolerance and plant growth promotion (Glick 1995). The zinc solubilizing bacteria had ability to produce auxin, therefore it might be the reason that better root growth could be due to auxin biosynthesis (Khalid et al., 2004; Kamilova et al., 2006). Ultimately, better yield obtained (Tariq et al., 2007). The mechanisms of acquisition of zinc by rhizobacterial strain from insoluble zinc (ZnO) compounds might be due to production of organic acids of microbial leading to solubilization of zinc and therefore influence the bioavailability of zinc, ultimately release of zinc in bioaccumulation of zinc inside cells of bacterial species had been reported (Agusto da Costa and Duta, 2001).

Similar decline in the rhizosphere pH with the inoculation of *Bacillus cereus* W9 was previously reported by Yu et al. (2011). Excretion of organic anions is often associated with proton extrusion, leading to a substantial lowering of rhizosphere pH (Dinkelaker et al., 1989; Neumann and Romheld, 2002), especially with the inoculation of PGPR. This decrease in pH might be resulted due to more availability of essential plant nutrients in the rhizosphere like phosphorus and zinc which ultimately improved growth and yield of maize. The bacterial strain (*Bacillus sp. AZ6*) used in the bio-activation of zinc had the ability to produce the organic acid. These are the acids i.e., cinamic acid, ferulic acid, caffeic acid, chlorggenic acid, syririgic acid and gallic acid were present in the metabolites of *Bacillus sp. AZ6*. As the production of organic acids can also directly facilitate the mobilization of zinc by reducing sorption of Zn by altering the surface charge characteristics of soil colloids, desorption of Zn ions from sorption sites (Jones, 1998).

BOZ4 contain more organic matter in the formulation along with same quantity of ZnO and zinc solubilizing bacteria as comparison to BOZ3, BOZ 2 and BOZ 1 formulation (Table 3.1). Application of BOZ 4 increased the yield and yield contributing parameters more as compared to other BOZ formulations. The more increase in the yield of maize with same quantity of zinc from insoluble zinc (ZnO) applied in the BOZ formulation was due to the more organic matter in BOZ 4 formulations. Bacteria survive for longer time due to organic matter. Carrier material provides the suitable micro-environment to increase the survival of applied bacteria (Roy et al., 2010). This is confirmed from the studies that organic matter is very crucial factor in nutrient mobility in soil. Various organic amendments are applied to soil to improve soil health, fertility and crop yields. The organic material can
improve the availability of Zn by releasing Zn with time and through changes in physicochemical properties of soil. These properties may increase soluble/available fraction of Zn in soil for plant uptake. Moreover, application of organic amendments also improves biological properties of soil (Tejada et al., 2006). For instance, microbial biomass and soil enzyme activities are substantially increased with the application of organic amendments (Blagodatsky and Richter, 1998; Liang et al., 2003). Application of insoluble source of zinc (ZnO) alone did not have any significant effect of yield of maize. Zinc oxide (ZnO) is insoluble source of zinc, the indigenous microorganism solubilizes this source but the concentration of available zinc is too small which did not increase growth of maize. Inoculation with zinc solubilizing bacteria enhances yield of maize under pot and field conditions at par compared to the control but less than ZnSO$_4$ and BOZ formulations. More yield as compared to control is due to the zinc solubilizing bacteria. Similar increases in dry matter accumulation and zinc acquisition through the inoculation of PGPR have been reported by Mader et al. (2010); Rana et al.(2012); He et al.(2010); Zhao et al. (2011) and Minaxi et al., (2012). Moreover, increase in available zinc in rhizospheric soil due to inoculation with zinc solubilizing bacteria might be correlated with increase in the mineralization or degradation of organic complexes and bound zinc and/or solubilization of zinc from recalcitrant sources such as carbonates, hydroxy carbonates and oxide of zinc into exchangeable zinc (Neumann and Romheld, 2002, 2002; Oburger et al., 2009).

At maturity, bio-activated zinc formulations (BOZ 4) significantly increased Zn concentration in maize shoots and grain as compared to available (ZnSO$_4$) and unavailable source of zinc (ZnO). As the bio-activated product contain PGPR, So these bacteria release zinc slowly. Similar increases in dry matter accumulation, zinc acquisition through the inoculation of PGPR have been reported by Mader et al. (2010), Rana et al.(2012), He et al.(2010), Zhao et al. (2011) and Minaxi et al., (2012). The increased zinc grain might be mainly due to high enzyme activities, significant drop in rhizosphere pH, and redistribution of native zinc pools resulting in increased zinc availability for crop acquisition. Present study confirms the significance of bio-activated zinc formulations to increase zinc concentration in maize grains above the minimum requirements needed for daily human requirements. The increased zinc concentration in maize grain as found in this study might be implicated to
overcome zinc malnutrition of the rural populations, wherein, zinc malnutrition is widespread.

Cereals are the most widely cultivated and consumed crops globally. A cereal provides a major food resource for man. It is the major source of energy and protein in the diet of many people. Several studies have been carried out on the nutritional composition of cereal (Ajayi and Korede, 1991; Addo 1983; Kushiro et al., 1992; Sule Enyisi et al., 2014) on most part of the world but little work is done on maize in Pakistan. In present study one aims was to investigate the nutritional and mineral composition of maize. Bio-activated zinc formulation (BOZ4) significantly increased the proximate and mineral composition (crude protein %, Ash %, crude fiber%, oil%, dry matter% and moisture %) of maize as compared to available (ZnSO4), unavailable (ZnO) sources of zinc and zinc solubilizing bacteria alone. Due to bio-activated zinc formulations zinc concentration increased in the soil solution, plants uptake more zinc in the grain.

In general, the amount of protein in zinc deficient plants is greatly reduced but the composition remains almost unchanged. The importance of zinc in protein synthesis suggests that relatively high zinc concentrations are required by meristematic tissue where cell division as well as synthesis of nucleic acid and protein is actively taking place. The mechanism by which zinc deficiency affects protein synthesis is considered to be due to a reduction in RNA and the deformation and reduction of ribosomes. In the meristem of rice seedlings, it has been found that the level of RNA and the number of free ribosomes was dramatically reduced by zinc deficiency. (Tiller, 1983). Zinc application along with nitrogen had synergistic effect on N and Zn uptake (Rahman et al., 2002). As the zinc increased, the nitrogen uptake also increases. A study on the effect of N rates on maize grain quality showed highest crude protein content (8%) at the higher rate of nitrogen 200 kg ha\(^{-1}\) as compared to lower rates (Bationo et al., 2004).

Dry matter was low with low nitrogen; application of zinc along with N had synergistic effect on N and Zn uptake (Rahman et al., 2002). As the zinc increased the nitrogen uptake increased, the dry matter also % also increased. The results are agreement with previous results of (Iritani and Weller, 1980; Sowokinos and Preston, 1988). The lower moisture content is important as it enables long storage by minimizing fungal contamination and spoilage of the maize/maize products. Maize bran is an important source of protein
supplement and energy for ruminant (Ghol, 1981). Proximate composition shows moisture content in the range of 9.201-10.908% (Ikram Ullah et al., 2010). Bio-activated zinc formulation increased crude fiber. Crude fiber was found to be the fourth largest chemical present in maize grain. The result of fiber content obtained in this study was in agreement with the findings of Ajabadenyi and Aebolu 2005, who reported fiber content in the range of 2.07 – 2.97, for maize grains. The percentage oil obtained for maize grains in this study was consistent and in agreement with other researchers (Matida et al., 1993; Ikenie et al., 2002).

Phytate to zinc ratio in maize grains was significantly decreased with the application of bio-activated zinc formulations. The phytate to zinc ratio is considered as a most important parameter to predict the relative bioavailability of zinc in food and feed (Cakmak et al., 2010; Zhang et al., 2012). A decrease in the phytate to zinc ratio with application indicates potential of bio-activated zinc formulations release more zinc. The increase in available zinc in bio-activated zinc formulation might be due to the chelation of zinc with zinc solubilizing bacteria in the incubation period at the time of formulating the product. These also improved nutritious quality of crops. In Pakistan maize is sown in two different seasons (March sowing and July sowing). In present study, two trials were conducted (Field trial I March, Field trial II July). There were found 2 to 10% difference in the results. The observed differences may possibly be due to environmental (Temperature) factors. Effect of bio-activated zinc formulation was almost same.

Zinc solubilizing bacteria solubilized the insoluble source of zinc (ZnO). With the introduction and best performing zinc mobilizer, four different bio-activated zinc formulations were prepared in combination with organic material. Our data suggest that the bio-activated zinc formulations (BOZ4) significantly improved the growth of maize under pot and field conditions over available (ZnSO₄), unavailable source of zinc (ZnO) inoculated zinc solubilizing bacteria and other BOZ formulations. The combine use of organic material enriched with cheap zinc source ZnO and zinc solubilizing bacteria could be novel approach. At the same time, it is cost effective, less time consuming and environmental friendly as compared to other strategies.
SUMMARY

Zinc is considered as an essential nutrient not only for improving crop production, but also for healthy life of human beings. Globally, Zn deficiency prevails in human beings due to soil and plant factors and proven to be the fifth largest cause of death in developing countries as adequate amount of zinc is essential for cereals. It is a well-established fact that more than 70% of Pakistani soils are categorized as zinc deficient, that ultimately results in Zn deficient crops as well. Zinc deficiency is common in the major staple cereal crops: rice, wheat and maize (human food). Maize is an important cereal crop of the world and has great value in livestock and poultry production. It is crucial to increase bioavailability of zinc in maize. Zinc availability can be increased by different application methods in these soils to enhance maize production. Zinc solubilizing capabilities appear to be well known and widespread characteristics within different bacterial taxa. A novel and promising approach in this respect is the use of zinc solubilizing microorganisms with organic amendment to solubilize unavailable source of zinc (ZnO), increased the availability of zinc. The present study was planned to formulate & evaluate bio-activated zinc for improving yield and quality of maize. The results of the study are summarized below.

Several zinc solubilizing bacteria were isolated from rhizosphere soil of maize grown in different areas. The plate assay results showed good potential of zinc solubilizing rhizobacteria to solubilize the insoluble ZnO. The rhizobacteria showing efficient zinc solubilizing potential were further tested in a broth assay supplemented with ZnO. The results implied that bacterial isolates reduced pH of the broth medium and solubilize insoluble source of zinc (0.1% ZnO) in it. On the basis of zinc solubilizing potential and maximum pH reduction in broth medium by the bacterial isolates, ten potential zinc solubilizing isolates were selected.

The bacterial isolates capable to solubilize ZnO were further screened for their plant growth promoting activity under axenic conditions. The results showed that all the bacterial isolates significantly increased the growth and physiology of maize seedling. Out of ten bacterial isolates, AZ6 was selected because it had the maximum ability to solubilize zinc, reduce pH and improve the growth of maize seedlings.

The selected bacterial isolate (AZ6) was later identified by using 16S rRNA sequences as Bacillus sp. AZ6. The Bacillus sp. AZ6 was characterized for its plant growth
promoting attributes like auxin production, ACC-deaminase activity, siderophores production and organic acid production. Results implied that the Bacillus sp. AZ6 had the ACC-deaminase activity and produced siderophores for the biocontrol purpose. Auxins productions were observed by the inoculation of Bacillus sp. AZ6 in the presence and absence of L-tryptophan. The organic acids produced by the Bacillus sp. AZ6 were cinamic acid, ferulic acid, caffeic acid, chlorggenic acid, syrirgic acid, gallic acid; detected on HPLC. These acids solubilized the insoluble source of zinc by lowering pH of broth media.

Most effective growth promoting and zinc solubilizing bacterial strain (Bacillus sp. AZ6) was used with the organic material to bio-activate the insoluble zinc with different formulations. Four different formulations were prepared using organic material (grinded orange peel), ZnO and zinc solubilizing bacteria.

These bio-activated zinc formulations were evaluated for temporal release of zinc in soil in a wire house experiment. It was noted in the experiment that with the application of bio-activated zinc formulations, zinc bioavailability was increased significantly as compared to control (without zinc), at 60th day of incubation. Different chemical, biological and bio-activated combinations of zinc release different amount of Zn during the incubation trial. The maximum zinc release was noted with the application of BOZ4 combination, during all the sampling times as compared to all other combinations. Different combinations varied in their potential for enhancing zinc bioavailability in soil and they were further evaluated in pot and field trials for improving yield and quality of maize crop.

The pot experiment was conducted to evaluate and compare the different formulations of bio-activated zinc with the ZnSO4 on maize crop. The results demonstrated that:

As the bio-activated zinc formulations contain organic material, ZnO and zinc solubilizing bacteria in different proportion. Most of the formulations significantly increased yield and yield contributing parameters of maize crop as compared to commercial ZnSO4 (a common source of zinc in Pakistan). The maximum yield of maize was recorded with the application of BOZ 4 (11%) and BOZ3 (7%) as compared to ZnSO4. Results implied that these BOZ formulations proved better than the common source of zinc (ZnSO4). Application of insoluble source of zinc (ZnO) alone did not have any influence on growth, physiological,
yield and quality parameters of maize whereas ZnSO₄ promoted these parameters significantly.

Quality parameters of maize grain, i.e., crude protein, crude fiber, ash content, dry matter and oil content were measured. Results depicted that all sources of zinc improved the quality of maize grain but BOZ formulations and ZnSO₄ proved better than all others. Overall BOZ 4 showed increased grain yield by 10-25% as compared to ZnSO₄. Inoculation with zinc solubilizing bacteria also promoted these quality parameters in most of the cases as compared to control (Without zinc).

Then, the results of pot trial were confirmed under field conditions by conducting experiments on maize in two seasons (Field trial I in March and Field trial II in July) at research area of the Institute of Soil & Environmental Sciences, University of Agriculture, Faisalabad.

In general, all the bio-activated zinc formulations improved growth, physiology and yield of maize. However, BOZ4 proved best that maximize in growth, physiology, quality and yield compared to control and commercial ZnSO₄. BOZ4 and BOZ 3 formulation also proved better in most of the parameters as compared to commercial ZnSO₄. BOZ4 increased maize grain yield 10% in field trial I and 12% more in field trial II as compared to available form of zinc (ZnSO₄). BOZ4 increased zinc concentration in grains and decreased phytate content as compared to available form of zinc (ZnSO₄). Furthermore, BOZ4 also improved quality of grain as a compared to available form of zinc (ZnSO₄).

In both the field trials BOZ 4& BOZ 3 formulations improved growth, physiology, yield and quality of maize crop significantly as compared to control and commercial ZnSO₄. Overall, both the trials showed similar results in most of the cases.

In conclusion, the application of bio-activated zinc formulations had a significant effect on growth, physiology, yield and quality of maize in pot and field conditions. In case of the different formulations of bio-activated zinc, the formulation BOZ 4 and BOZ 3 produced better results regarding growth, physiology, yield and quality parameters of maize in comparison with ZnSO₄, control (no zinc), and other BOZ formulations. Among the bio-activated zinc formulations, i.e., BOZ4 improved 8-12% more grain yield as compared ZnSO₄ in pot and field condition. The combined use of organic material enriched with cheap zinc source ZnO and zinc solubilizing bacteria could be a novel approach at the same time, it
is cost effective, less time consuming and environmental friendly as compared to other strategies. It is concluded that bio-activation of ZnO is an effective strategy for economical supply of the Zn for improving yield and quality of maize. The farmer’s community can get maximum profit from their limited resources.
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