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Inoculation with *Lysinibacillus fusiformis* Strain YJ4 and *Lysinibacillus sphaericus* Strain YJ5 Alleviates the Effects of Cold Stress in Maize Plants

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Abstract

Low temperature is an important abiotic variable that inhibits plant growth and yield by restricting plant distribution on land. Cold-tolerant plant growth-promoting rhizobacteria (PGPR) improve nutrient absorption and availability in plants through biochemical and physiological mechanisms. Furthermore, they increase the tolerance of plants to cold stress. Different strains of bacteria were isolated from the roots of *Suaeda nudiflora*. These isolates were identified using 16SrDNA as *Lysinibacillus fusiformis* strain YJ4 and *Lysinibacillus sphaericus* strain YJ5 and were used to study their role in alleviating the harmful effect of cold stress. The two bacterial strains have the ability to solubilize phosphorus and to produce gluconic acid, phytohormones, catechol and hydroxymate siderophores. The present study aimed to study the effect of inoculating maize seeds with PGPR and its use to alleviate the adverse effects of cold stress. The results showed that cold stress (4 °C) reduces germination, growth criteria, photosynthetic pigments (i.e., chl a, chl b, and carotenoids), photosynthetic rate, membrane stability index, phytohormones (auxin and gibberellin), and mineral contents (N, P, K, and Ca) while increasing conductivity, malondialdehyde (MDA), lignin, cell viability, osmolytes (proline, glycine betaine, and soluble sugars), phenolic content, abscisic acid, 1-aminocyclopropane-1-carboxylic acid (ACC) content and the antioxidant defense system in maize plants. Besides, the lignification, osmolytes, phenolic content, phytohormones, the enzymatic antioxidant defenses (i.e., superoxide dismutase, catalase, and phenylalanine ammonia-lyase), and mineral contents of maize plants increased after inoculation with *L. fusiformis* and *L. sphaericus* alone or in combination as compared to normal and cold stress conditions. In conclusion, the inoculation with *L. fusiformis* and *L. sphaericus* in maize plants induced resistance of osmotic and oxidative stress caused due to exposure to cold stress by upregulation of osmolytes, phenolics, phytohormones, and antioxidant enzymes. Also, *L. sphaericus* strains is more effective in tolerance to cold stress than *L. fusiformis*.

Keywords Antioxidant enzyme · Cold stress · Phytohormones · Proline · PGPR · Glycine betaine · Sugars · Minerals

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Die Inokulation mit dem *Lysinibacillus fusiformis*-Stamm YJ4 und dem *Lysinibacillus sphaericus*-Stamm YJ5 mildert die Auswirkungen von Kältestress bei Maispflanzen

Zusammenfassung

Niedrige Temperaturen sind eine wichtige abiotische Variable, die das Pflanzenwachstum und den Ertrag hemmt, indem sie die Verteilung der Pflanzen auf dem Boden einschränkt. Kältetolerante pflanzenwachstumsfördernde Rhizobakterien (plant growth-promoting rhizobacteria, PGPR) verbessern die Nährstoffaufnahme und -verfügbarkeit in Pflanzen durch biochemische und physiologische Mechanismen. Außerdem erhöhen sie die Toleranz der Pflanzen gegenüber Kältestress. Verschiedene Bakterienstämme wurden aus den Wurzeln von *Suaeda nudiflora* isoliert. Diese Isolate wurden anhand der 16SrDNA als *Lysinibacillus fusiformis*-Stamm YJ4 und *Lysinibacillus sphaericus*-Stamm YJ5 identifiziert und zur Untersuchung ihrer Rolle bei der Abschwächung der schädlichen Auswirkungen von Kältestress verwendet. Die beiden Bakterienstämme sind in der Lage, Phosphor zu solubilisieren und Gluconsäure, Phytohormone, Catechin und Hydroxymat-Siderophore zu produzieren. Ziel der vorliegenden Studie war es, die Wirkung der Beimpfung von Maissaatgut mit PGPR und dessen Einsatz zur Abschwächung der negativen Auswirkungen von Kältestress zu untersuchen. Die Ergebnisse zeigten, dass Kältestress (4 °C) die Keimung, die Wachstumskriterien und die photosynthetischen Pigmente (Chl a, Chl b und Carotinoide), die Photosyntheserate, den Membranstabilitätsindex, die Phytohormone (Auxin und Gibberellin) und den Mineralstoffgehalt (N, P, K und Ca) reduziert, während die Leitfähigkeit, Malondialdehyd (MDA) Lignin, Zelllebensfähigkeit, Osmolyte (Prolin, Glycinbetain und lösliche Zucker), Phenolgehalt, Abscisinsäure, Gehalt an 1-Aminocyclopropan-1-carbonsäure (ACC) und das antioxidative Abwehrsystem in Maispflanzen erhöht wurden. Außerdem stiegen die Lignifizierung, die Osmolyte, der Phenolgehalt, die Phytohormone, die enzymatische antioxidative Abwehr (d. h. Superoxid-Dismutase, Katalase und Phenylalanin-Ammoniak-Lyase) und der Mineralstoffgehalt der Maispflanzen nach der Inokulation mit *L. fusiformis und L. sphaericus* allein oder in Kombination im Vergleich zu normalen und Kältestressbedingungen. Zusammenfassend lässt sich sagen, dass die Inokulation mit *L. fusiformis* und *L. sphaericus* in Maispflanzen die Resistenz gegen osmotischen und oxidativen Stress, der durch Kältestress verursacht wird, durch die Hochregulierung von Osmolyten, Phenolen, Phytohormonen und antioxidativen Enzymen induziert. Außerdem sind die *L. sphaericus*-Stämme bei der Toleranz gegenüber Kältestress effektiver als *L. fusiformis*.

Schlüsselwörter Antioxidative Enzyme · Kältestress · Phytohormone · Prolin · PGPR · Glycinbetain · Zucker · Mineralien

Introduction

Agricultural production of economically important crops is severely constrained by various environmental variables, such as salinity, drought, high temperature and low (cold) temperature (Naeem et al. 2020; Ghonaim et al. 2021; Boinot et al. 2022). Such groups of abiotic factors have a remarkable role in reducing agricultural production worldwide. Among these factors, low temperature is one of the most detrimental factors for agricultural productivity (Anderson et al. 2012). Low temperature imparts miscellaneous effect on the plant similar to salinity and integrated adaptive responses require to be induced by the plant under cold stress. Such abiotic factor causes a chain of biochemical, morphological, physiological and molecular changes, which adversely limit the plant productivity (Ashrostaghi et al. 2022).

Cold stress causes several morphological and physiological changes in plants, which are either directly or indirectly connected to lower photosynthetic efficiency (Mesa et al. 2022; Jha 2019a), alters the membrane lipids, antioxidant enzymes, total protein content, stress-related gene expression, increases proline content, total phenolics, and sugar content, reduces ion leakage from the cell membrane and decreases plant growth (Kong et al. 2018; Zhou et al. 2018). Low temperature affects other aspects of photosynthesis, such as sucrose production in the cytosol, resulting in a buildup of phosphorus-related intermediates in the chloroplast. It induces a reduction of accessible inorganic phosphate and, as a result, lower inorganic phosphate cycling between the cytosol and chloroplast, obstructing the regeneration of ribulose 1.5 bis-phosphatase, which supports CO_2 fixation by obstructing ATP synthesis (Gao et al. 2018). Plants induce various adaptations, such as production of osmoprotectants such as glycine betaine (GB) and proline (Pro), to overcome cold-induced osmotic stress and maintain water uptake (Mohamed and Abdel-Hamid 2013; Mohamed and Abd-El Hameed 2014; Arbona et al. 2017). (). Temperatures near 0 °C cause the formation of ice crystals, which causes membrane damage and electrolyte release. This causes a significant rise in necrosis, which results in the triggering of cell death and plant death (Karabudak et al. 2014; Galluzzi et al. 2018).

Plant growth promoting rhizobacteria (PGPR) establish favorable ecological relationships with plants, stimulating their growth through direct and indirect mechanisms. The

former includes biological nitrogen fixation, synthesis of organic acids and siderophores, modulation of phytohormone synthesis, and activity of the aminocyclopropane-1-carboxylic acid deaminase enzyme (Hussain et al. 2022). The indirect mechanisms include growth inhibition of phytopathogens through competition for space and nutrients, antibiosis by secondary metabolites, volatile organic compounds, and lytic enzymes, induction of plant immune responses, and the improvement of soil physicochemical properties (Basit et al. 2021; Santoyo et al. 2021). Plants can survive in cold temperatures by having a variety of adaptations, including a relationship with plant growth-promoting rhizobacteria (PGPR), which stimulate plant growth in extreme environments. The rhizosphere contains a complex microbial diversity. However, some plant growth-promoting bacteria (PGPB) are distinguished for promoting plant growth and stimulating their tolerance to cold stress (Chandran et al. 2021). The majority of these relationships are symbiotic, in which bacteria endophytic live in plant tissue without causing harm to the host plant (Jha 2019b). Cold-tolerant plant growth-promoting rhizobacteria improve nutrient absorption and availability in plants through biochemical and physiological mechanisms (Hassan et al. 2021; Jha 2019b).

Maize (*Zea mays* L.) is an herbaceous perennial annual plant that is the world's third-largest grain crop. Animals and humans both feed maize. It is used in the production of maize oil, starch, and fructose, as well as in the bread sector. Maize is grown in more nations than any other crop, and because of its adaptability to a variety of environmental factors, it will become the world's greatest crop within a few years. (Mohamed et al. 2019). Despite that successful adaptation to different climate conditions, some limitations to maize cultivation remain, the susceptibility to low temperatures being the major one in the temperate climate. Maize is a cold-sensitive plant, and often, yield is limited in cool, humid regions. Thus, low temperature weakens the seedling and may also cease the grain filling prematurely at the end of the growth cycle resulting in lower and inconsistent grain production in mountainous and temperate areas. Injury to plant cells or tissue under chilling stress during the early seedling stage or low temperatures at the reproductive stage in maize may vary depending upon the stress duration and its extent (Wijewardana et al. 2016). Low temperature stress causes damage to macromolecules, cellular structures, and membranes due to the excessive production of reactive oxygen species (ROS) in maize plants (Hussain et al. 2019). In defense, plants produce more antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), and proline (Hussain et al. 2019).

The aim of this work was to examine the plant growth-promoting characteristics of *L. fusiformis* strain YJ4 and *L. sphaericus* strain YJ5 that allow them to tolerate cold stress through various metabolic adaptations such as regulating osmolytes, antioxidant activities, and plant growth hormones. In addition, *L. fusiformis* strain YJ4 and *L. sphaericus* strain YJ5 could ameliorate the negative impact of cold stress on plants, improving the growth of maize plants.

Materials and Methods

Isolation of Plant Growth Promotion Bacteria

In this study, useful plant-associated bacteria were isolated from the roots of *Suaeda nudiflora* wild mosque plants collected from various locations around Mount Abu near the Gujarat-Rajasthan border in December (Jha and Subramanian 2011). Bacteria were isolated in a semi-solid NFb medium with Fluconazole (0.015% w/v) and then moved to NFb (nitrogen-fixing bacteria) agar plate with Fluconazole and bromothymol blue using the serial dilution procedure. Plates are incubated for 96 h at 15 °C ± 1 °C in a BOD (Bio-Oxygen Demand) incubator, and purified pure cultures are kept at 4 °C on the same medium. The cold-tolerant endophytic PGPR was chosen for future research based on its temperature range of growth and ability to promote plant growth.

Molecular Identification of Bacteria

The molecular identification of selected bacteria was done by ribotyping where genomic DNA was used for PCR amplification of 16SrDNA with the help of 16S universal primer 8F: 5′ AGAGTTTGATCCTGGCTCAG3′ and 1510R: 5′ GGCTACCTTG TTACGTA3′. The band obtained on agarose were eluted and sequenced, the nucleotide sequences obtained were BLAST and submitted to the NCBI data base.

Quantitative Estimation Plant Growth-promoting Characteristics

Freshly prepared 10% ascorbic acid was combined with cold 0.42% ammonium molybdate in 1N H_2SO_4 in a 1:6 ratio and kept on a cold bath for 1 h to determine phosphate solubilization (Ames 1966). At 820 nm, the absorbance was measured. Cultures grown in Pikovskaya's broth at 30 °C, were centrifuged at 12,000 g. The supernatant was collected, and 1 mL of the diluted sample was used to perform the assay. To the sample, 2.3 mL of the above freshly prepared reagent mixture was added, and the tubes were incubated for 20 min at 45 °C in a water bath. Titratable acidity (TA) was determined by titrating 1 mL of culture filtrate against 10 mM NaOH in presence of phenolphthelein

(Whitelaw et al. 1999). For determination of gluconic acid produced by strains, 1 mL of strains supernatant was used and complete to 5 mL with distilled water. A pinch of Eriochrome T dye was used, in addition to NH_4Cl and $MgSO_4$. This solution was titrated with 0.05 M ethylene diamine tetraacetic acid (EDTA) until blue colour appear according to Welcher (1958) method. Estimation of hydroxymate type siderophores was carried out by Mayer and Abdallah (1978) method and catechol groups was estimated by Rioux et al. (1983) colorimetric assay method. Concentration of siderophore was calculated by the following formula:

$$\text{Siderophore concentration} = \frac{\text{ODx}\,1500 \times 1000}{16500}\text{mgL} - 1$$

The bacterial strains were grown in N-broth containing 0.2% yeast extract and 1% glucose and incubated for 24 hrs. After the incubation period, the strains were centrifuged, and the supernatant was used for the estimation of IAA by using Salkowski reagent (0.5 M $FeCl_3$ in 35% perchloric acid). The contents were mixed by shaking and allowed to stand at room temperature for 30 min till the development of pink color and the wavelength of the mixture was read at 530 nm using spectrophotometer according to Kamnev et al. (2001) method. Also, the strains supernatant was used for estimation of GA_3 by using zinc acetate, potassium ferrocyanide and 30% HCl was added. The mixture was read at 254 nm after incubation for 1 h at 20 °C according to Holbrook et al. (1961) method.

Experimental Procedure

Co-inoculation of Maize Seedlings with PGPR and Cold Stress Treatment

According to Jha et al. (2021), the maize seedling was inoculated with selected endophytic PGPR. The seeds of a specific maize variety, Pioneer 30 V92, were received from Gujarat's Main Maize Station. To rule out contamination, the healthy seeds were sterilized and put on a tryptophan glucose yeast extract agar medium. The seeds that were fully free of contamination were then inoculated with PGPR. Certified seeds of maize variety Pioneer 30 V92 were inoculated with isolated selected bacteria by transferring the contamination free germinating seeds from the Petri dish to culture tubes containing 400 µL Hoagland's nutrient medium, 400 µL micronutrients, 1% agar in 40 mL distilled water and bacterial inoculum of the isolated bacteria at a concentration of 6×108 cfu mL^{-1}. To obtain a mixture of both bacterial cultures, an equal volume of both the cultures were mixed in the medium to give a concentration of 6×10^8 cfu mL^{-1} and all the tubes were incubated at 27 °C in a 12 h light/dark cycle in a growth chamber for two weeks for better association. After two weeks growth stage, the maize seedling inoculated with selected bacteria were transferred to pots containing sterilized sand-perlite (1:1) and kept in a growth chamber maintained at 4 °C to 8 °C in presence of cool white fluorescent light, photon flux of 70 µmol $m^{-2}s^{-1}$ and the seedlings were always transferred at 12 hrs light/dark cycle in a growth chamber. Each treatment has three replicates. The plants were irrigated with tap water and with Hoagland nutrient solution once a week. The growth parameters were recorded after two weeks of transfer in the greenhouse.

The confirmation of root association of the endophytic PGPR with maize has been done by staining the root with 2, 3, 5-triphenyl tetrazolium chloride stain for overnight (Jha and Subramanian 2018). The presence of cold tolerant endophytic PGPR in the root has been observed in the cross-sections of the stained root under an image analyzer microscope (Carl Zeiss) as red colored cell.

Morphological Criteria

After one month of sowing the plants were collected to determine morphological criteria such as root length, shoot length and dry weigh of plant. In addition, the leaves were used for all biochemical constituents.

Determination of Photosynthetic Parameter and Rate of Photosynthesis

Fresh leaves (0.5 g) of maize plants are used to extract chlorophyll by shaking them in 80% acetone until they are totally bleached, as described by Kamble et al. (2015). Using a spectrophotometer, the extract's supernatant is used to quantify chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoid at 663, 645, and 470 nm absorbance, respectively. The photosynthetic rate was determined using fresh leaves by a Li-Cor 6400 open-system portable photosynthesis meter.

The chlorophyll a, b and a + b (total chlorophyll) contents were calculated out by applying the following (Arnon 1949) formulae:

$$\text{mg chlorophyll a/g tissue} = \frac{12.7\,(A663) - 2.69\,(A645) \times V}{1000 \times W}$$

$$\text{mg chlorophyll b/g tissue} = \frac{22.9(A645) - 4.68(A663) \times V}{1000 \times W}$$

$$\text{mg carotenoids/g tissue} = \frac{1000(A470) - 2.27(Chla) - 81.4(Chlb)}{227}$$

- Where, A = absorbance at specific wavelength
- V = final volume of chlorophyll extract in 80% acetone
- W = fresh weight of tissue extracted

Determination of Cell Membrane Stability (Solute Leakage)

Cell membrane stability (CMS) in leaves was determined in 500 mg of leaves per treatment, according to Sullivan and Ross (1979) using a Conductivity meter (DIGICOND IV, Buenos Aires. Argentina). CMS was determined according to the following equation:

$$\text{CMS} = \frac{1-(T1/T2)}{1-\left(\frac{C1}{C2}\right)} x100$$

T and C refer to conductivity of treated and control sampled. T1 and C1 represent the electrolyte leakage (dS m-1) after incubating at 25 °C for 4 h, and T2 and C2 represent the total electrolyte concentration measured after heating in boiling water for 60 min and cooled to room temperature. T1 and T2 correspond to the first and second solution conductivity determinations of treated samples, and C1 and C2 are the respective values for the controls.

Determination of Lipid Peroxidation

By crushing the leaves (200 mg) into a fine powder under liquid nitrogen, lipid peroxidation (as determined by MDA content) was detected in plant tissue. The tissue was mixed in 800 µL of cold 5% (w/v) tri chloroacetic acid (TCA). The mixture was centrifuged at 12,000 g for 30 min was mixed with thiobarbituric acid (TBA) and the wavelength was read at 532 nm and 600 nm using a spectrophotometer according to the method of Rao and Sresty (2000). The amount of MDA was calculated using an extinction coefficient of 155 mM cm^{-1}.

Determination of Lignin

Leaves were washed and placed in a forced air circulation oven at 65 °C for 7 days, until a constant mass was reached. The leaves were then ground using liquid nitrogen and stored at ±4 °C. For total lignin leaves samples were homogenized in 50 mM sodium phosphate buffer, pH 7.0, purified in 1% Triton X-100, 1M NaCl, and acetone, and centrifuged for 15 min.The final pellet was dried and considered to be the protein freed from cell walls and lignin was quantified using the thioglycolic acid according to the method of Kovácik and Klejdus (2008).

Determination of Cell Viability

Sanevas et al. (2007) method was used to measure cell viability. Leaves of maize plants were stained for 15 min at room temperature with 0.25% (w/v) Evans Blue, then rinsed for 30 min in distilled water and then put in 1% (w/v) SDS following incubation for 1 h at 55 °C. The extract was read at 600 nm using a spectrophotometer.

Estimation of Proline

Bates et al. (1973) technique was used to measure proline content in fresh maize leaves (0.5 g) after mixing in 10 mL of 3% sulfosalicylic acid before being filtered. 2 mL of supernatant was mixed with 2 mL of acid ninhydrin (3% v/v) and 2 mL of glacial acetic acid, and then 4 mL of toluene was added. the absorbance was measured at 520 nm using a spectrophotometer.

Estimation Glycine Betaine

Glycine betaine (GB) was measured according to the method of Grieve and Grattan (1983). Half gram of dried maize leaves was mixed with 20 mL of deionized water for 24 h at 25 °C. The mixture was then filtered, and the supernatant were diluted (1:1) with 2N H_2SO_4 and mixed with KI-I_2 reagent (20% KI-8.5% I_2) and 1, 2-dichloroethane was added. The absorbance was read at 365 nm by using a spectrophotometer.

Determination of Sugars

One gram of leaves methanolic phase was used for the quantification of total soluble sugar according to Irigoyen et al. (1992). Soluble sugars were analyzed by 0.1 mL of the alcoholic extract reacting with 3 mL freshly prepared anthrone (200 mg anthrone 100 mL 72% (w:w) H_2SO_4) and placed in a boiling water bath for 10 min according to Irigoyen method. After cooling, the absorbance was determined at 620 nm in a spectrophotometer. The calibration curve was made using glucose in the range of 20–400 µg mL^{-1}.

Determination of Total Phenolic Content

Total phenolic content in leaves of maize plants was determined using Folin-Ciocalteu assay according to the method of Shahidi and Wanasundara (1992). Test tube containing either 500 µL of crude extracts (diluted 400-fold with distilled deionized water) was prepared; 500 µL of 10% Folin-Ciocalteu phenol reagent (in DDW) was added into each test tube and mixed. After 20 min, 350 µL of 1M Na_2CO_3 solution was added into the reaction mixture and incubated

for 20 min at room temperature, the absorbance was determined at 750 nm using a spectrophotometer.

Estimation of Indole Acetic Acid Production

The reaction mixture comprised of 15 mL of leaf extract and ferric chloride-perchloric acid reagent was added in 1:2 ratio for estimation of IAA produced by the cultures. The tubes were incubated at room temperature for 25 min. The wavelength of the mixture was read at 530 nm using a spectrophotometer according to Kamnev et al. (2001) method.

Estimation of Gibberellic Acid

The reaction mixture comprised of 15 mL of leaf extract and 2 mL of zinc acetate. The mixture was then incubated at room temperature for 2 min and 2 mL of potassium ferrocyanide was added. The mixture was then centrifuged at low speed for 15 min. To 5 mL of supernatant, 5 mL of 30% HCl was added. The reaction mixture was incubated at 20 °C for 75 min. The wavelength of the mixture was read at 254 nm according to Holbrook et al. (1961).

Determination of Abscisic Acid

Fresh leaves of maize plants were ground in 10 mL of 80% v/v methanol. This solution was kept under constant agitation of 150 rpm at 4 °C for 12 h. Afterwards, the samples were filtered with hydrophobic paper of 0.22 µm porosity, and the extract was concentrated with a rotary evaporator at 50 °C to eliminate the methanol and further extraction was performed four times with 10 ml ethyl acetate resulting in organic phase, which contained free ABA and was quantified at 265 nm with a UV-VIS spectrophotometer according to the method of Materán et al. (2009).

1-aminocyclopropane-1-carboxylic Acid (ACC Deaminase) Activity Assay

To determine the ACC deaminase activity in leaves extract the amount of F-ketobutyric acid (F-KA) generated from the cleavage of ACC was monitored using spectrophotometer (Penrose et al. 2001). The amount of F-KA produced during this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of F-ketobutarate.

Extraction and Determination of Antioxidant Enzyme

Leaves of maize plants (2 g) were mixed with 4 mL of ice-cold 50 mM Tris-acetate buffer pH 6.0. The mixture was centrifuged at 12,000 g for 20 min and the supernatant was complete to know volume and kept measuring antioxidant enzymes. Phenylalanine ammonia lyase (PAL; EC 4.3.1.24) activity was measured following the method of Dickerson et al. (1984) by using 1 mM L-phenylalanine and the absorbance of the toluene phase containing Trans-cinnamic acid was read at 290 nm against the blank of toluene. Catalase activity (CAT; EC 1.11. 1.6) was measured the initial rate of disappearance of H_2O_2 according to the method of Ramalingam and In-Jung (2013). Superoxide dismutase (SOD, EC 1.15.1.1) activity was estimated spectrophotometrically as inhibition of photochemical reduction of NBT at 560 nm according to Costa et al. (2010) method.

Determination of Inorganic Ion Concentrations

Before mineral analysis, the maize leaves were dried and ground. After 6 h of digestion in nitric-perchloric acid (5:3), the phosphorus concentration was measured using a colorimetric method according to Mohamed et al. (2016). One gram of plant material was digested in a tri-acid mixture of sulphuric acid (H_2SO_4), nitric acid (HNO_3), and perchloric acid ($HClO_4$) in a 9:3:1 ratio to determine plant K, N, and Ca. Using a special filter on digital flame photometry, the digested material was filtered, and its aliquots were examined. After the Kjeldahl digestion, the N concentration was measured using colorimetry.

Statistical Analysis

One-way ANOVA was used to evaluate the data (analysis of variance). All of the treatments were repeated three times. At the P 0.05 level, differences were judged significant. Duncan's test was used to compare the means.

Results

Molecular Identification of Bacterial Isolates

Morphological and biochemical characteristics, as well as molecular analysis, are used to identify the isolates in this study. The isolates' molecular study included amplification of the 16S rDNA gene, sequencing with universal primers, and BLAST analysis. After separation of the generated PCR product using 16S rDNA specific primer on an agarose gel, both isolates had defined bands of 1.5 Kb (Fig. 1). Both sequences were utilized in phylogenetic profiling and were submitted to a gene bank. *Lysinibacillus fusiformis* strain YJ4 has been identified as HM756642, and *Lysinibacillus sphaericus* strain YJ5 has been identified as HM756643 (Fig. 2).

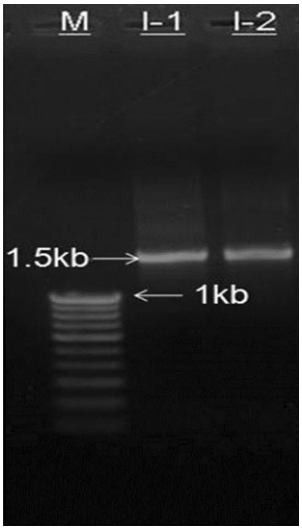

Fig. 1 Agarose gel showing the amplified 16S rDNA of two bacterial isolates. M is 100 bp marker, L-1 and 2 are 16S rDNA amplicons of isolates YJ4 and YJ5 respectively

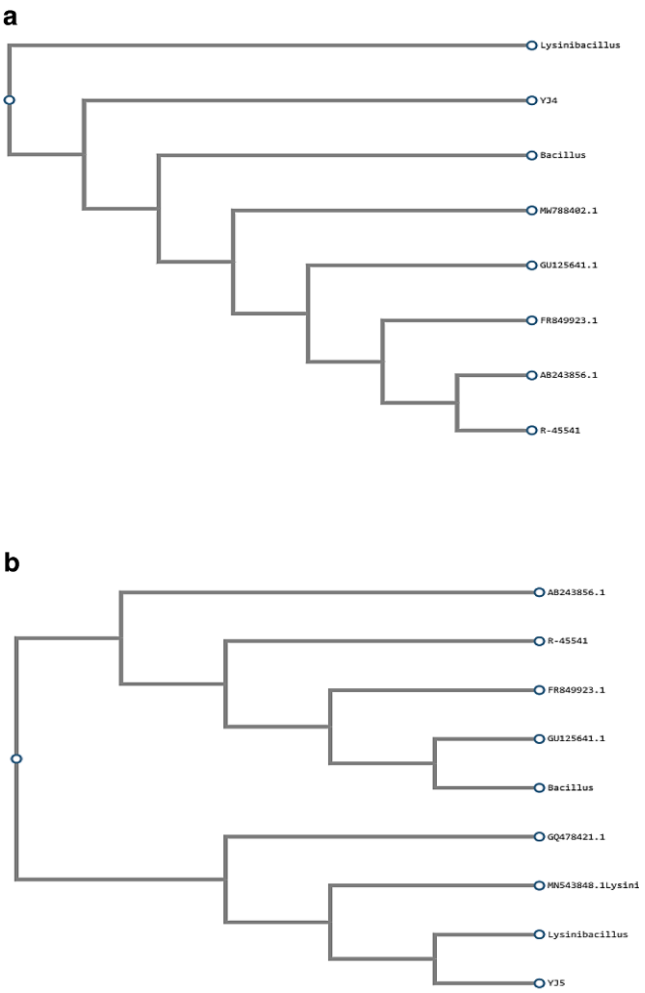

Fig. 2 Phylogenetic tree showing the identification of *L. fusiformis* and *L. sphaericus*

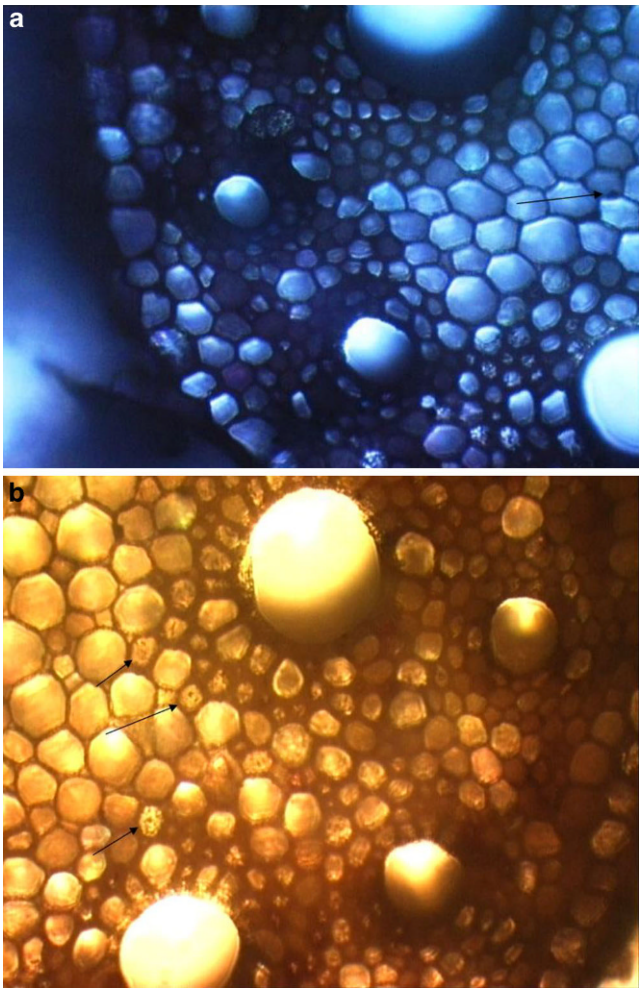

Fig. 3 **a** Photomicrograph of a section of maize plant root showing the association of bacteria in root cortex as dark spots due to TTC staining. **b** Photomicrograph of a section of maize plant root showing the association of bacteria in root cortex as red spots due to TTC staining

Localization of Bacteria in the Maize Root *in vivo*

L. fusiformis strain YJ4 and *L. sphaericus* strain YJ5 were discovered as endophytic root-related bacteria. After TTC staining, a dark red stain was noticed in the transverse section of the root cortical region under the microscope (Fig. 3a,b).

Characterization of the Two Selected Endophytic Bacterial Strains

In terms of phosphate solubilizing ability, the two bacterial strains *L. fusiformis* strain YJ4 and *L. sphaericus* strain YJ5 were shown to be effective. In the current research, the phosphate released and the titratable acidity by *L. sphaericus* was about 278.4 µg P mL^{-1}, 8.7×10^{-2} higher than the other strains which released 231.5 µg P mL^{-1} and 8.3 × 10-2,

Table 1 Changes in pH, phosphate released, titratable acidity, gluconic acid concentration, catechol and hydroximate concentrations in the two bacterial strains during an incubation period of 12 days

Strains	pH	Phosphate released (μg P mL^{-1})	Titratable acidity ($\times 10^{-2}$)	Gluconate ($\times 10^{-4}$ g %)	Concentration of catechol siderophore (μg mL^{-1})	Concentration of hydroximate siderophore (μg mL^{-1})
L. fusiformis	5.8 ± 0.05	231.5 ± 1.42	8.3 ± 1.01	4.86 ± 0.11	11.5 ± 0.03	3.27 ± 1.01
L. sphaericus	5.7 ± 0.02	278.4 ± 3.01	8.7 ± 0.21	5.23 ± 0.13	14.2 ± 2.01	3.97 ± 0.12

Values are mean of three replications and represents mean ± S.D

Table 2 Production of phytohormones auxin and gibberellic acid by the two bacterial strains during different incubation periods

Strains	Auxin Production by the strains in μmol mL^{-1}		
	24 hrs	**48 hrs**	**72 hrs**
L. fusiformis	3.2 ± 0.03	10.7 ± 1.03	12.05 ± 0.11
L. sphaericus	5.2 ± 1.05	11.6 ± 0.01	15.75 ± 2.01
	Gibberellic acid Production by strains μmol mL^{1}		
	24 hrs	**48 hrs**	**72 hrs**
L. fusiformis	2.4 ± 0.02	3.8 ± 0.01	5.1 ± 1.21
L. sphaericus	3.7 ± 1.02	4.8 ± 0.05	6.8 ± 2.01

Values are mean of three replications and represents mean ± S.D

respectively. As demonstrated in Table 1, both strains were capable of dissolving phosphorus with the formation of gluconic acid. The synthesis of catechol and hydroxymate siderophores was also studied in the two strains. *L. sphaericus* generated more catechol (14.2 g mL^{-1}) and hydroxymate (3.97 g mL^{-1}) than the other strain, *L. fusiformis*, which generated 11.5 g mL^{-1} and 3.27 g mL^{-1}, respectively (Table 1). Furthermore, both bacterial strains produced more IAA and gibberellic acid throughout time (24, 48, and 72 h). The production of IAA and gibberellin increased by *L. sphaericus* higher than *L. fusiformis* (Table 2). The most pronounced production of auxin (15.75 and 12.05 μmol mL^{-1}) and gibberellin (6.8 and 5.1 μmol mL^{-1}) were detected after 72 h of incubation periods in *L. sphaericus* and *L. fusiformis*, respectively.

Effect of Endophytic Bacterial Strains on Plant Growth Under Cold Stress

In a greenhouse, the influence of the PGPR on maize growth was studied, and the results revealed that the PGPR has a beneficial effect on plant growth. Table 3 shows that cold stress (4 °C) reduced germination rate (45%), root length (34.3%), shoot length (40.9%), and plant dry weight (49.5%) when compared to non-stressed plants (27 °C). In addition, inoculation of maize plants with endophytic bacterial strains (*L. fusiformis* and *L. sphaericus*) alone or in combination caused a significant boost in the germination and morphological criteria over normal temperature and cold stress conditions. Inoculation with a combination of the two bacterial strains caused a significant boost in germination (22.4% and 26.5%), root length (26.8% and 54.5%), shoot length (21.9% and 16.4%) and dry weight of plants (16.5% and 23.9%) under control and stress conditions,

Table 3 Effect of endophytic bacterial strains on plant growth in maize plant under cold stress

Treatment	Germination %	Root length (cm)	Shoot length (cm)	Dry weight (g $Plant^{-1}$)
Normal				
No inoculation	63.8 ± 0.3^{d}	20.1 ± 0.03^{c}	57.7 ± 0.8^{c}	6.14 ± 0.04^{c}
Inoculation with *L. fusiformis*	68.4 ± 0.5^{c}	23.1 ± 0.05^{b}	61.3 ± 0.9^{b}	6.61 ± 0.05^{b}
Inoculation with *L. sphaericus*	72.2 ± 0.6^{b}	24.2 ± 0.06^{b}	64.3 ± 0.9^{b}	7.17 ± 0.07^{a}
Inoculation with *L. fusiformis* + *L. sphaericus*	79.1 ± 0.7^{a}	26.3 ± 0.07^{a}	71.1 ± 1.0^{a}	7.23 ± 0.07^{a}
Cold stress				
No inoculation	35.1 ± 0.1^{g}	13.2 ± 0.0^{f}	34.1 ± 0.6^{e}	3.10 ± 0.02^{f}
Inoculation with *L. fusiformis*	39.3 ± 0.3^{f}	16.0 ± 0.03^{e}	37.4 ± 0.8^{d}	3.44 ± 0.02^{e}
Inoculation with *L. sphaericus*	40.9 ± 0.3^{f}	18.2 ± 0.05^{d}	38.3 ± 0.7^{d}	3.56 ± 0.03^{de}
Inoculation with *L. fusiformis* + *L. sphaericus*	44.4 ± 0.4^{e}	20.4 ± 0.06^{c}	39.7 ± 0.9^{d}	3.84 ± 0.04^{d}

Values are the means of five replicates. Values with different letters are significantly different at $P < 0.05$ (Duncan's Test)

respectively. Besides, inoculation with *L. sphaericus* was more effective in germination and morphological criteria than with *L. fusiformis.*

Effect of Endophytic Bacterial Strains on Photosynthetic Pigments Under Cold Stress

Inoculating the maize plants with *L. fusiformis* and *L. sphaericus* alone or in combination resulted in a higher level of Chl a, Chl b, carotenoid, and total photosynthetic pigments as well as a higher photosynthetic rate in comparison to control under normal temperature conditions (27 °C) and cold stress conditions (4 °C) (Table 4). However, when the maize plants were exposed to cold stress conditions (4 °C), there was a dramatic drop in Chl a (7.2%), Chl b (52.3%), carotenoid (37.3%), total photosynthetic pigments (23.3%), and photosynthetic rate (25.0%) was detected in non-inoculated control plants (Table 4). The most pronounced increases in Chl a (21.8% and 29.7%), Chl b (42.1% and 33.3%), carotenoid (35.2% and 37.5%), total photosynthetic pigments (34.4% and 27.0%) and photosynthetic rate (29.6% and 44.4%) were detected in maize plants inoculated with a combination of two bacterial strains as compared to control and cold stressed plants, respectively.

Effect of Endophytic Bacterial Strains on Membrane Stability Index, Conductivity, MDA, Lignin Deposition and Cell Viability Under Cold Stress

The membrane stability index (MSI) of maize plants exposed to cold stress (4 °C) decreased significantly by about 51.4% as compared to plants grown under normal conditions (Table 5). In the country, exposure to cold stress showed a significant improvement in the conductivity (46.4%), MDA (26.4%), lignin deposition (16.3%) and cell viability (47.5%) of maize plants when the plants were

Table 4 Effect of endophytic bacterial strains the photosynthetic pigment content and rate of photosynthesis of maize under cold stress

Treatment	Chl a (mg g^{-1})	Chl b (mg g^{-1})	Carotenoid (mg g^{-1})	Total chl (mg g^{-1})	Rate of photosynthesis (μmol $m^{-2}s^{-1}$)
Normal					
No inoculation	0.69 ± 0.02e	0.44 ± 0.01d	0.51 ± 0.02c	1.64 ± 0.2d	24 ± 2.0d
Inoculation with *L. fusiformis*	0.78 ± 0.04cd	0.57 ± 0.01c	0.54 ± 0.02b	1.89 ± 0.2c	27 ± 3.0c
Inoculation with *L. sphaericus*	0.82 ± 0.04b	0.66 ± 0.01b	0.58 ± 0.03b	2.06 ± 0.3b	30 ± 3.0b
Inoculation with *L. fusiformis* + *L. sphaericus*	0.86 ± 0.03a	0.68 ± 0.01a	0.70 ± 0.03a	2.24 ± 0.3a	32 ± 4.0a
Cold stress					
No inoculation	0.64 ± 0.03f	0.21 ± 0.01g	0.32 ± 0.01f	1.17 ± 0.2h	18 ± 1.0f
Inoculation with *L. fusiformis*	0.77 ± 0.02d	0.24 ± 0.01f	0.36 ± 0.01e	1.37 ± 0.2g	20 ± 1.0e
Inoculation with *L. sphaericus*	0.79 ± 0.03c	0.26 ± 0.01ef	0.38 ± 0.01e	1.43 ± 0.3f	23 ± 2.0d
Inoculation with *L. fusiformis* + *L. sphaericus*	0.83 ± 0.04b	0.28 ± 0.01e	0.44 ± 0.01d	1.55 ± 0.3e	26 ± 2.0c

Values are the means of three replicates. Values with different letters are significantly different at $P < 0.05$ (Duncan's Test)

Table 5 Effect of endophytic bacterial strains on membrane stability index, conductivity, MDA and lignin deposition in maize under cold stress

Treatment	Membrane stability index (MSI%)	Conductivity (dS cm^{-1})	MDA (nmol g^{-1} FW)	Lignin (mg g^{-1}FW)	Cell viability (Evan blue Conc mg $cell^{-1}$)
Normal					
No inoculation	84.6 ± 4.0^{d}	70.2 ± 4.0^{g}	326 ± 5.0^{d}	1.47 ± 0.03^{g}	21.9 ± 0.2^{c}
Inoculation with *L. fusiformis*	89.2 ± 3.0^{b}	82.9 ± 4.0^{f}	291 ± 6.0^{e}	1.62 ± 0.05^{f}	18.4 ± 0.1^{d}
Inoculation with *L. sphaericus*	94.1 ± 4.0^{a}	85.3 ± 5.0^{f}	289 ± 6.0^{e}	1.68 ± 0.05^{e}	17.8 ± 0.2^{de}
Inoculation with *L. fusiformis* + *L. sphaericus*	98.7 ± 5.0^{a}	95.9 ± 4.0^{e}	267 ± 5.0^{f}	1.72 ± 0.06^{d}	17.2 ± 0.3^{e}
Cold stress					
No inoculation	41.1 ± 2.0^{g}	102.8 ± 5.0^{d}	412 ± 6.0^{a}	1.71 ± 0.04^{d}	32.3 ± 0.3^{a}
Inoculation with *L. fusiformis*	48.4 ± 3.0^{f}	110.3 ± 4.0^{c}	387 ± 7.0^{b}	1.86 ± 0.04^{c}	22.2 ± 0.5^{c}
Inoculation with *L. sphaericus*	53.7 ± 3.0^{e}	114.1 ± 5.0^{b}	379 ± 7.0^{b}	1.91 ± 0.05^{b}	24.6 ± 0.4^{b}
Inoculation with *L. fusiformis* + *L. sphaericus*	59.7 ± 4.0^{d}	121.5 ± 5.0^{a}	361 ± 5.0^{c}	1.99 ± 0.05^{a}	25.3 ± 0.6^{b}

Values are the means of three replicates. Values with different letters are significantly different at $P < 0.05$ (Duncan's Test)

exposed to cold stress over normal temperature conditions. In addition, the level of MDA and cell viability stained by Evan blue was observed to be minimum in the case of plants inoculated with *L. fusiformis* and *L. sphaericus* alone or in combination, while the level of the membrane stability index, the conductivity, and lignin deposition were observed to be maximum under normal and stress conditions. The inoculation of maize plants with *L. fusiformis* and *L. sphaericus* in combinations resulted in the reduction of MDA (20.3% and 12.4%) and cell viability (25.5% and 21.7%) and boosted the membrane stability index (15.8% and 45.8%), the conductivity (31.0% and 18.2%), and lignin deposition (15.4% and 16.4%) under control and cold stress conditions, respectively (Table 5).

Effect of Endophytic Bacterial Strains on Osmolytes and Phenolic Content Under Cold Stress

Exposure of maize plants to cold stress (4 °C) caused a substantial increase in proline (46.2%), glycine betaine (50%), soluble sugars (56.8%) and phenolic content (5.7%) as compared to plants grown under normal conditions (Fig. 4). Inoculation of maize plants with bacterial strains alone or in combination resulted in a significant boost in osmolyte content and phenolic content compared to control and cold stress plants. The inoculation with *L. fusiformis* and *L. sphaericus* in combinations caused a substantial boost in proline (68.2% and 68.4%), glycine betaine (57.1% and 55.6%), soluble sugars (67.6% and 42.0%) and phenolic content (32.7% and 34.8%) as compared to non-stressed and cold stressed plants, respectively.

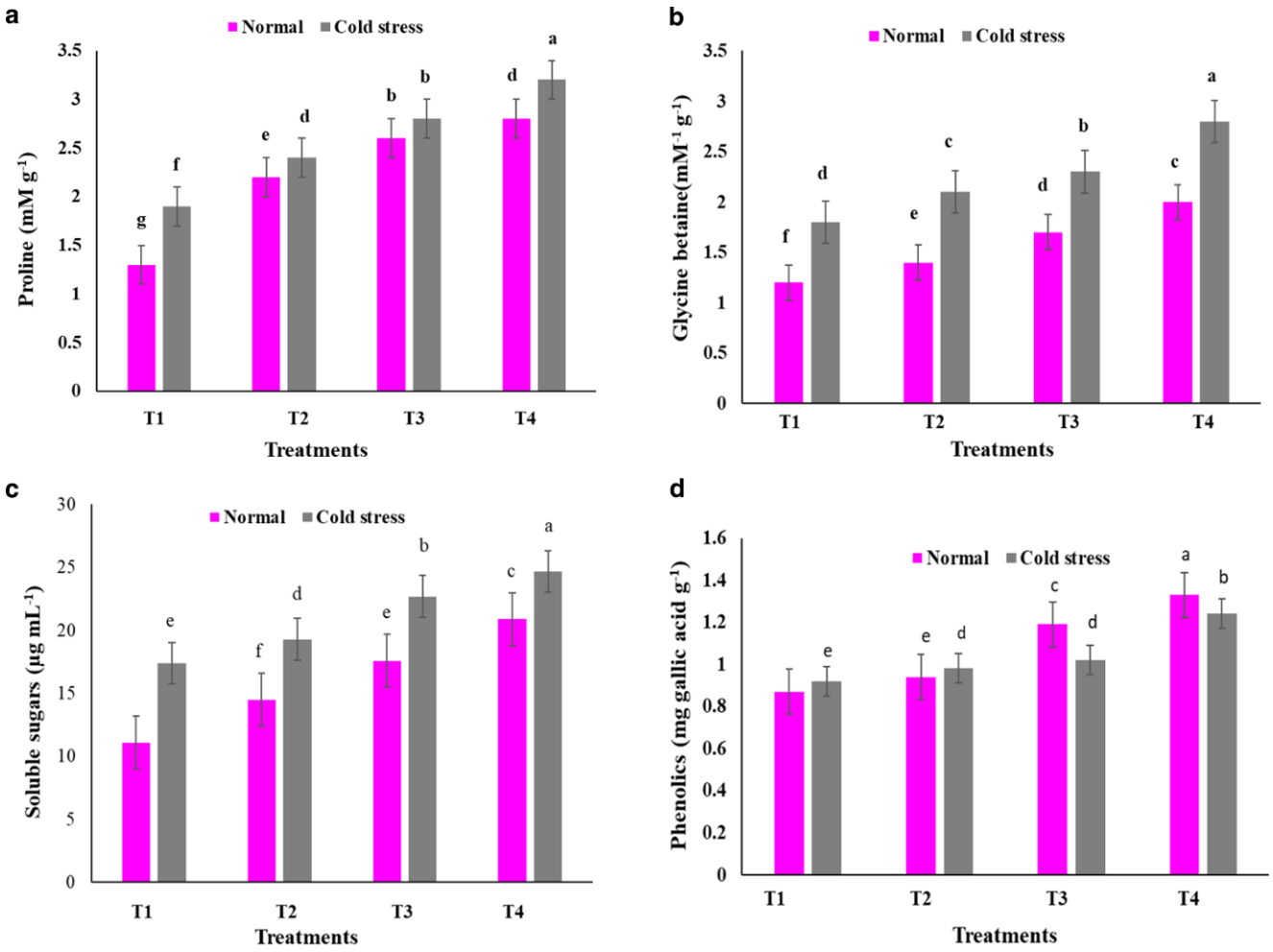

Fig. 4 Effect of endophytic bacterial strains on osmolytes content like proline (**a**), glycine-betaine (**b**), sugar (**c**) and phenolics (**d**) content in maize under cold stress. Values are the means of three replicates. Values with different letters are significantly different at $P < 0.05$ (Duncan's Test). (*T1* Non inoculation, *T2* Inoculation with *L. fusiformis*, *T3* Inoculation with *L. sphaericus*, *T4* Inoculation with *L. fusiformis* + *L. sphaericus*)

Effect of Endophytic Bacterial Strains on Phytohormones and ACC Deaminase Under Cold Stress

When compared to non-stressed maize plants, there was a considerable drop in the level of endogenous phytohormones such as auxin and gibberellin, as well as the buildup of abscisic acid (ABA) and ACC deaminase (Fig. 5). Cold stressed plants inoculated with *L. fusiformis* and *L. sphaericus* alone or in combination exhibited the maximum level of auxin, gibberellin, and ACC deaminase content and the lowest ABA content in contrast to non-stressed and cold stressed plants. Under control and cold stress conditions, maize plants inoculated with *L. fusiformis* and *L. sphaericus* in combination had higher levels of auxin (18.1% and 41.4%), gibberellin (76.8% and 65%), ACC deaminase (45.7% and 47.2%), and lower levels of ABA (23.3% and 27.1%).

Effect of Endophytic Bacterial Strains on Antioxidant Enzyme Activity Under Cold Stress

The phenylalanine ammonia lyase (21.4%), superoxide dismutase (32.2%), and catalase (59.3%) activities significantly increased in maize plants under cold stress (Fig. 6). In addition, the inoculation with *L. fusiformis* and *L. sphaericus* alone or in combinations caused a significant boost in the antioxidant enzyme activity of maize plants under non-stressed and cold stressed conditions. Besides, the higher activities of phenylalanine ammonia lyase (27.2% and 42.9%), superoxide dismutase (25.2% and 24.3%) and catalase (50.9% and 34.9%) were detected in plants inoculated with *L. fusiformis* and *L. sphaericus* in combinations under control and cold stress conditions, respectively.

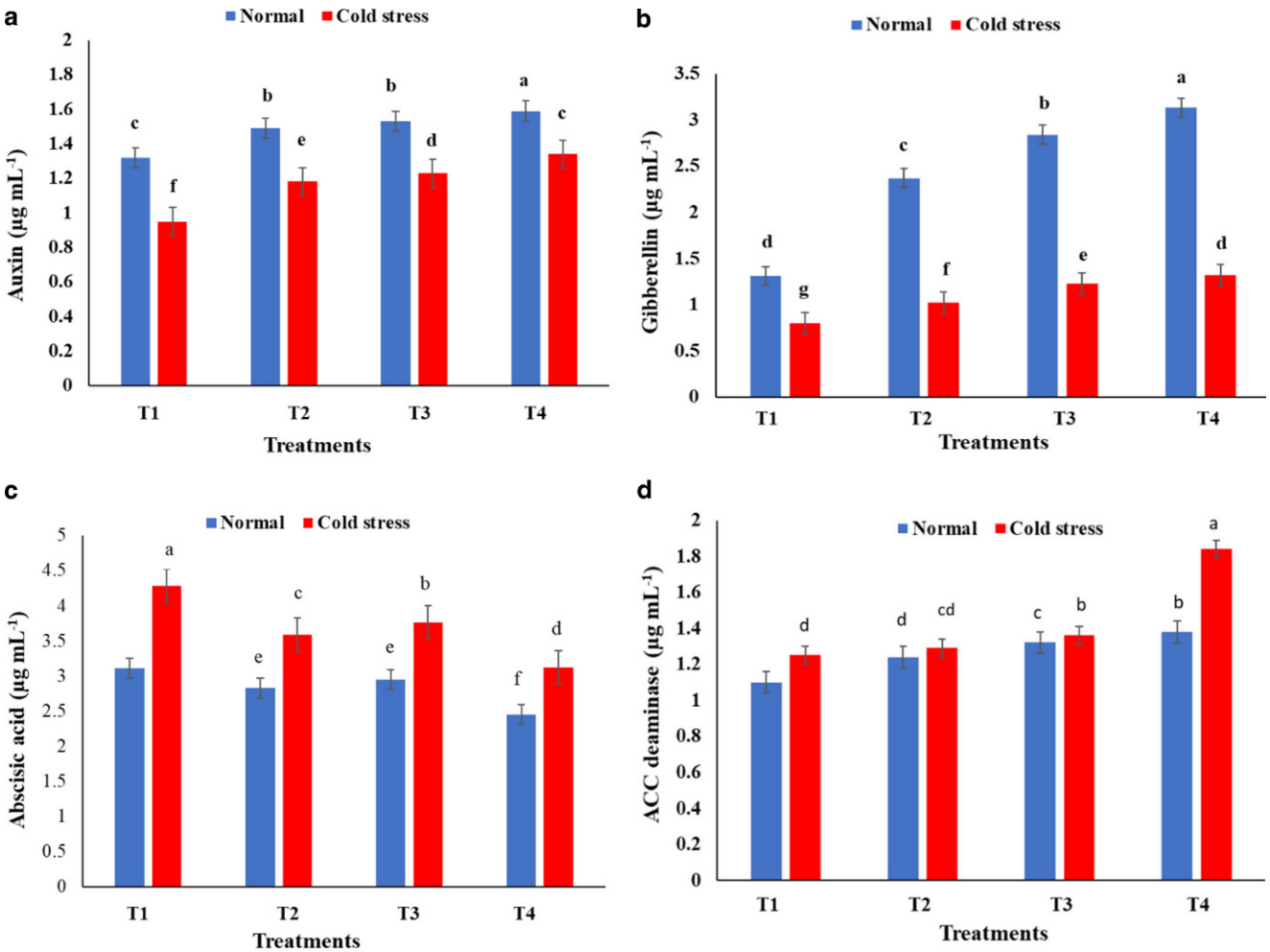

Fig. 5 Effect of endophytic bacterial strains on phytohormones like auxin (**a**), gibberellin (**b**), abscisic acid (**c**) and ACC deaminase (**d**) in maize under cold stress. Values are the means of three replicates. Values with different letters are significantly different at $P < 0.05$ (Duncan's Test). (*T1* Non inoculation, *T2* Inoculation with *L. fusiformis*, *T3* Inoculation with *L. sphaericus*, *T4* Inoculation with *L. fusiformis* + *L. sphaericus*)

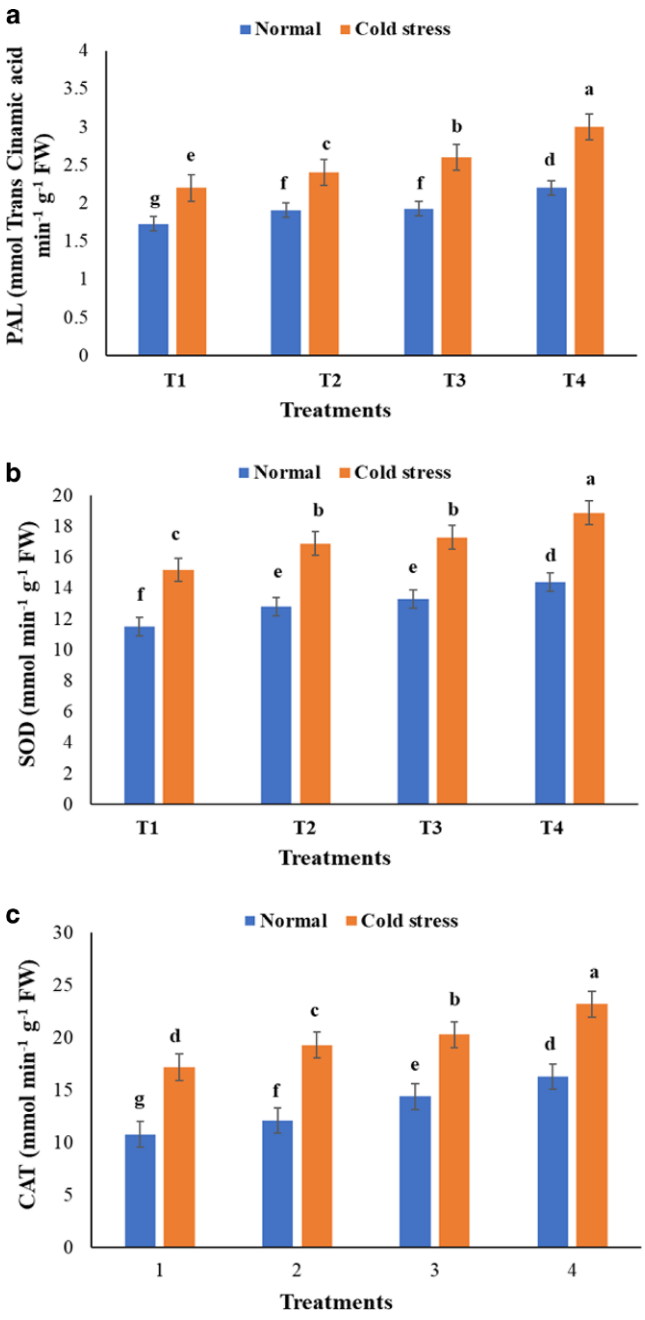

Fig. 6 Effect of endophytic bacterial strains on antioxidant enzyme activity like PAL (**a**), SOD (**b**) and CAT (**c**) in maize under cold stress. Values are the means of three replicates. Values with different letters are significantly different at $P < 0.05$ (Duncan's Test). (*T1* Non inoculation, *T2* Inoculation with *L. fusiformis*, *T3* Inoculation with *L. sphaericus*, *T4* Inoculation with *L. fusiformis* + *L. sphaericus*)

Effect of Endophytic Bacterial Strains on Mineral Contents Under Cold Stress

Inoculating the maize plants with *L. fusiformis* and *L. sphaericus* alone or in combination resulted in a higher level of N, P, K and Ca content in comparison to control under normal temperature conditions (27 °C) and cold stress conditions (4 °C) (Table 6). However, exposure of maize plants to cold stress caused a significant decrease in N (19.5%), P (13.6%), K (42.2%), and Ca (28.6%) content compared to non-stressed plants. The higher values of N (69.5% and 47.0%), P (18.5% and 20.6%), K (18.5% and 16.1%), and Ca (15.9% and 10.6%) content were reported in maize plants inoculated with *L. fusiformis* and *L. sphaericus* in combinations under control and cold stress conditions, respectively.

Discussion

Beneficial microorganisms, particularly plant growth-promoting rhizobacteria (PGPR), have been implicated in helping plants withstand abiotic stressors and maintain productivity, according to several studies (Aly et al. 2013, 2017). Beneficial soil bacteria thrive in the rhizosphere as symbiotic partners with plants or as endophytes within host plants. They help plants grow by secreting phytohormones, enzymes, and biological nitrogen fixation, as well as solubilizing minerals and mineralizing organic phosphate, generating organic matter like amino acids and improving the bioavailability of nutrients in the rhizosphere via modifying permeability and converting nutrients (Jha et al. 2014). Lowering ethylene levels, producing and accumulating suitable solutes like proline and glycine betaine, and lowering ROS generation are some of the primary methods by which PGPR helps plants cope with abiotic stress (Sofy et al. 2021a). As a result, the usage of PGPR is appropriate for reducing crop plant oxidative and osmotic stress and can be considered a key strategy of sustainable agriculture operations (Mohamed and Gomaa 2012).

The cold-resistant endophytic PGPR was chosen for further experimentation in the current study based on its growth in low temperatures and its ability to promote plant growth. In terms of phosphate solubilizing ability, the two bacterial strains, *L. fusiformis strain* YJ4 and *L. sphaericus* strain YJ5, were shown to be effective. Phosphorus is one of the most important nutrients for plants, second only to nitrogen in terms of demand. The majority of phosphorus in soil is in the form of insoluble phosphates, which plants cannot use (Pradhan and Sukla 2005). Also, the phosphate released, the titratable acidity, the synthesis of catechol and hydroxymate siderophores, the syntheis of IAA and gibberellin, and the generation of gluconic acid by *L. sphaericus* was higher than *L. fusiformis* strain as shown in Tables 1 and 2. Similar to our outcomes, *Bacillus pumilus* and *Pseudomonas pseudoalcaligenes* were found to be capable of dissolving phosphorus by generating gluconic acid, as well as auxins and gibberellins (Jha et al. 2014). Siderophores are low molecular weight compounds that scavenge iron, which is widespread in the environment

Table 6 Effect of endophytic bacterial strains on minerals concentration in maize under cold stress

Treatment	N (mg kg^{-1})	P (mg kg^{-1})	K^+ (mg kg^{-1})	Ca^{2+} (mg kg^{-1})
Normal				
Non inoculation	124.4 ± 3.0^{d}	214.2 ± 6.0^{e}	571.0 ± 6.0^{c}	163.1 ± 1.0^{c}
Inoculation with *L. fusiformis*	234.4 ± 4.0^{b}	239.1 ± 6.0^{c}	613.1 ± 6.0^{b}	173.4 ± 3.0^{b}
Inoculation with *L. sphaericus*	273.8 ± 5.0^{a}	246.0 ± 7.0^{b}	622.3 ± 7.0^{b}	183.6 ± 3.0^{ab}
Inoculation with *L. fusiformis* + L. *sphaericus*	287.2 ± 5.0^{a}	258.4 ± 7.0^{a}	684.2 ± 7.0^{a}	188.0 ± 4.0^{a}
Cold stress				
Non inoculation	100.1 ± 5.0^{e}	185.1 ± 5.0^{g}	330.2 ± 7.0^{e}	116.4 ± 4.0^{f}
Inoculation with *L. fusiformis*	138.3 ± 4.0^{d}	201.3 ± 6.0^{f}	373.2 ± 8.0^{d}	125.1 ± 4.0^{e}
Inoculation with *L. sphaericus*	147.1 ± 4.0^{cd}	223.2 ± 7.0^{d}	383.4 ± 8.0^{d}	128.7 ± 3.0^{e}
Inoculation with *L. fusiformis* + *L. sphaericus*	156.9 ± 5.0^{c}	235.2 ± 7.0^{c}	393.5 ± 8.0^{d}	136.3 ± 4.0^{d}

Values are the means of three replicates. Values with different letters are significantly different at $P < 0.05$ (Duncan's Test)

as complex, and become available to microorganisms that assist in its availability to plants through roots, thereby causing plant growth promotion (El-Mahdy et al. 2021).

In the current research, cold stress decreased germination and morphological criteria of maize plants as compared to control plants, but inoculation with *L. fusiformis* and *L. sphaericus* alone or in combination decreased the harmful effect of cold stress in maize (Table 3). Low temperature freezes the cell contents of the plant, which hamper metabolic activity, causing cell damage and interrupts the movement of water and nutrients which result in reduction in germination, morphological criteria and dry weight of plants (Subramanian et al. 2016). The two bacterial strains have a good influence on plant growth and have the ability to tolerate cold stress in maize. Generally, the two bacterial strains have the ability to absorb and solubilize phosphorus and potassium from the soils and also have the ability to fix and transport nitrogen, so it caused improvement in plant growth through the improvement of several metabolic processes like photosynthesis (Mohamed and Gomaa 2012; Helmi and Mohamed 2016). Also, the two bacterial strains have the ability to generate hormones, so they increased the level of phytohormones in plants, which boosted cell division, water uptake, and nutrient availability, resulting in plant growth improvement (Singh et al. 2020). In addition, the two bacterial strains employed in this work may have the genes for ACC deaminase, which catalyzes the conversion of ACC to ammonia and alpha ketobutyrate, lowering ethylene levels and boosting plant growth and development according to Sofy et al. (2021a). These findings are in accordance with those of Zubair et al. (2019), who found that under cold stress, inoculating wheat seedlings and wheat plants with psychrophilic *Bacillus* CJCL2 and RJGP41improved vigor index, fresh/dry weight, and shoot length as compared to non-stressed plants.

Cold stress reduces chlorophyll, carotenoids and photosynthetic rates in maize plants (Table 4). Chlorophyll is an important and critical bioactive compound in photosynthesis, acting as both a light absorber and a light energy transmitter (Dey et al. 2021). Chlorophyll formation is hindered in low-temperature stressed plants, resulting in a reduction in the light gathering (Xu et al. 2010). The inhibition in the chlorophyll content under low temperatures is possibly due to the boost in the efficiency of the chlorophyll degrading enzyme (chlorophyllase), and a decline in 5-aminolevulinic acid production (Dey et al. 2021). Furthermore, cold stress caused a drop in protein spot P8 expression, which was linked to the breakdown of RuBisCo activase, resulting in a reduction in photosynthetic efficiency in the leaf and inhibiting growth in potato plants. P48 expression was downregulated in potato leaves during low-temperature stress, suggesting that the photosynthetic rate was lowered, and chloroplasts were disrupted, resulting in limited chlorophyll production and reduction in growth and photosynthesis. (Li et al. 2021).

In addition, inoculation with *L. fusiformis* and *L. sphaericus* strains caused a significant boost in chlorophyll content and photosynthetic rate over cold stressed plants. Chlorophyll content is influenced by the nitrogen level, as the levels of N_2 boosted in plant due to inoculation with PGPR, because PGPR having the ability for nitrogen fixing which caused enhancement of N2 concentration in plant resulting to direct enhancement in chlorophyll a, b and total chlorophyll (Alexander et al. 2019). In addition, *L. fusiformis* and *L. sphaericus* strains have the ability for the production of siderophores (Table 1) which are capable of chelating Fe and transported into the cell, consequently boosting iron concentrations and improving Fe bioavailability and transport which related to the increase the synthesis of chlorophyll in the plants (Mohamed and Gomaa 2012).

Under cold stress conditions, the membrane stability decreased, while the conductivity, MDA, lignin and cell viability increased compared to non-stressed plants (Table 5). Temperatures below 4 °C induce the nucleation of ice crystals responsible for dehydration in cell and block all enzymatic activity as well as rupture cell membrane and results

in electrolyte leakage from the affected cell and decreased the membrane stability (Jha 2019a). Cold stress also alters the physical properties of the cell wall and normally causes damage to structure and function of plasma membrane by altering the overall lipid composition (Szechyńska-Hebda et al. 2017). Electrolyte leakage is connected to K+ efflux from the plant cell, which is regulated by plasma membrane cation conductivity, and it occurs in response to stress, such as cold stress (Jha et al. 2022). Cold sensitive plant initiates ROS generation in their cells due to alteration in the functioning of electron transport chains, which activate lipid peroxidation in plant cell. Free radicals generated due to altered electron transport chain and enzyme inactivation can initiate lipid peroxidation process in the plant cell. Malondialdehyde (MDA) is a byproduct of the peroxidation of polyunsaturated fatty acids in plant cells. The rise in free radicals caused a boost in MDA content (Liu et al. 2013; El-Beltagi et al. 2019). Elevated ion leakage shows severe damage to the cell membrane and has been utilized as a key criterion in determining the plant stress tolerance ability (Jha 2019c). Lignification happens during plant growth exposed to low temperatures, and the formation of lignin is boosted to help in the acclimation of plants to cold stress through modifying the cell wall and reinforcing it, minimizing freezing harm and cell rupture. Freezing can cause extracellular ice production during extreme cold exposure, resulting in cell dehydration and perhaps cell rupture (Takahashi et al. 2019). The hardness of cell walls may play a role in cell resistance to freeze caused dehydration. Lignification is a multi-step process that requires a variety of phenolic compounds and enzymes (Jha 2017a, b).

Inoculation with *L. fusiformis* and *L. sphaericus* strains increased membrane stability, conductivity, lignin content but caused reduction in the lipid peroxidation and cell viability in maize compared to cold stress plants (Table 5). Our findings are consistent with those of Zubair et al. (2019) and Tiwari et al. (2017) who discovered that cold stress and abiotic stress caused an increment in proline content, stress-related genes, and reduction in MDA content when wheat and rice plants inoculated with Bacillus strains and *Bacillus amyloliquefaciens,* respectively. Furthermore, under freezing stress, inoculating *Arabidopsis thaliana* with *Burkholderia phytofirmans* PsJN caused cell wall thickening as well as increasing cell wall rigidity, decreasing cell wall pore size and prevent cell rupture (Su et al. 2015). Furthermore, the role of COR genes in the stabilization of membranes and proteins under freeze-caused dehydration may help to clarify the bacterized plant's ability to maintain cell structure at low temperatures (Thomashow 1999).

The osmolytes contents (proline, glycine betaine and soluble sugars) and phenolics compound in maize plants increased under cold stress and inoculation with *L. fusiformis* and *L. sphaericus* alone or in combinations (Fig. 4). Plants modify various cellular and molecular processes under cold stress, like the production of compatible solutes or osmolytes to maintain the cellular machinery from cold stress to mount their growth and development. Osmolytes are osmoprotectant solutes that help cells maintain water balance without interfering with normal metabolism (Jha et al. 2011). These organic compounds' primary role is to control osmotic equilibrium. These osmotic solutes aid the plant's ability to withstand extreme osmotic stress during cold stress in their life cycle, and they are neutral molecules that protect proteins, enzymes, and other cell membranes from osmotic stress in order to keep cellular metabolism functioning (Zubair et al. 2019). More considerable proline production during stressful circumstances was believed to account for some of the plants' enhanced tolerance to cold stress by reducing ROS-induced oxidative damage (Ben Rejeb et al. 2014). The major amino acid functioning as a protective molecule against cold in cold-tolerant plants is proline, and the increased proline level in tissues is the process by which plants acclimatize to endure cold stress. Furthermore, due to enhanced production or a slower degradation rate, the buildup of this amino acid has been seen in several plants under cold stress (Ritonga and Chen 2020). Soluble sugars defend plant cells from environmental stresses like cold stress by functioning as osmoprotectants, providing nutrition, and interacting with the lipid bilayer (Ben Rejeb et al. 2014). Also, *Burkholderia phytofirmans* PsJN increases Grapevine cold resistance by influencing cold gene expression, causing an increment in proline content, phenolic compound content, and carbohydrate metabolism alteration (Fernandez et al. 2012; Theocharis et al. 2012).

Cold stress caused inhibition of auxin and gibberellin content but caused accumulation of abscisic acid and ACC deaminase in maize plants (Fig. 5). On the other hand, inoculation with endocytic bacterial stains caused improvement on phytohormones and reduction in ABA content. The potential of *L. fusiformis* and *L. sphaericus* strains to synthesize phytohormones like auxin and gibberellic acid is investigated (Table 1) which positive role in development of cold stress tolerance in maize. Through the synthesis of phytohormones within the root zone, PGPR can effectively aid the expansion and proliferation of their plant host; these hormones boost the density, length, and spread of the root hairs in the soil that help in the enhancement of nutrient and water uptake and transport (Mohamed and Gomaa 2012). According to Sofy et al. (2021a), ABA concentrations in plants are elevated during osmotic stress, and inoculation *Bacillus subtilis* and *Pseudomonas fluorescens* can reduce ABA concentrations in plants to relieve osmotic stress. These findings are consistent with those of Zubair et al. (2019), who found that under low-temperature stress and inoculation with Bacillus spp. CJCL2 and RJGP41, a decline in plant ABA levels. In addition, PGPBs can di-

rectly or indirectly increase ACC deaminase activity, which promotes plant growth and development (Mohamed and Gomaa 2012). This method is dependent on PGPBs consuming ACC before it is oxidized by plant-produced ACC oxidases. As a result of their ability to reduce ethylene levels, PGPBs could be a good source of growth promoters and stress resistance (Santoyo et al. 2016). By increasing the activity of ACC deaminase and hence controlling ethylene concentrations, PGPBs boosted plant growth in wheat (Amna et al. 2019).

L. fusiformis and *L. sphaericus* strains are able to activate as well as regulated the activities of different antioxidant enzyme catalase, superoxide dismutase and phenylalanine ammonia lyase in maize plant to help the plant for their survival under cold stress (Fig. 6). Environmental stress such as cold stress led to generation of excess of ROS in plants due to disruption of cellular homeostasis. When the amount of ROS produced surpasses the cell's immune defenses, oxidative stress occurs, resulting in enzyme activity reduction, lipid peroxidation, nucleic acid degradation, protein oxidation, activation of the leading apoptosis pathway, and cell death (Jha and Subramanian 2018). Antioxidant enzymes can help prevent chilling harm by adequately removing the excess ROS generated during cold stress. Many studies have shown that over-expressing different antioxidant enzymes can improve stress resistance, and SOD is an essential antioxidant enzyme that protects chloroplasts, mitochondria, plasma membranes, peroxisomes, apoplast, endoplasmic reticulum, and cell walls from abiotic stress because it is the first and most effective line of defense against ROS at such locations (Jha and Subramanian 2015). Antioxidant enzymes have been shown to improve plant responses to freezing stress as in previous research according to Theocharis et al. (2012) and Ding et al. (2011). Also, in cold stressed tomato plantlets treated with endophytic *Pseudomonas* sp. strains OB155 and OS261, antioxidant enzymes SOD, CAT, APX, POD, and GR activity increased significantly (Subramanian et al. 2015). The regulation of cellular ROS levels requires the expression of these enzymes. SOD and CAT are the first line of defence for the antioxidative mechanism in plants. They play an important function in intracellular H2O2 signaling and inhibit the development of more dangerous ROS (Moustafa-Farag et al. 2020). SOD catalyzes the conversion of O_2 to H_2O_2 and O_2 molecules in the first step. Superoxide radicals are more hazardous than hydrogen peroxide. POD enzymes, on the other hand, accelerate the transformation of H_2O_2 to H_2O and O_2. Then, in various organelles and antioxidant cycles, H_2O_2 is detoxified by POD and CAT (Abd El-Rahman and Mohamed 2014). CAT is an antioxidant enzyme that has a higher potential for quickly removing H_2O_2 and is more involved in H_2O_2 detoxification (removes H_2O_2 by breaking it down to produce H_2O and oxygen and oxidizes H^+ donors via peroxide consumption), which is necessary for cold stress resistance (Skyba et al. 2012; Auh and Scandalios 1997). The deamination of phenylalanine to trans-cinnamate is catalyzed by phenylalanine ammonia-lyase (PAL), a critical step at the intersection of the phenolics and lignin synthesis pathways (Sofy et al. 2021b). The PAL activity and lignification's has significantly been enhanced, both in the presence and absence of the cold stress in inoculated plant and it get more intensified under cold stress.

Mineral contents like N, P, K and Ca in maize plants significantly reduced under cold stress, while the inoculation with *L. fusiformis* and *L. sphaericus* strains alone or in combinations caused stimulation in the mineral contents over cold stressed plants (Table 6). Hussain et al. (2018) found that nutrient uptake was decreased may be due to the reduction in root length, limitation of hydraulic conductivity, the reduction of root branching, and the increment in the root thickness under cold stress. By boosting root area, root porosity, and mineral nutrient absorption, PGPR can directly improve nutrient availability in the root system and/or stimulate ion transport mechanisms in the root (Jha et al. 2012). Among major mineral nutrient nitrogen, phosphorus and potassium are necessary for amino acids production and proteins activation are the most essential nutrient for plants, which is stimulated by bacteria. Bacteria can boost N_2 fixation, which is regulated by the nif gene and other fundamental genes; they can also boost plant growth, yield, and sustain nitrogen content in the soil, as well as enhance soil characteristics (Damam et al. 2016). Phosphate is a structural and signaling chemical that is necessary for photosynthesis, energy conservation, and carbon metabolism (Abu-Shahba et al. 2021). Potassium controls cell expansion, plasma membrane potential and transport, pH value, and many other catalytic processes as the cell's primary osmoticum (Jha 2017a). Reduction in plant growth, turgor loss, increased sensitivity to cold stress and pathogens, and chlorosis and necrosis are all symptoms of potassium, nitrogen and phosphorus deficiency (Rajawat et al. 2020; Vijayraghavan and Soole 2010). Plants linked with PGPR have evolved many adaptation strategies to cope with changes in nutrient availability, such as alterations in ion transporter expression, increased root development to explore more soil volume, and acidity of the surrounding soil to mobilize more mineral nutrients. Microorganisms are the only ones that can mineralize and solubilize the organic or insoluble forms of phosphate and potassium (Kour et al. 2020). The fact that particular PGPB produces ACC-deaminase, an enzyme that improves the uptake of key nutrients like N, P, and K, hence enhancing plant growth under abiotic stress, could also explain the growth promotion (Vaishnav et al. 2016).

Conclusion

Because the crop growing cycle in most regions of the world is subject to freezing temperatures, cold-tolerant plant-growth-promoting bacteria (PGPBs) are of major agronomic value. In this state, PGPR is metabolically active and creates a variety of metabolites, including plant growth regulators, which promote plant growth and make nutrient intake easier. Plants with PGPRs grow faster and are more resistant to cold stress. The mechanism of plant-microbe interaction, particularly under stress conditions, is extremely complex, and it will take a lot of effort to establish such a system. Finding cold-active microorganisms capable of promoting plant growth under cold stress would be extremely useful in agriculture all over the world. The present study concludes that inoculation of *L. fusiformis* and *L. sphaericus* strains alone or in combinations alleviates cold stress in maize plants by producing osmolytes (proline, glycine betaine and soluble sugars), phytohormones (auxin, gibberellin, abscisic acid and ACC deaminase) phenolics and antioxidant enzymes (PAL, SOD and CAT). The pot inoculation study with *L. fusiformis* and *L. sphaericus* strains significantly enhanced the growth and biomass of maize plants compared to non-inoculated plants under cold stress. These promising bacterial strains also reduce the levels of electrolyte leakage and MDA content but boosted lignin content to relieve maize plants from cold stress. In conclusion, *L. sphaericus* strains is more effective in tolerance to cold stress than *L. fusiformis*.

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Conflict of interest Y. Jha and H.I. Mohamed declare that they have no competing interests.

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