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SOIL AND AGRICULTURAL MICROBIOLOGY - RESEARCH PAPER

Alleviation of cold stress in wheat with psychrotrophic phosphorus solubilizing *Acinetobacter rhizosphaerae* EU-KL44

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Abstract

Low-temperature stress can seriously impair plant physiology. Chilling injury leads to a complex array of cellular dysfunctions, and symptoms include chlorosis, sterility, loss of vigor, wilting, and even death of the plants. Furthermore, phosphorus limitations additionally halt the growth of plants. Low-temperature adaptive plant growth–promoting microbes through various direct and indirect mechanisms help in the survival of plants under stress conditions. The present investigation deals with isolation of P-solubilizing psychrotrophic bacteria from diverse cultivars of wheat grown in the Keylong region of Himachal Pradesh. A total of 33 P-solubilizing bacterial isolates were obtained. P-solubilizers were screened for different plant growth–promoting (PGP) attributes of K and Zn solubilization, production of IAA, siderophores, and different hydrolytic enzymes. Among 33 P-solubilizers, 8 efficient strains exhibiting multiple PGP attributes were used as bioinoculants for wheat under low-temperature stress in different in vitro and in vivo experiments. The psychrotrophic bacterial isolates positively influenced the growth and physiological parameters as well as nutrient uptake and yield of wheat and efficiently alleviated low-temperature stress. The potential of low-temperature stress adaptive and PGP microbes can be utilized in agricultural sector for amelioration of low-temperature stress and plant growth promotion. The present study deals with the isolation of psychrotrophic P-solubilizers with multiple PGP attributes and their role in alleviation of cold stress in wheat.

Keywords Diversity · Low-temperature stress · Plant growth–promoting microbes · P-solubilization · Wheat

Introduction

Extreme environments including drought, salinity, and high and low temperature greatly affect the productivity of several crop plants of commercial importance [1]. Low temperature is among the major factor limiting the geographical distribution of many species and agricultural productivity [2]. The effect of low-temperature stress on plants depends on the degree of severity and the extent of exposure. Injuries are known to occur through chilling at temperatures between 15 and 0°C [3]. The exposure of the crops to low temperatures disrupts cellular homeostasis, leads to the production of reactive oxygen species (ROS), and damages carbohydrates, DNA, lipids, and proteins eventually leading to cell death [4]. Plants possess tolerance mechanisms to cope with such non-freezing temperatures by a phenomenon referred to as cold acclimation [5]. The primary mechanisms involved in cold acclimation include the accumulation of cytosolic Ca^{2+}, alterations in the expression of cold-related genes, activation of ROS scavenger systems, changes in protein and sugar synthesis, and accumulation of osmolytes. But, many a time, plants need the support of the microbiome they harbor for reducing their burden to combat low-temperature stress. In fact, the association of plants with microbes plays a key role in the existence of both partners in stress conditions.

Another major problem faced by the plants is the unavailability of phosphorus in sufficient amounts for their uptake. Phosphorus is among the essential macronutrients for plants and thus supplied in the form of phosphatic fertilizers. However, a large portion of soluble Pi supplied in chemical form is immobilized rapidly and also becomes

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unavailable to plants. P-solubilizing microbes are efficient in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization) [6]. A range of P-solubilizing microbes have been reported from wheat [7–9]. Psychrotrophic P-solubilizers have been also reported [10, 11].

Numerous tools of biotechnology have been also broadly applied for crop improvement under abiotic stress conditions and nutrient limitations of which the role of plant growth–promoting microbes (PGPMs) in the alleviation of stress has become of paramount importance [12]. Diverse plant growth–promoting microbial genera including *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Brevundimonas*, *Enterobacter*, *Exiguobacterium*, *Flavobacterium*, *Jeotgalicoccus*, *Klebsiella*, *Kluyvera*, *Kocuria*, *Leclercia*, *Lysinibacillus*, *Methylobacterium*, *Mrakia*, *Naganishia*, *Paenbacillus*, *Pantoea*, *Planococcus*, *Pontibacillus*, *Providencia*, *Pseudomonas*, *Rhodotorula*, *Sporosarcina*, *Staphylococcus*, *Stenotrophomonas*, and *Virgibacillus* have been reported from low-temperature environments [1, 9, 13].

PGPMs positively influence plants under stress conditions and promote their growth by diverse mechanisms. These mechanisms include the production of different plant growth regulators, siderophores, and solubilization of insoluble and unavailable various macro- and micronutrients. Additionally, there is a production of ACC deaminase enzyme which lowers the levels of ethylene and accumulation of osmolytes for water uptake and ROS scavenging enzymes [14]. The utilization of PGPMs as bioinoculants to mitigate the negative effects of low temperatures is an innovative and cost-effective strategy and has drawn the attention of the scientific community throughout the world [15]. These beneficial PGPMs if judiciously formulated and allowed to colonize the target crops will surely work for the well-being of the crops and enhance the yield under all conditions [16].

Wheat is the major cereal crop of India and its products play a progressively more significant role in managing India's food economy. It is the staple food of millions of Indians, particularly in the northern and northwestern parts of the country. It is nutrient-rich and provides balanced food. India is the second largest producer as well as the consumer of wheat [17]. The major producers of wheat are Bihar, Gujarat, Haryana, MP, Punjab, Rajasthan, and Uttar Pradesh. In HP, wheat is cultivated as both rabi and kharif crops. Sirmaur district is among the major producers of wheat in Himachal Pradesh. The total area under the crop is about 29.8 million hectares in the country. Wheat requires an ideal winter temperature of 10 to 15°C. Longer durations of very low temperatures can cause damage to wheat which is most sensitive during the reproductive period. Factors such as temperature reached and the duration of the cold stress are directly related to the amount of damage inflicted. The selection and successful application of low-temperature adaptive microbes with P-solubilizing capability will encourage the development of climate-specific bioinoculants. Cold-tolerant P-solubilizers also exhibit the possibility of being an alternative to phosphatic fertilizers needed to increase agricultural productivity, especially for the regions disturbed by low temperatures during the winter season.

Materials and methods

Sample collection

Keylong, 32.58°N, 77.03°E, in Great Himalayas located 120 km from Indo-Tibetan border at an elevation of 10,100ft was chosen for sample collection. Seven different wheat varieties (HS240, UP2338, Hahn^{WR}, PBW 343YR, Pavon 1-96-1, HD2967, and PBW 343-Lr24-GPC-B_1) with their rhizospheric soil were collected from the Keylong region in sterile polyethene bags and stored in ice packed boxes and brought to Baru Sahib for further analysis.

Area of study and isolation of rhizospheric microbes

Baru Sahib, 30.7537° N, 77.2965° E, Valley of Divine Peace, located in a remote corner of Sirmaur district, Himachal Pradesh, India, was chosen as the area of study. The culturable microbes were isolated from the wheat rhizospheric region by standard serial dilution plating technique using different growth media [18]. The pure cultures were maintained on slants of nutrient agar and glycerol stock at 4°C and −80°C respectively.

Screening of rhizospheric microbes for P-solubilization attributes and other plant growth–promoting attributes

The isolates were screened qualitatively for P-solubilization on Pikovskaya agar supplemented with three different insoluble forms of phosphorus (rock phosphate (RP), apatite (AP), and tricalcium phosphate (TCP)) [19]. The microbial isolates solubilizing all three insoluble sources of phosphorus were further screened for multiple PGP attributes. Potassium solubilization was done by the method described by Hu et al. [20]; zinc solubilizing ability was carried by the method by Saravanan et al. [21]. IAA production was conducted according to the method of Bric et al. [22]. The siderophore production was analyzed on chrome-azurol-S (CAS) agar medium by Schwyn and Neilands [23]. The production of ammonia (NH_3) was examined in peptone water as described by Cappucino [24]. The microbial isolates were also screened for enzyme production such as amylases by the method of Castro et al. [25], cellulase activity by Zhou

et al. [26], and pectinases and proteases by Kanekar et al. [27] respectively.

On the basis of P-solubilization and other attributes of plant growth promotion, the selected isolates were quantified for phosphorus on tricalcium phosphate by the method of Murphy and Riley [28]. The amount of phosphorus solubilized was expressed in mg/L.

Identification of microbial isolates

Bacterial genomic DNA was extracted by the method described earlier [8] with minor modifications in the protocol. DNA samples were subjected to PCR amplification of conserved gene (16S rRNA) gene and carried out as described by Yadav et al. [1] followed by purification by Quiaquick purification kit (USA).

Accession numbers

The selected P-solubilizers were identified and partial 16S rDNA sequences were submitted to NCBI GenBank and accession numbers were assigned.

Evaluation of plant growth–promoting ability under in vitro and in vivo conditions

Seed germination plate assay

The seed germination bioassay was done on wheat. The selected psychrotrophic P-solubilizing strains were tested for their capability to inhibit/promote seedling growth using the method described by Elliott and Lynch [29]. The selected strains were grown for 48h in their respective growth medium containing at least 10^7 cells mL^{-1}. The seeds were surface-sterilized with 0.1% $HgCl_2$ followed by consecutive washing with sterile distilled water. The seeds of wheat were kept for 10min in the culture medium. Sterilized soft agar was poured into the sterilized plates and 10 seeds were placed in each soft agar plate along with control plates which contained seeds treated with a respective sterilized medium. After 3 days, the root radical length and shoot length were recorded.

Plant growth promotion under in vitro conditions

The evaluation of the PGP ability of selected psychrotrophic P-solubilizers on wheat was determined under greenhouse conditions. The experiment was carried out in the month of January when the temperature was observed to be 20°C during daytime and less than 5°C during nighttime. The efficient strains were grown in nutrient broth at 10°C. Seeds of wheat were dipped in inoculums for 1h. The seeds were surface-sterilized before putting in inoculums. The seeds were kept in a 48-h grown culture containing at least 10^7 cells mL^{-1} for 1h prior to sowing. Six seeds were sown in each pot and autoclaved water was given according to need. After the emergence of the first leaf, plant density was reduced to 4 plants per pot.

Plant growth promotion under in vivo conditions

The evaluation of the PGP ability of selected psychrotrophic P-solubilizers on wheat was determined under field conditions. The experiment was carried out in two plots in the month of January when the temperature was observed to be 20°C during daytime and less than 5°C during nighttime. Both plots consisted of 12 blocks each of $1.5m^2$ area. All blocks consisted of six rows with each row consisting of 15 plants at a distance of about 25 cm. A complete randomized block design was used for the field experiment. The experiment was done in triplicates. The selected psychrotrophic P-solubilizing bacterial strains were grown in a growth medium for 48h under shaking conditions (10^7 cells $mL^{-1)}$. Wheat seeds were surface-sterilized and coated with inoculums and sugar solution (1:1 ratio) before sowing. The control seeds were coated with sterilized growth media and sugar solution. The inoculums were sprayed on the wheat plants of plot I after 30 days of sowing and plot II was left unsprayed until harvesting.

Determination of plant growth, physiological parameters, nutrient uptake, and yield

Growth parameters

Different growth parameters including shoot and root length and fresh and dry weight were studied in each experiment.

Physiological parameters

The proline content of the leaves was determined according to the method described by Bates et al. [30]. Glycine betaine (GB) estimation was done according to Grieve and Grattan [31] using dried leaf powder. Total soluble sugars (TSS) were determined by the method of Irigoyen et al. [32]. The level of lipid peroxidation was done according to Heath and Packer [33]. Superoxide dismutase (SOD) activity was determined by Dhindsa et al. [34] and glutathione reductase (GR) activity was assayed by Smith et al. [35].

Nutrient uptake in plant and yield

For the analysis of iron, zinc, and phosphorus, the grains were collected after harvesting and washed in deionized water and dried at 80°C in a hot air oven for 5h. After drying, 0.5g of seed samples was weighed and digested with

5mL concentrated nitric acid and 2mL H_2O_2 in a microwave digester (Anton Paar, GmbH, Austria) at a set temperature and pressure until a clear solution is obtained. For iron and zinc analysis, the digested samples were then transferred to the 50-mL graduated Falcon tubes, and the final volume was made to 25 mL with deionized water for further analysis by atomic absorption spectrophotometer (AA240FS, Agilent Technology, CA, USA) [36]. The P content after digestion was analyzed by the ammonium molybdate method [28]. The nutrient concentrations were expressed in mg/kg of dry weight. The yield of wheat was expressed in q ha^{-1}.

Statistical analysis

The various data obtained in the study were subjected to statistical analysis using Student's *t*-test. Mean comparisons were conducted using the least significant difference (LSD) test (P¼ 0.05) and critical difference (CD5% and CD1%). The standard error (SE) and LSD results were calculated.

Results

Isolation of rhizospheric microbes

The population of heterotrophic rhizospheric microbes was enumerated from different varieties of wheat cultivated in Keylong, Himachal Pradesh. Nutrient agar supported the highest population of bacteria whereas the least microbial population was supported by ammonium mineral salt agar. A total of 100 bacterial morphotypes were finally selected for further screening.

Screening of rhizospheric microbes for P-solubilization attributes

Among 100 isolates, 33 solubilized phosphorus. Among 33 P-solubilizers, 8 isolates solubilized all three insoluble sources of phosphorus. Among selected 8 isolates, strain EU-KL44 exhibited multiple plant growth–promoting attributes including the solubilization of minerals (K and Zn), production of Fe chelating compounds, IAA, and different hydrolytic enzymes (Table 1).

The amount of P-solubilized was observed in the range of 80.3±0.009 to 572.0±0.001mg L^{-1}. The highest amount of phosphorus was solubilized by EU-KL44 and the lowest by the strain EU-KL59 (Table 1).

Identification of microbial isolates

16S rRNA sequence analysis of the strain EU-KL44 showed 99.02% similarity to *Acinetobacter rhizosphaerae* in the existing database of the National Center of Bioinformatics. The partial 16S rRNA gene sequence was submitted to NCBI GenBank and the assigned accession number MN733449. The strain EU-KL44 was deposited to the culture collection facility of NBAIM, Mau, Uttar Pradesh, India.

Evaluation of plant growth–promoting ability under in vitro and in vivo conditions

Seed germination plate assay

Eight psychrotrophic P-solubilizing strains were evaluated for their capability to inhibit/promote seedling growth. All the isolates positively influenced the root radical length and shoot length. Strain EU-KL44 showed the highest increment in the shoot and the root length followed by strain EU-KL78 as compared to untreated control (Table 2).

Plant growth promotion under in vitro conditions

On the basis of seed germination plate assay, three efficient strains of *Acinetobacter rhizosphaerae* EU-KL44, EU-KL78, and EU-KL86 were used as inoculants for wheat under greenhouse conditions. All the strains showed variations in influencing the growth of the wheat. The strains

Table 1 Quantification of P and other PGP attributes of P-solubilizers

P-solubilizers	(TCP) mg/L	K	Zn	IAA	Sid	Amm	Amy	Pec	Pro	Cellu
EU-KL39	146.0±0.011	-	-	-	-	-	-	-	+	-
EU-KL44	572.0±0.001	+	+	+	+	+	+	+	-	-
EU-KL59	80.3±0.009	-	+	-	-	-	-	-	-	-
EU-KL78	568.4±0.036	+	-	-	+	-	-	-	-	-
EU-KL86	481.2±0.003	-	-	+	-	+	-	-	-	-
EU-KL87	108.5±0.001	-	-	-	-	-	-	+	+	-
EU-KL88	421.0±0.002	-	+	-	-	+	-	-	+	-
EU-KL90	493.5±0.027	-	-	+	-	-	-	-	-	-

K potassium, *Zn* zinc, *IAA* indole acetic acid, *Sid* siderophores, *Amm* ammonia, *Amy* amylase, *Pec* pectinase, *Pro* protease, *Cellu* cellulase

Table 2 Effect of inoculation of different strains of psychrotrophic P-solubilizing bacteria on growth parameters of wheat under cold stress conditions

Treatments	Root length (cm)	Shoot length (cm)
EU-KL39	2.8±0.003	1.1±0.013
EU-KL44	4.0±0.002	1.8±0.011
EU-KL59	2.5±0.003	0.8±0.007
EU-KL78	2.9±0.002	1.4±0.005
EU-KL86	2.9±0.003	1.5±0.007
EU-KL87	2.6±0.023	1.1±0.012
EU-KL88	2.7±0.013	1.0±0.017
EU-KL90	2.2±0.016	0.8±0.022
Control	1.6±0.022	0.7±0.013

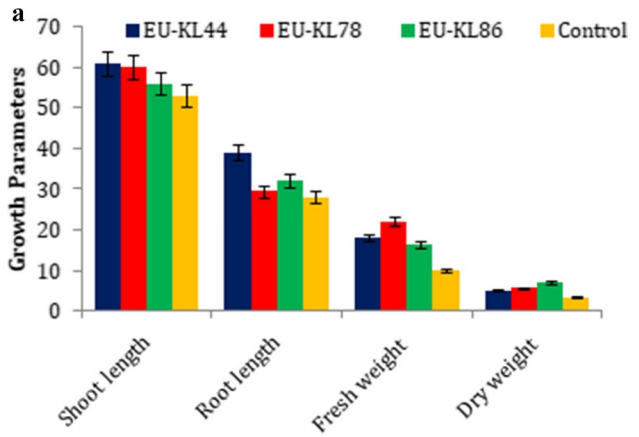

Fig. 1 a Effect of inoculation of different strains of psychrotrophic P-solubilizing bacteria on growth parameters of wheat under cold stress conditions [biomass (gm); length (cm)]. b Effect of inoculation of different strains of psychrotrophic P-solubilizing bacteria on proline, glycine betaine, sugar content, lipid peroxidation, and enzymatic activities of wheat under cold stress conditions

efficiently improved both the physiological and growth parameters under low-temperature conditions.

Determination of plant growth and physiological parameters

Growth parameters

Acinetobacter rhizosphaerae EU-KL44 showed the highest increment in the shoot length and root length whereas the strain EU-KL78 showed the highest increment in the fresh weight and the highest increment in dry weight was observed in EU-KL86-treated seeds as compared to the untreated control under low-temperature conditions (Fig. 1a).

Physiological parameters

All the treatments showed differences in their activities in influencing the physiological parameters of the wheat in low-temperature conditions. The highest content of proline was observed in *Acinetobacter rhizosphaerae* EU-KL44 (0.035 µmol g^{-1} of fresh weight leaf)-treated plants followed by strain EU-KL78 (0.031 µmol/g^{-1} of fresh weight leaf)-treated plants when compared to uninoculated control plants which showed 0.023 µmol/g^{-1} of proline content. The lowest content of TBARS was observed in *Acinetobacter rhizosphaerae* EU-KL44 (3.2 nmol g^{-1} of fresh weight leaf)-treated plants as compared to the control plants which showed TBARS content of about 4.0 nmol g^{-1}. EU-KL86-treated plants showed the highest content of total soluble sugars (0.053 mg g^{-1} of fresh weight leaf) followed by *Acinetobacter rhizosphaerae* EU-KL44-treated plants (0.048 mg g^{-1} of fresh weight leaf) as compared to untreated control plants. The highest content of glycine betaine was observed in EU-KL86-treated plants followed by *Acinetobacter rhizosphaerae* EU-KL44-treated plants. The highest activity of SOD was observed in *Acinetobacter rhizosphaerae* EU-KL44 (97 units mL^{-1}) treatment whereas control plants showed 80 units mL^{-1} of SOD activity. GR activity was also enhanced in bacterial-treated wheat plants. The highest activity was observed in *Acinetobacter rhizosphaerae* EU-KL44 and strain EU-KL78 treatments as compared to untreated control (Table 3, Fig. 1b).

Plant growth promotion under in vivo conditions

On the basis of plant growth promotion under in vitro conditions, *Acinetobacter rhizosphaerae* EU-KL44 was selected for in vivo conditions under low-temperature conditions. The strain positively influenced the growth and physiological parameters of wheat under low temperatures. The differences in each studied parameter were observed in plants sprayed with inoculums after 30 days and plants left unsprayed till harvesting.

Determination of plant growth, physiological parameters, nutrient uptake, and yield

Growth parameters

The sprayed and unsprayed plants did not show much variation in their growth parameters but the increment of growth parameters in both cases was higher than in untreated control plants. In comparison, plants left unsprayed showed slightly higher shoot and root length and fresh and dry weight (Fig. 2a).

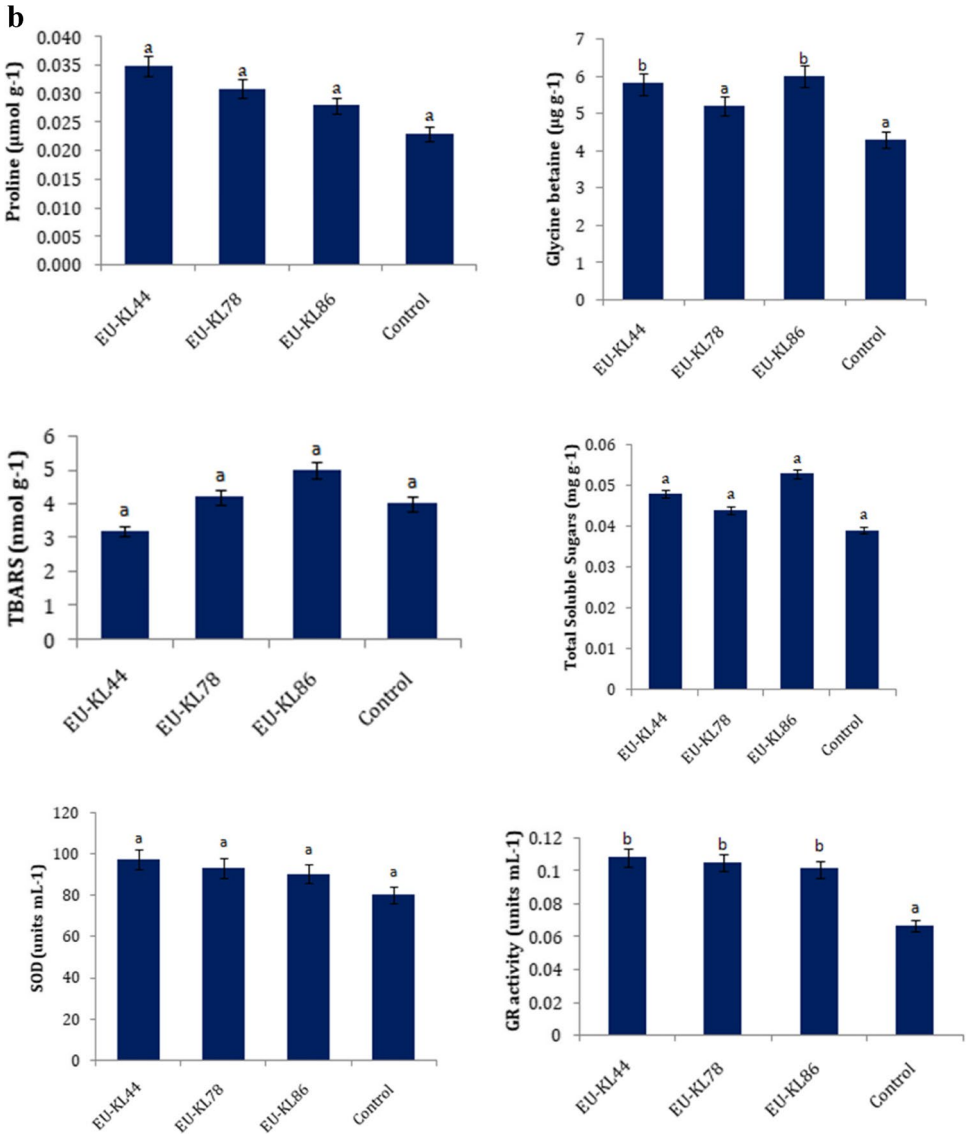

Fig. 1 (continued)

Table 3 Effect of inoculation of different strains of psychrotrophic P-solubilizing bacteria on physiological parameters of wheat under cold stress conditions

Treatments	Pro (µmol g^{-1})	GB (µg g^{-1})	TBARS (nmol g^{-1})	TSS (mg g^{-1})	SOD (units mL^{-1})	GR (units mL^{-1})
EU-KL44	0.035^{a}	5.8^{b}	3.2^{a}	0.048^{a}	97^{c}	0.108^{b}
EU-KL78	0.031^{a}	5.2^{a}	4.2^{a}	0.044^{a}	93^{b}	0.105^{b}
EU-KL86	0.028^{a}	6.0^{b}	5.0^{a}	0.053^{a}	90^{b}	0.101^{b}
Control	0.023^{a}	4.3^{a}	4.0^{a}	0.039^{a}	80^{a}	0.067^{a}
LSD	0.024	1.38	3.26	0.014	3.72	0.011
SE	0.007	0.43	1.02	0.015	0.96	0.003
$CD_{5\%}$	0.38	0.27	0.31	0.004	1.65	1.654
$CD_{1\%}$	0.70	0.49	0.57	0.029	3.03	3.037

Pro proline, *GB* glycine betaine, *TBARS* thiobarbituric acid reactive substance, *TSS* total soluble sugars, *SOD* superoxide dismutase, *GR* glutathione reductase, *LSD* least significant difference, *SE* standard error, $CD_{5\%}$ critical difference at 5% table value, $CD_{1\%}$ critical difference at 1% table value [numerical values are mean ± standard deviation of mean (SDm) of three independent observations]

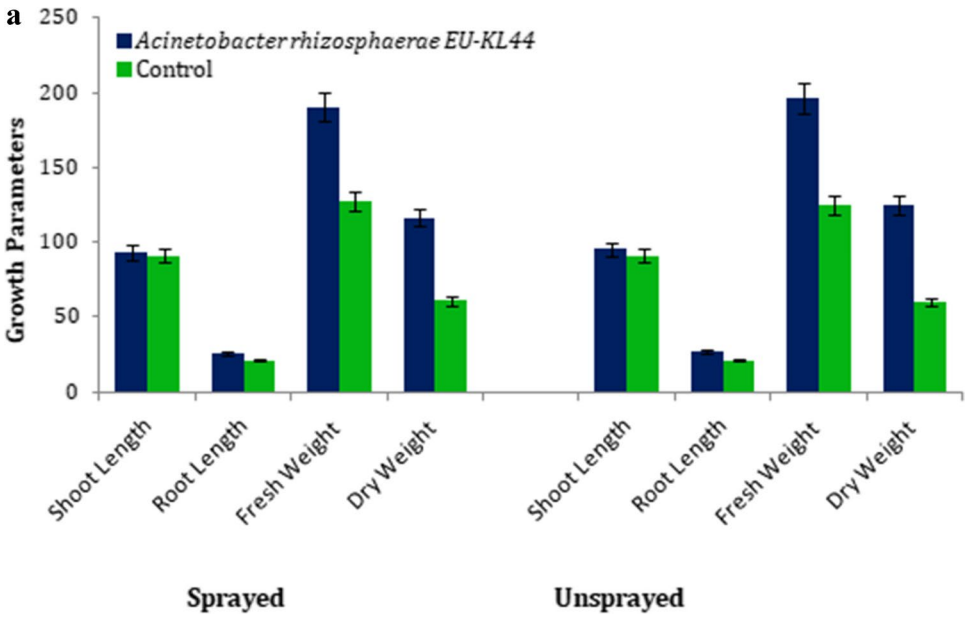

Fig. 2 **a** Effect of inoculation of *Acinetobacter rhizosphaerae* EU-KL44 on growth parameters of wheat under cold stress conditions [biomass (gm); length (cm)]. **b** Effect of inoculation of *Acinetobacter rhizosphaerae EU-KL44* on proline, glycine betaine, sugar content, lipid peroxidation, and enzymatic activities as well as nutrient uptake of wheat under cold stress conditions. **c** Effect of inoculation of *Acinetobacter rhizosphaerae* EU-KL44 on yield of wheat under cold stress conditions

Physiological parameters

The proline content was higher in wheat plants sprayed with inoculum of *Acinetobacter rhizosphaerae* EU-KL44 (0.046 µmol g^{-1} of fresh weight leaf) as compared to untreated control plants (0.017 µmol g^{-1} of fresh weight leaf) whereas unsprayed plants showed the 0.026 µmol g^{-1} of proline content in their leaves.

TBARS content was observed lowest in wheat plants sprayed with inoculum of *Acinetobacter rhizosphaerae* EU-KL44. The wheat plants sprayed with inoculum of *Acinetobacter rhizosphaerae* EU-KL44 showed 0.064 mg g^{-1} of total soluble sugars whereas total soluble sugar content in unsprayed plants was observed to be 0.058 mg g^{-1} of fresh weight of leaf sample. The untreated controls of both sprayed and unsprayed plants could increase the sugar content up to 0.048 mg g^{-1} and 0.045 mg g^{-1} respectively under cold-temperature conditions. *Acinetobacter rhizosphaerae* EU-KL44 increased the glycine content in both sprayed and unsprayed samples though the content was observed to be higher in samples sprayed with inoculum. The glycine content was observed to be 12.9 µg g^{-1} in sprayed samples whereas unsprayed showed glycine content of 9.2 µg g^{-1}. The activity of SOD did not show much variation in both sprayed and unsprayed wheat samples and reached up to 117 units mL^{-1} and 118 units mL^{-1} in sprayed and unsprayed samples respectively. *Acinetobacter rhizosphaerae* EU-KL44 efficiently increased the GR activity in both sprayed and unsprayed wheat plants as compared to control plants under cold stress (Table 4, Fig. 2b).

Nutrient uptake in plant and yield

The inoculation with *Acinetobacter rhizosphaerae* EU-KL44 efficiently improved the nutrient content and increased the yield of wheat under cold temperature conditions. The content of phosphorus in the wheat plants sprayed with inoculum after 30 days reached up to 44.90 mg kg^{-1} and in the case of unsprayed samples the content was 37.0 mg kg^{-1}. The P content in untreated control plants reached up to 30 mg kg^{-1} and 34 mg kg^{-1} in sprayed and unsprayed samples respectively. Fe content in wheat samples in sprayed and unsprayed samples was observed to be 35 mg kg^{-1} and 32 mg kg^{-1} respectively. Zn content was 39.7 mg kg^{-1} and 32.7 mg kg^{-1} in sprayed and unsprayed samples respectively (Fig. 2b).

A 34–35% increase in yield was observed in *Acinetobacter rhizosphaerae* EU-KL44-treated wheat plants (Fig. 2c) (Table 5).

Discussion

Plants are frequently exposed to a multiplicity of environmental stresses. Low temperature constitutes the chief factor in influencing the growth, development, and productivity of plants. It is a well-known fact that about two-thirds of the world's cumulative terrestrial area is annually affected by below-freezing point temperatures and a three-fourth portion of the Earth's biosphere's temperature is below 5°C [37]. There is an estimation that 50% of the loss in global productivity of agricultural crops is due to abiotic stresses. Chilling

Fig. 2 (continued)

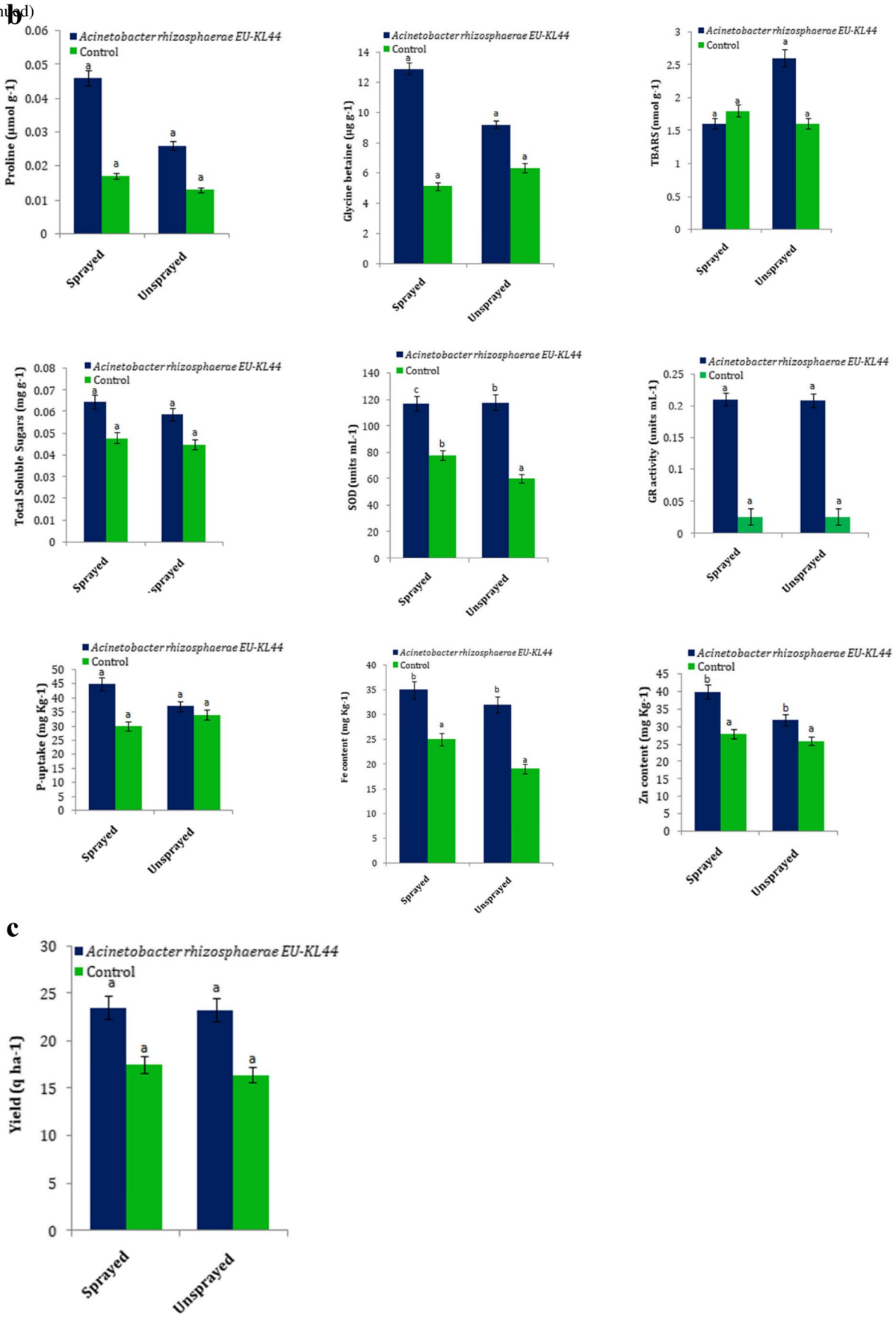

Table 4 Effect of inoculation of *Acinetobacter rhizosphaerae* EU-KL44 on physiological parameters of wheat under cold stress conditions

Treatments	Pro (µmol g^{-1})		GB (µg g^{-1})		TBARS (nmol g^{-1})		TSS (mg g^{-1})		SOD (units mL^{-1})		GR (units mL^{-1})	
	S	US	S	US	S	US	S	US	S	US	S	US
EU-KL44	0.046^a	0.036^a	12.9^a	9.2^a	1.6^a	2.6^a	0.064^a	0.058^a	117^c	118^b	0.211^a	0.209^a
Control	0.017^a	0.013^a	5.14^a	6.34^a	1.8^a	1.6^a	0.048^a	0.045^a	78^b	60^a	0.026^a	0.029^a
LSD	0.074	0.168	16.59	22.07	8.81	3.79	0.19	0.19	24.7	12.6	1.05	0.97
SE	0.005	0.013	1.84	2.45	0.69	0.29	0.01	0.03	4.90	0.90	0.08	0.07
$CD_{5\%}$	0.83	0.88	0.88	0.83	3.13	0.49	0.49	0.26	0.33	0.38	0.30	0.67
$CD_{1\%}$	4.20	4.45	4.45	4.20	15.7	2.48	2.4	6.5	1.65	2.42	1.52	3.36

S sprayed, *US* unsprayed

Pro proline, *GB* glycine betaine, *TBARS* thiobarbituric acid reactive substance, *TSS* total soluble sugars, *SOD* superoxide dismutase, *GR* glutathione reductase, *LSD* least significant difference, *SE* standard error, $CD_{5\%}$ critical difference at 5% table value, $CD_{1\%}$ critical difference at 1% table value [numerical values are mean ± standard deviation of mean (SDm) of three independent observations]

Table 5 Effect of inoculation of *Acinetobacter rhizosphaerae* EU-KL44 on nutrient uptake and yield of wheat under cold stress conditions

Treatments	P (mg kg^{-1})		Fe (mg kg^{-1})		Zn (mg kg^{-1})		Yield (q ha^{-1})	
	S	US	S	US	S	US	S	US
EU-KL44	44.9^a	37.0^a	35^b	32^b	40^b	32^b	23.6^b	23.3^a
Control	30.0^a	34.0^a	25^a	19^a	28^a	26^a	17.6^a	16.5^a
LSD	9.59	19.6	9.52	3.80	1.01	1.26	1.04	6.14
SE	7.5	6.4	7.49	2.99	7.99	9.99	8.20	4.84
$CD_{5\%}$	0.52	0.39	0.35	0.40	0.34	0.29	0.39	0.44
$CD_{1\%}$	2.60	19.79	1.78	2.03	1.71	1.46	1.97	2.22

S sprayed, *US* unsprayed

P phosphorus, *Fe* iron, *Zn* zinc, *LSD* least significant difference, *SE* standard error, $CD_{5\%}$ critical difference at 5% table value, $CD_{1\%}$ critical difference at 1% table value [numerical values are mean ± standard deviation of mean (SDm) of three independent observations]

leads to disturbances in enzymatic activity, impairment of photosynthesis, membrane rigidification, accumulation of ROS, and leakage across membranes. Confronted to fluctuations in temperatures, plants have evolved mechanisms to readjust their biochemical makeup to adapt and survive [38]. The microbiomes of diverse cold habitats play an indispensable role in the growth and adaptation of plants to low temperatures. These microbiomes are naturally gifted with different strategies to allow higher tolerance and in addition promote plant growth under abiotic stress conditions of low temperature. The identification of psychrotrophic microbes with the capability to support plant growth at low temperatures is a worldwide trend in the field of agricultural inoculation technology [39]. The present study deals with the isolation of psychrotrophic P-solubilizing bacteria from wheat grown in the Keylong region of the Great Himalayas, their PGP attributes, and their role in the alleviation of cold stress in wheat.

In the present investigation, 100 psychrotrophic bacteria were isolated from different wheat cultivars grown in Keylong. In high-altitude agriculture, the psychrotrophic microbes are of vast significance due to their survival and adaptation to cold conditions. A huge diversity of psychrotrophic bacteria has been explored including *Acinetobacter* sp., *Aeromonas* sp., *Alcaligens* sp., *Flavobacterium* sp., *Micrococcus* sp., *Moraxella* sp., *Pseudomonas* sp., *Vibrio* sp., and *Xanthomonas* sp. from Antarctic marine waters [40], *Janthinobacterium lividum* from Alaskan soil [41], *Arthrobacter* sp., *Bacillus* sp., *E. coli*, *Micrococcus* sp., *Paenibacillus* sp., *Pseudomonas* sp., and *Staphylococcus* sp. from the soil of Jammu region [42]. Przemieniecki et al. [43] reported psychrotrophic *Arthrobacter* sp. ad *Pseudomonas* sp. from root zone of winter wheat. Verma et al. [9] explored the diversity of psychrotrophic bacteria associated with wheat and total morphotypes which belonged to genera *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Brevundimonas*, *Enterobacter*, *Exiguobacterium*, *Flavobacterium*, *Klebsiella*, *Kocuria*, *Kluyvera*, *Leclercia*, *Methylobacterium*, *Pantoea*, *Planococcus*, *Providencia*, *Pseudomonas*, *Stenotrophomonas*, and *Staphylococcus*.

Among 100 isolates, 33 were efficient in solubilizing different insoluble forms of phosphorus. Many Indian soils have a low content of organic phosphorus and thus their bioavailability is also low. On average, the phosphorus content in soil is about 0.05%, from which 0.1% is accessible to plants. The poor mobility and low concentration of plant-available phosphorus in soil demand the application of chemical phosphatic fertilizers. The application of chemical fertilizers to agricultural land though has improved soil fertility and increased crop yield but simultaneously disturbed the P cycling event [44] and also has led to other adverse effects on the environment. P-solubilizing microbes play an amazing role in the nutrition of plants by increasing the phosphate uptake by plants and reducing the demands of chemical phosphatic fertilizers.

P-solubilizers in addition to increasing the bioavailability of P also stimulate the growth of the plants by solubilizing many other unavailable macro- and micronutrients, producing plant growth regulators and various hydrolytic enzymes. In the present study, 8 efficient P-solubilizing psychrotrophic bacteria also possessed multiple PGP activities such as solubilization of K and Zn, production of siderophores, IAA, ammonia, and various hydrolytic enzymes. Verma et al. [9] reported P-solubilizing psychrotolerant bacteria with multifarious PGP attributes allied with wheat from the northern hills zone of India. Yadav et al. [1] reported psychrotrophic Bacilli with P-solubilizing ability and other PGP traits from the cold desert of the northwestern Indian Himalayas. P-solubilizers are thus efficient bioinoculants for agricultural crops to increase yield and reduce the use of chemical fertilizers.

P-solubilizers are also known to be good stress alleviators for which P-solubilizers possess specific mechanisms to support the growth and adaptation of the plants. Cold stress disturbs the natural soil nutrient cycling and reduces soil fertility [45]. Exposure to cold stress interrupts cellular homeostasis in plants and ROS including hydrogen peroxide and singlet oxygen are among the major products of stress-induced cellular changes [46]. ROS damage biomacromolecules ultimately leading to total cell death in plants [47]. An effectual solution for protecting the plants from cold stress and enhancing their growth includes the application of cold-adapted and cold-loving PGP microbes as bioinoculants which are capable of tolerating the low temperature. PGP microbes use a range of mechanisms such as increasing the accumulation of the proline, glycine betaine, and sugars, activities of the ROS scavenging enzymes, and decreasing lipid peroxidation to improve the adaptability and growth of plants under stress conditions.

The accumulation of compatible solutes protects plants from stress through diverse mechanisms including cellular osmotic adjustment, protection of membrane integrity, detoxification of ROS, and protection and stabilization of proteins [48]. Proline is a water-soluble amino acid that accumulates in plants under different conditions of abiotic stress. It has been well established that the accumulation of proline within plants under stress conditions plays a noteworthy role in developing stress tolerance capacity simultaneously acting as an osmoregulatory molecule [49]. Proline is also known to be a way to store carbon and nitrogen. It is also known to play an additional role of molecular chaperone stabilizing the structure of proteins [50]. Proline accumulation is more significant in photosynthetic tissues though it also takes place in roots [51]. Glycine betaine is another important cryoprotective which protects the enzyme and protein activities and stabilizes membranes. It is also known to play a very important role in the adjustment and protection of the thylakoid membrane thus maintaining photosynthetic efficiency [48]. The tolerance to cold stress has been associated with the accumulation of glycine betaine in numerous plant species [52]. Sugars serve as important osmoprotectants and also protect the cellular membranes through interacting with the lipid bilayer from damage caused due to freezing [53]. In the present study, the accumulation of all three important compatible solutes viz. proline and glycine betaine was observed in wheat exposed to cold stress and inoculated with efficient psychrotrophic bacteria. In the study of Mishra et al. [2], the alleviation of cold stress in wheat inoculated with psychrotolerant pseudomonads has been reported. The study showed an increase in proline content in wheat along with other metabolites. The study of Subramanian et al. [4] showed increased tolerance capacity of tomato plants with inoculation of psychrotolerant bacteria and exposure to cold stress. The tomato plants showed a reduction in membrane damage, antioxidant enzyme activation, and proline accumulation. Thus, the increased concentrations of these three osmoprotectants may have protected the wheat plants from cold stress.

In the present study, the activities of ROS scavengers including SOD and GR and less lipid peroxidation were also observed in inoculated wheat. The abiotic stress is associated with the generation of ROS which are deleterious chemical entities with the capability to induce cellular damage by degrading the proteins and inactivating the enzymes. ROS also interfere in various pathways which are of metabolic importance. Loss of crop productivity under abiotic stress has been related to high ROS production [54]. Cold stress also enhances the production of peroxidized membranes. SOD and GR play a critical role in the detoxification of ROS and limit oxidative stress in plants. SOD provides protection against the toxic effects of oxidative stress by acting as a scavenger of superoxide radicals [55]. The involvement of antioxidative enzymes in protecting plants from stress conditions has been documented through various studies [56]. GR also plays an essential role in cell defense against reactive oxygen metabolites and confers abiotic stress tolerance

in plants [57]. GR efficiently maintains the intracellular glutathione pool in the reduced state and functions as an antioxidant and facilitates ROS scavenging [58]. The study of Tiryaki et al. [59] reported less lipid peroxidation and stimulated activities of SOD as well as GR in beans inoculated with psychrotolerant bacteria.

The psychrotrophic P-solubilizing strain EU-KL44 identified as *Acinetobacter rhizosphaerae* by 16S rRNA gene sequencing efficiently alleviated the negative effects of cold stress by diverse mechanisms in wheat plants and promoted their growth and enhanced yield. *Acinetobacter rhizosphaerae* has been reported as a P-solubilizer from cold deserts of the Himalayas by Gulati et al. [60]. But much work has not been done on inoculation of *Acinetobacter rhizosphaerae* and mitigation of cold temperature stress in crops. Probably to the best of our knowledge, this is the first report on the utilization of psychrotrophic P-solubilizing *Acinetobacter rhizosphaerae* as a bioinoculant for the alleviation of cold stress in wheat. Thus, psychrotrophic P-solubilizing microbes exhibiting PGP attributes can be isolated from regions of low temperature and utilized for the development of biofertilizers for the mitigation of cold stress in mountain agriculture.

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Declarations

Competing interests The authors declare no competing interests.

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