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**Research Article**

# Isolation and identification of *Bacillus cereus* strains from the rhizosphere zone of *Artemisia absinthium* L. and their effect on biochemical indices of *Artemisia absinthium* L. under salinity stress

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## Abstract

***Artemisia absinthium* (L.) is known as wormwood and grand wormwood. The use of growth-promoting bacteria reduces environmental stress and improves plant growth. Some *Bacillus* spp. found in the rhizosphere of plants, which can produce auxin hormone and have phosphate dissolution ability, which improves plant growth. This study aimed to isolate *Bacillus* spp. from the rhizosphere of *A. absinthium* L. and to evaluate the ability of the isolated strains for auxin production and phosphate dissolution as well as their effect on biochemical indices of *A. absinthium* L. under salinity stress. Soil samples were collected from the rhizosphere of *A. absinthium* L. and *Bacillus* spp. were isolated and purified from them, utilizing an initial heat shock for bacterial spore selection and then cultivation of spores on nutrient agar. In the next step, quantitative measurements of auxin production capacity and inorganic phosphate dissolution were performed on the purified isolates, and then the selected isolates that had more auxin production and phosphate dissolution were biochemically and molecularly identified. Finally, the effect of selected bacteria on some biochemical characteristics of the *A. absinthium* plant under salt stress was investigated. The effect of four levels of inoculation (no inoculation, inoculation with *B. cereus* B strain, *B. cereus* E strain, and co-inoculation with *B. cereus* B strain and *B. cereus* E strain) was investigated on the biochemical indices of *A. absinthium* under three levels of salinity stress (control, 75 mM, and 150 mM). The production of malondialdehyde (MDA), protein, proline, superoxide dismutase enzyme (SOD), catalase (CAT), peroxidase (POX), and total phenol increased with increasing salinity. The results showed that the inoculation of *Bacillus* bacteria under salinity stress decreased proline and MDA and increased the amount of protein, total phenol and antioxidant enzymes (superoxide dismutase, catalase, and peroxidase). The best results were obtained by the combined inoculation of rhizosphere bacteria, *B. cereus* B and E strains, under salinity stress (75 mM NaCl), in which the greatest decrease in proline (90%) and MDA (90%) as well as the greatest increase in protein (9%), total phenol (180%), and antioxidant enzymes including superoxide dismutase (SOD) (50%), catalase (CAT) (40%), and peroxidase (POX) (70%) was obtained compared to the control.***

**Keywords: *Artemisia absinthium*, Antioxidant enzymes, Rhizosphere bacteria, Auxin hormone, Salinity stress**

## Introduction

Plant growth-promoting rhizobacteria (PGPR) are microorganisms forming aggregations in soil around rhizospheres. These microorganisms can improve plant growth and facilitate their absorption and use of minerals. PGPR can improve plant growth, reduce plant disease and finally increase crop yield (Huang *et al.*, 2017). PGPR improves plant growth through two direct and indirect mechanisms (Olanrewaju *et al*., 2017). One of these mechanisms is the production of the hormone indole-3-acetic acid (IAA), which is one of the physiologically active auxins involving plant growth and development (Mohite, 2013; Mano and Nemoto, 2012). Auxin influences cell division, cell elongation, cell differentiation, and the function of cells and tissues in all evolved plants (Ljung, 2013). Another mechanism is phosphate dissolution by rhizospheric bacteria. Phosphate constitutes about 0.2% of the dry weight of a plant. This nutrient is among the mineral nutrients that generally limit the growth of plants, after nitrogen. On average, soil phosphorus content is around 0.05% (W/W); however, only 0.1% of this element is available

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to plants and can be used by them (Alori *et al*., 2017). Agricultural ecosystems can benefit from phosphate dissolution by rhizospheric and phosphate solubilizing bacteria (PSB) (Wan *et al.,* 2020). PSB can convert phosphate into a bioavailable form through dissolution and mineralization processes (Behera *et al.,* 2017).

Salinity is one of the biggest global problems with a negative effect on crop production through water stress, toxicity due to increased absorption of ions such as sodium and chloride, and nutritional imbalance. In addition, salinity negatively affects plant growth and development with oxidative stress, producing reactive oxygen species (Isayenkov and Maathuis, 2019). Saline soil is one of the main abiotic stresses affecting plant growth in arid and semi-arid regions of the world, such as Iran (Moradi and Piri, 2018). Plants develop various mechanisms such as activation of antioxidant enzymes and synthesis of antioxidant compounds, ion homeostasis, polyamine accumulation, biosynthesis of compatible solutions and osmotic protectors, producing nitric oxide, and hormone modulation to tolerate salinity (Numan *et al.,* 2018). Plants primarily deal with oxidative stress through internal defense mechanisms, including various antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX)) and non-enzymatic antioxidants (phenolic acid) (Hasanuzzaman *et al.,* 2020; Munawar *et al.,* 2021). PGPR increased proline and the activity of peroxidase and catalase in mung bean plants under salt stress (Islam *et al.,* 2016). In previous research, application of *B. pumilus*, *B. safensis* and *Zhihengliuella halotolerans* increased phenol and the activity of antioxidant enzymes in wheat plants of *Quds variety* under salt stress (Mosleh Arani *et al*., 2021). Also, under salt stress, inoculation of *Cucumis sativus* with *Pseudomonas fluorescens* and *B. subtilis* increased SOD activity (Saberi-Riseh *et al*., 2020). Proline content also has increased in common bean (*Phaseolus vulgaris*) plants inoculated with PGPR and *Triticum aestivum* inoculated with PGPRs under salinity stress (Islam *et al*., 2016). It has been observed that PGPRs reduced the content of MDA in *Fragaria* × *ananassa* plants under drought stress (Erdogan *et al.,* 2016). The plant *A. absinthium* L. belongs to the genus of Artemisia (Beigh and Ganai, 2017). Artemisia is a part of the largest and most widespread genus of plants in the Asteraceae family, which has more than 400 species. In traditional medicine, *A. absinthium* is used to treat stomach pain, stimulate the heart, improve memory, and restore reduced mental function (Mohammadian *et al.,* 2016), as well as an antiparasitic, antimalarial, antiseptic and anti-inflammatory agent (Shahnazi *et al.,* 2015). This study aimed to evaluate the effect of *Bacillus* bacteria on the amount of protein, proline, malondialdehyde, and phenol and the activity of antioxidant enzymes in *A. absinthium* as a medicinal plant under salt stress.

## Material and methods

**Isolation and identification of bacteria from soil samples by biochemical test:** At first, soil samples were taken from the rhizosphere of *A. absinthium* plant collected from three areas of Zarin Shahr (southwest of Isfahan province), Qahdarijan (southwest of Isfahan city), and Dorcheh (southwest of Isfahan city) to isolate and identify *Bacillus* bacteria. Then, 10 gr of soil from the three regions were poured into an Erlenmeyer flask containing 100 ml of sterile physiological serum and placed on a shaker at 110 rpm for 15 min. In the next step, the characteristic of sporulation was used to isolate *Bacillus* from the rhizosphere region. For this purpose, first, dilutions of $10^{-1}$ to $10^{-8}$ were prepared from soil suspensions. Then, the series of dilutions were placed in a hot water bath at 80°C for 10 min. Then, 100 µl of each tube content was spread on the surface of sterile nutrient agar and incubated for 24 hours under aerobic conditions. In the next step, a Gram slide was prepared from each colony and after observing Gram-positive or Gram-variable spore-bearing *Bacillus*, the desired bacterial colonies were purified on nutrient agar. In order to identify the different strains of *bacillus*, Gram staining was done and examined by a microscope. In the next step, the isolates catalase production, sugar fermentation, citrate utilization, urea production, starch hydrolysis, Vogue-Prosquare (VP), and methyl red (MR) test results were examined (Kones *et al*., 2020).

**Quantitative measurement of auxin production capacity:** At first, 1 ml of each 6 fresh bacterial suspensions was inoculated into 6 Erlenmeyer flasks containing 25 milliliters of LB-Tryptophan culture medium, then it was placed on a shaker with a speed of 100 at 28 °C for 72 h, and in the next step, 1.5 ml of the suspensions inside each Erlenmeyer flask was taken and centrifuged for 10 min at 10000 rpm. After this period, one ml of the clear supernatant solution was added to 2 ml of Salkowski's reagent, and the samples were kept at laboratory temperature for 20 min. In the next step, their absorbance was read at a wavelength of 530 nm. Finally, the amount of auxin produced was calculated by comparing the read absorbance intensity with the standard graph prepared from indole acetic acid (Torres-Rubio *et al.,* 2000).

**Investigating the dissolution of inorganic phosphate:** First, suspensions of each of the six bacteria with a concentration of $10^9$ CFU/mL were prepared, and then 50 µl of the suspension of the mentioned bacteria were added separately to 25 mm of Sperber's liquid culture medium. The sample was placed on a shaker for five days at 27 °C and a speed of 138 per min. The bacterial suspension was centrifuged at 4000 rpm for 10 min after five days. In the next step, 1 ml of the supernatant solution was removed and mixed with 3 ml of distilled water and 1 ml of ammonium molybdate vanadate reagent. After 20 min, the light absorption intensity at 430 nm wavelength was read. The amount of phosphorus dissolved in the culture medium was calculated in comparison with the standard curve (Sperber, 1958).

**Molecular identification of isolated bacteria:** In



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**Table 1. Sequences of the used universal primers (27F/1492R)**

| Primer name | General primer sequence | Product length | Nucleotide quantity (bp) | Melting temperature (ºC) |
|---|---|---|---|---|
| 27F | 5'-AGAGTTTGATCCTGGCTCAG-3' | 1464 bp | 20 | 56 |
| 1492R | 5'-GGTTACCTTGTTACGACTT-3' | | 19 | 52 |

order to extract DNA from the selected isolates, a fresh culture was prepared from them, and in the next step, DNA was extracted from the selected isolates using phenol/chloroform method (Wright *et al*., 2017). Then, all the extracted DNAs were analyzed quantitatively and qualitatively with a spectrophotometer and 1.5% agarose gel electrophoresis. In the next step, PCR was performed using the general primers of the 16S rRNA gene (Table 1). The PCR product was electrophoresed to confirm the amplification with general primers. The PCR products were sequenced, and the results of PCR product sequencing were checked with Chromas Software Version 2.6.6. In the next step, the sequence reads were checked in the NCBI online database. The phylogeny tree was drawn in Mega Software Version 6 based on the neighbor-joining model and the aligned sequence of the 16S rRNA gene.

**Cultivation and treatment of the plant:** Seeds were purchased from Pakan Bazr Seed Company (Iran) and were cultivated in greenhouse conditions with a temperature of 25 ± 2 °C and a time efficiency of 8 hours of darkness and 16 hours of lighting in pots with a width of 20 cm and a height of 20 cm. The pots contained sterile peat moss, cocopeat, and perlite in proportions of 50%, 30%, and 20%, respectively, and were equally fertilized with food solutions. After the plants reached the three-leaf stage, the soil around the plant in the root zone was inoculated with a concentration of $10^9$ CFU/ml in two levels (without bacterial inoculation and co-inoculation with the isolated bacilli). After two weeks, The plants were subjected to salinity treatment at three levels (0, 75, and 150 mM NaCl) in three replications every other day for two weeks. Plant leaf samples were taken after 21 days from the last treatment to check biochemical indicators.

**Preparation of enzyme extract:** First, 0.1 gr of fresh leaf tissue from each group was ground on ice in 1 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 0.01 polyvinyl pyrrolidine and 1 mM EDTA. The resulting extract was centrifuged at 10000 g at 4°C for 30 min. In the last step, the supernatant solution was used to perform protein assay tests and check the activity of antioxidant enzymes (Gapinska *et al.*, 2008).

**Protein measurement:** First, 50 µl of enzyme extract was added to 190 µl of Bradford solution and incubated for 5 min. Finally, the solution was measured at a wavelength of 595 nm and calculated using the standard curve of the amount of protein in mg/g of plant tissue. Bovine serum albumin was used as a standard to calculate the protein content of the samples (Bradford, 1976).

**Measurement of SOD activity:** The activity level of the SOD was measured through its ability to prevent the photoreduction of nitro blue tetrazolium chloride. A total of 3 ml of reaction mixture containing potassium phosphate 50 mM (pH 7.8), methionine 130 mM, nitroblue tetrazolium chloride 75 µM, ethylene ediaminetetra acetic acid 0.1 mM, riboflavin 360 µM, and also 30 µl of crude extract were stirred. In the next step, the spectrophotometer tubes were placed under a 15-watt fluorescent lamp at a distance of 35 cm for 10 min. The reaction was stopped by turning off the lamp, and then the absorbance of the reaction mixture was read at 560 nm. One unit of superoxide dismutase enzyme activity was considered to prevent the photoreduction of nitro blue tetrazolium chloride by 50%. The specific activity of the enzyme was reported by the number of absorption units per minute per mg of protein (Dhindsa *et al.*, 1981).

**Measurement of POX activity:** Initially, 100 µl of guaiacol (20 mM), 100 µl of enzyme extract, and 100 µl of hydrogen peroxide (40 mM) were added to 2.7 ml of potassium phosphate buffer (25 mM) with pH = 6.8. Enzyme activity started by adding hydrogen peroxide to the reaction mixture. The increase in light absorption after adding hydrogen peroxide was measured at a wavelength of 470 nm. The number of absorption units per minute per mg of protein was reported using the extinction coefficient of 66.2 mM/cm and the amount of enzyme activity (Chance and Maehly, 1995).

**Measurement of CAT activity:** The amount of CAT enzyme was measured by slight modification. For this purpose, 2.5 ml of 50 mM phosphate buffer (pH = 7) was poured on 10 mmol of $H_2O_2$ and 0.3 ml of enzyme extract, and then its activity was measured after 1 min by a spectrophotometer at a wavelength of 240 nm (Aebi, 1984).

**Measurement of proline:** First, 0.2 g of frozen plant tissue (stem and leaf) was rubbed in 10 ml of 3% sulfosalicylic acid solution, and the resulting extract was centrifuged for 5 min at 10000 rpm. Then, 2 ml of the supernatant was mixed with 2 ml of ninhydrin reagent and 2 ml of pure acetic acid and placed in a hot water bath at 100 °C for 1 h. The tubes containing the mixture were immediately cooled in ice, and 4 ml of toluene was added to the mixture, and the tubes were shaken. Then, two separate layers were formed by keeping the tubes fixed for 15 to 20 s. The absorbance of the upper color layer, which included toluene and proline, was determined at 520 nm, and the proline standard curve was used to calculate the amount of proline. The results were calculated in terms of mg/g fresh weight of the plant (Bates, 1973).

**MDA measurement:** 0.1 g of fresh leaves of the plant were ground in a Chinese mortar on ice and at 4 °C. Then, 1.5 ml of 1% trichloroacetic acid (TCA) was



[ DOI: DOI: 10.22034/13.64.37 ]

added to the resulting mixture and vortexes. The solution was centrifuged at 10000 rpm for 10 min at 4 °C. In the next step, 1 ml of 0.5% thiobarbituric acid (TBA) solution plus 20% TCA was added to 0.5 ml of the supernatant solution. Then, the reaction mixture was placed in a water bath at 95 °C for 30 min. In the last step, after 10 min of centrifugation, the samples were centrifuged at, 10000 g. Then, the absorbance of the resulting supernatant was measured at two wavelengths of 532 and 600 nm, and the concentration of malondialdehyde was calculated using the extinction coefficient of 155 $mM.cm^{-1}$ (Heath and Packer, 1969).

**Measurement of total phenolic compounds:** First, 0.1 g of the dry aerial part of the plant was rubbed with 5 ml of 95% ethanol and then placed in the dark for 24-72 h. Then, 1 ml of 95% ethanol was added to 1 ml of supernatant solution, and then the volume of the solution was increased to 5 ml using distilled water. In the next step, 0.5 ml of 50% folin's reagent and 1 ml of 5% sodium carbonate were added. The mixture was placed in the dark for 1 h, and then the absorbance of each sample was read at a wavelength of 725 nm. The concentration of total phenolic compounds was calculated in terms of mg of gallic acid per gram of dry weight of the plant and using the standard curve (Ronald and Laima, 1999).

**Statistical Analyses:** Data was extracted in factorial format in a completely random design with 3 replications. Data analysis was done using SPSS statistical software, and the averages were compared using Duncan's method at the significant level lower than 5%.

## Results and discussion

**Identification of *Artemisia absinthium* using macroscopic, microscopic, and biochemical methods:** The macroscopic, microscopic, and biochemical techniques were used to evaluate the results of the identification process of rhizosphere bacteria, which were isolated from the *A. absinthium* L. plant's rhizosphere. A total of 6 bacterial strains were isolated from the rhizosphere of *A. absinthium* L. The outcomes of microscopic and macroscopic analysis and biochemical assays are presented in Tables 2 and 3, respectively.

**Screening of *Bacillus* isolates regarding the ability to produce auxin and dissolve phosphate:** At this stage, *Bacillus* isolates with the highest auxin production and phosphate dissolution were identified. According to the average data comparison in Figure 1, E bacteria produces more auxin than other bacteria. Figure 2 shows that bacteria B can dissolve more phosphate than other bacteria. These two isolate were finally analyzed for molecular identification.

**Molecular identification of selected *Bacillus* strains isolated from Artemisia rhizosphere (PCR):** The PCR products obtained from amplifying the 16S rRNA gene of the selected isolates were taken on the gel after DNA extraction, and PCR and 1464 pb bands can be seen in Figure 3.

**Results of sequencing:** The sequences obtained from the PCR product in the selected isolates were blasted in NCBI. It was observed that the two strains were different from the previously known strains, so they were submitted to the GenBank and were deposited in it with the accession numbers OQ410446 and OR473625 for *Bacillus cereus* B isolate and *Bacillus cereus* E isolate. The genetic relationship of these strains with other strains was determined by using MEGA7 software and are shown in the phylogenetic trees (Figure 4).

**The effect of rhizosphere bacteria and sodium chloride salinity stress on biochemical indicators:** The amount of protein, proline and malondialdehyde (MDA) of the plant increased with increasing salinity stress. The combined treatment with rhizosphere bacteria and salinity stress increased the amount of protein compared to the control, which was not statistically significant. The combined treatment with rhizosphere bacteria and salinity stress caused a decrease in proline compared to the control, and the biggest reduction in proline was related to the combined treatment of rhizosphere bacteria (E and B) and salinity stress by 150 mM sodium chloride, which was statistically significant compared to the control. The combined treatment with rhizosphere bacteria and salinity stress decreased the amount of MDA compared to the control, and the most significant decrease in the amount of MDA was related to the combined treatment with rhizospheric bacteria (E and B) and sodium chloride 75 mM (Figure 5).

**The effect of *Bacillus* bacteria and sodium chloride salinity:** The activities of the superoxide dismutase, catalase, and peroxidase enzymes, and phenol increased with increasing salinity stress, which was significant compared to the control. The combined treatment of rhizosphere bacteria and salt stress increased the activities of the superoxide dismutase enzyme (including rhizosphere bacteria B and 75 mM sodium chloride), catalase enzyme, peroxidase enzyme, and phenolic compound content (Table 4 and 5).

In this study, the salinity stress increased protein in the *Artemisia absinthium* L. plant. Proteins may be made as new proteins in response to salt stress or may be present in low amounts and increase when plants are exposed to salt stress (Zhang *et al.*, 2013). Combined treatments of rhizosphere bacteria and salinity stress increased the amount of protein compared to the control. Researchers have stated that inoculating radish seeds with *Bacillus subtilis* and *Pseudomonas fluorescens* bacteria significantly increased the total free amino acids and crude protein content compared to uninoculated seeds under salinity stress (Mohamed and Gomaa, 2012). Inoculation of *Pisum sativum* with *Bacillus subtilis* RhStr_71, *Bacillus safensis* RhStr_223, and *Bacillus cereus* RhStr_JH5 under salinity stress increased the amount of protein (Gupta *et al.*, 2021).

Inoculation of rice (*Oryza sativa* L.) with three



[ DOI: DOI: 10.22034/13.64.37 ]

**Table 2. *Bacillus* isolates from both microscopic and macroscopic perspectives**

| Isolate code | Starch | Cat | Glu | Urea | Citrate | MR | VP | Luc | Suc | Gz | Mal | Man |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bacterium A | + | + | + | - | - | - | - | - | - | - | + | - |
| Bacterium B | + | + | - | - | - | - | - | - | - | - | - | + |
| Bacterium C | + | + | + | - | - | - | - | - | - | - | - | - |
| Bacterium D | - | + | + | - | - | - | - | - | - | - | + | - |
| Bacterium E | + | + | + | - | - | - | - | - | + | + | + | + |
| Bacterium F | + | + | - | - | - | - | - | - | + | + | + | + |

**Sugar fermentations (Man: Mannitol, Mal: Maltose, Gz: Glucose, Suc: Sucrose, Luc: Lactose), VP: Voges Proskaeur, MR: Methyl red, Cat: Catalase**

**Table 3. The microscopic and macroscopic characteristics of *Bacillus* isolates**

| Isolate code | Macroscopic properties | | | | Microscopic properties | |
|---|---|---|---|---|---|---|
| | Size of colony | Type of colony | Colony margin | Type of Surface | Color | Microscopic form |
| Bacterium A | Medium | Rough | Rough | Flat | Light milky | Small rods |
| Bacterium B | Small | Rough | Rough | Flat | Light milky | Almost medium rods |
| Bacterium C | Coarse | Rough | Rough | Flat | Light milky | Medium rods |
| Bacterium D | Medium | supple | Flat | Embossed | Dark milky | Large rods |
| Bacterium E | Small | supple | Flat | Embossed | Dark milky | Large rods |
| Bacterium F | Small | supple | Flat | Embossed | Dark milky | Large rods |

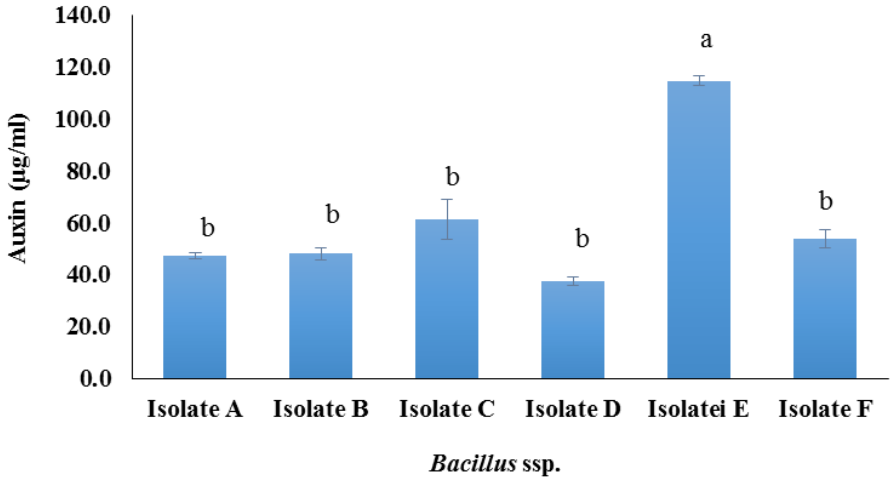


**Figure 1. The average produced auxin by the isolated strains isolated from the rhizosphere of *Artemisia absinthium* plant**

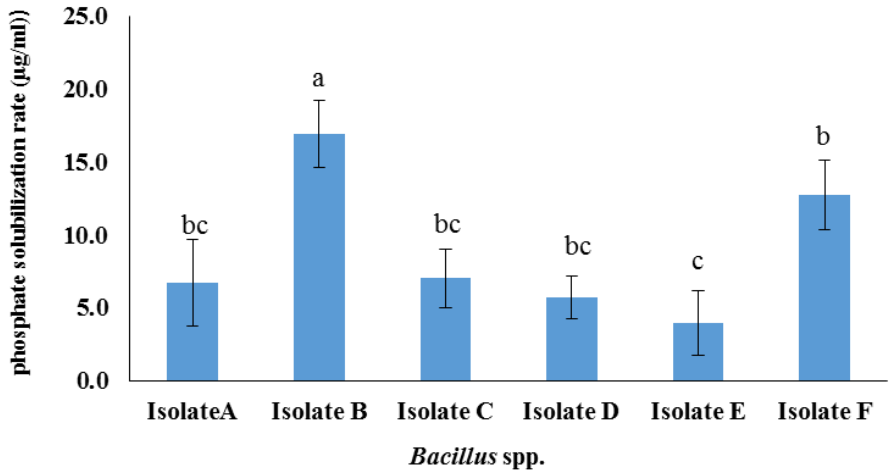


**Figure 2. The average phosphate dissolution by Bacillus strains isolated from the rhizosphere of *Artemisia absinthium* plant**

strains of *Bacillus* spp., ALT11, ALT12, and ALT30, under salt stress (70 and 140 mM NaCl) increased the amount of total protein (Khan *et al.*, 2021). Proline is one of the most important osmolytes, produced in plants



[ DOI: DOI: 10.22034/13.64.37 ]

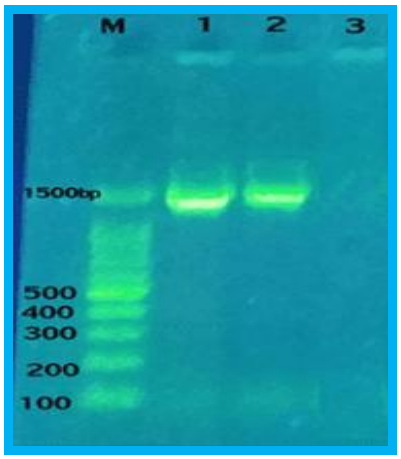


**Figure 3. Gel electrophoresis results of PCR products for molecular identification of selected isolates using primers 27F and 1492R marker: Column M, strain B: Column 1 and strain: E Column 2; Negative control: Column 3. PCR products with a length of, 1464 bp are observed in wells number one and two.**

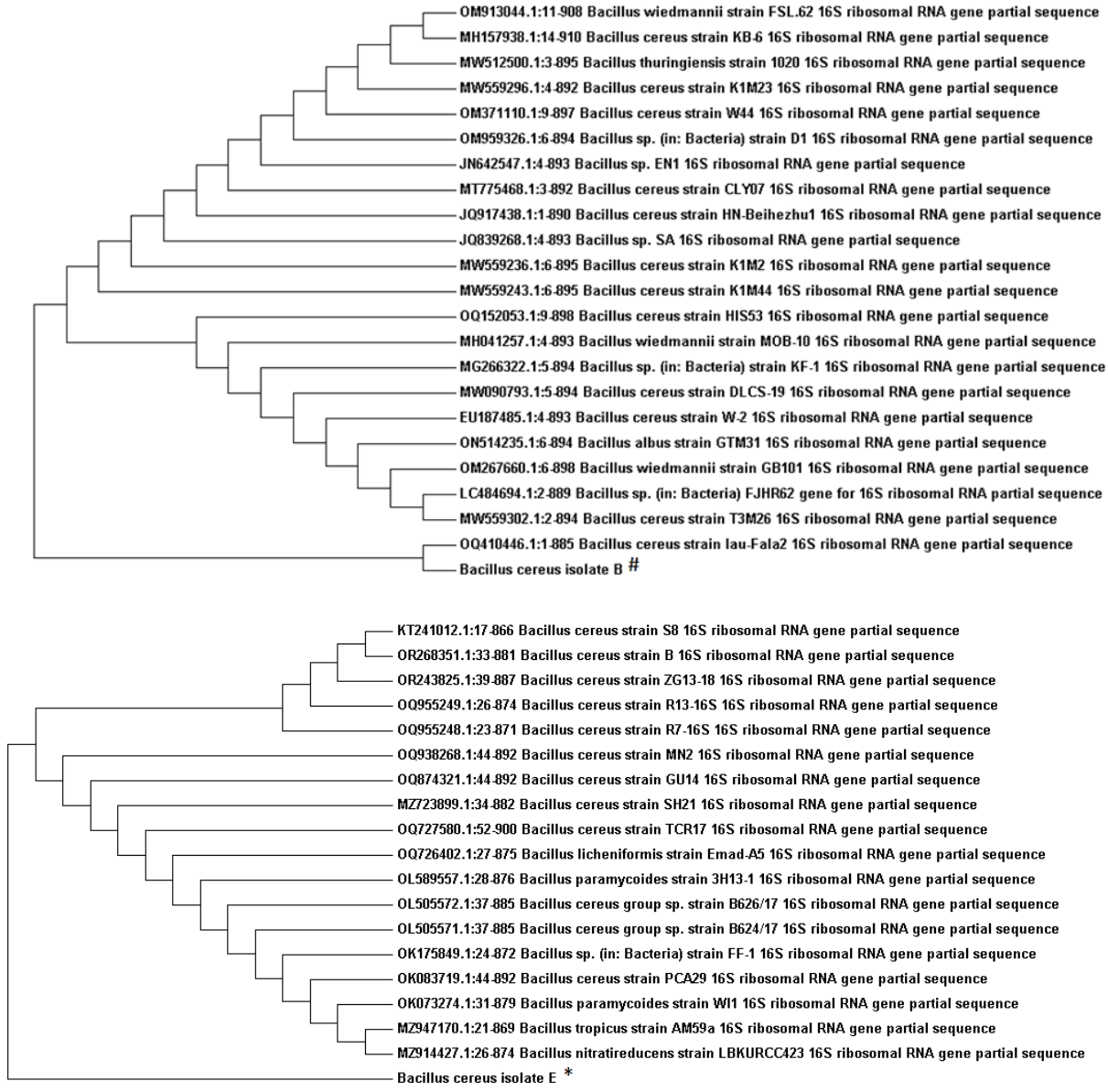


**Figure 4. Drawing a phylogenetic tree and the position of B and E isolated in it, which are defined by markers # and *, respectively.**



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**Table 4. Analysis of variance effect of rhizosphere bacteria and salinity stress (sodium chloride) on the biochemical indices of *A. absinthium* L.**

| Sources of change | df | Protein | Proline | MDA | SOD | Cat | POX | Total |
|---|---|---|---|---|---|---|---|---|
| Salinity stress | 2 | 262.597$^{**}$ | 277.120$^{**}$ | 0.0031$^{**}$ | 0.001$^{*}$ | 63.9849$^{**}$ | 0.006 $^{*}$ | 50.287$^{**}$ |
| Bacteria | 3 | 82.472$^{*}$ | 906.918$^{**}$ | 0.0011$^{**}$ | 0.003$^{**}$ | 117.4425$^{**}$ | 0.031$^{**}$ | 4.687$^{**}$ |
| Bacteria×Salinity stress | 6 | 38.069$^{ns}$ | 1084.873$^{**}$ | 0.003$^{*}$ | 0.000$^{*}$ | 17.7805$^{*}$ | 0.002 $^{*}$ | 0.519$^{*}$ |
| Error | 24 | 219.167 | 889.475 | 0.001 | 0.0001 | 53.362 | 0.011 | 0.183 |
| Coefficient of variation | | 383.139 | 14.895 | 0.010 | 7.743 | 5.74 | 0.001 | 10.703 |

**** Significant at P≤ 0.01, * Significant at P≤ 0.05, and ns non-significant**

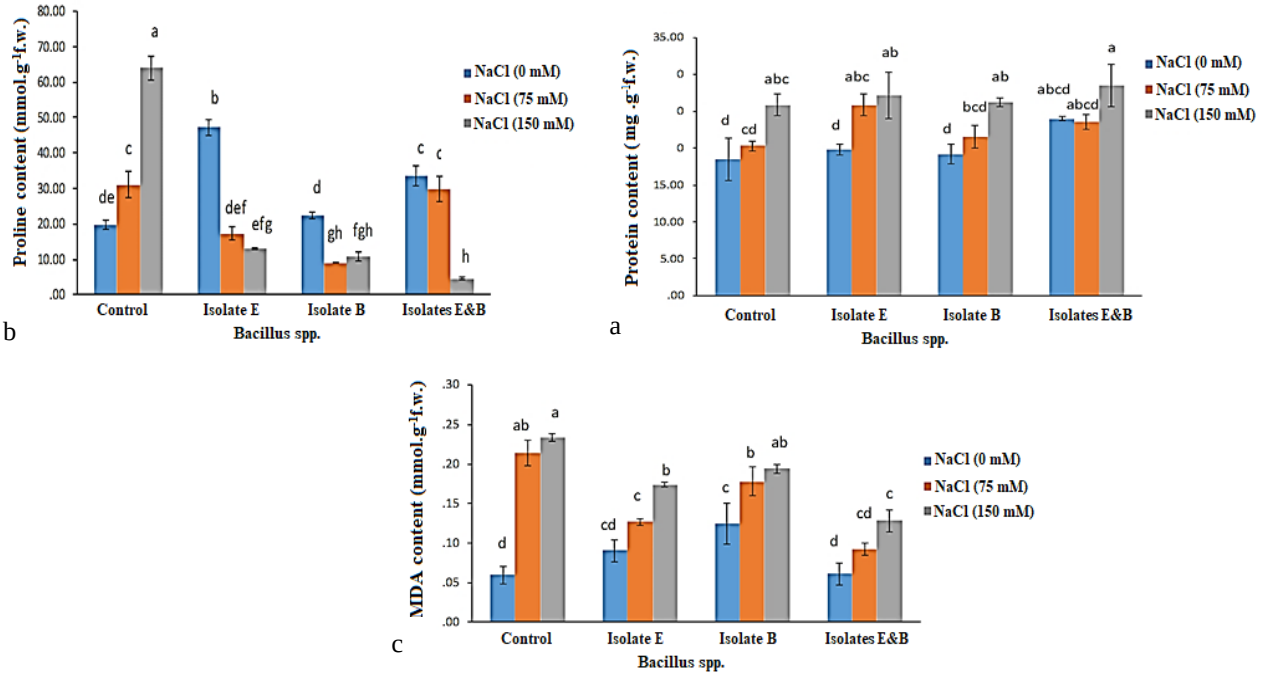


**Figure 5. Mean comparison effect of rhizosphere bacteria and salinity stress (sodium chloride) on the production of protein (a), proline (b), and malondialdehyde (c) *in Artemisia absinthium* plant**

**Table 5. Mean comparison of the effect of rhizosphere bacteria (B, E, and E & B) on the activities of superoxide dismutase enzyme, catalase enzyme, peroxidase enzyme and phenolic compounds contents in *Artemisia absinthium* plant under salinity stress (0, 75, 150 mM NaCl).**

| Bacterial isolates | Salinity (mM) | Superoxide dismutase enzyme (U.mg$^{-1}$. protein) | Catalase enzyme (U.mg$^{-1}$. protein) | Peroxidase enzyme (U.mg$^{-1}$. protein) | Phenolic compound content (mg. g$^{-1}$ DW) |
|---|---|---|---|---|---|
| - | 0 | 0.031 ± 0.0059$^{e}$ | 1.847 ± 0.4391$^{c}$ | 0.064 ± 0.3828$^{ef}$ | 0.759 ± 0.3828$^{f}$ |
| - | 75 | 0.045 ± 0.0014$^{de}$ | 2.671 ± 0.0556$^{de}$ | 0.081 ± 0.3828$^{def}$ | 1.729 ± 0.3828$^{de}$ |
| - | 150 | 0.050 ± 0.0067$^{cd}$ | 8.384 ± 1.4029$^{bc}$ | 0.086 ± 1.0793$^{ef}$ | 4.866 ± 1.0793$^{b}$ |
| E | 0 | 0.060 ± 0.0150$^{bc}$ | 6.550 ± 0.6551$^{cd}$ | 0.123 ± 0.2472$^{cde}$ | 1.645 ± 0.2472$^{de}$ |
| E | 75 | 0.071 ± 0.0105$^{b}$ | 11.791 ± 1.4166$^{ab}$ | 0.122 ± 0.23811$^{cde}$ | 1.663 ± 0.23381$^{df}$ |
| E | 150 | 0.066 ± 0.0143$^{b}$ | 12.729 ± 3.6370$^{a}$ | 0.126 ± 0.0375$^{cd}$ | 4.344 ± 0.0375$^{b}$ |
| B | 0 | 0.030 ± 0.0050$^{e}$ | 4.372 ± 0.6557$^{cde}$ | 0.045 ± 0.2531$^{f}$ | 1.378 ± 0.2531$^{ef}$ |
| B | 75 | 0.032 ± 0.0051$^{e}$ | 6.815 ± 0.9017$^{cd}$ | 0.104 ± 0.2766$^{cde}$ | 2.010 ± 0.2766$^{de}$ |
| B | 150 | 0.050 ± 0.0058$^{cd}$ | 7.3863 ± 1.901$^{c}$ | 0.111 ± 0.2284$^{cde}$ | 5.757 ± 0.2284$^{a}$ |
| (E & B) | 0 | 0.064 ± 0.0107$^{bc}$ | 7.982 ± 1.5684$^{bc}$ | 0.162 ± 0.2493$^{bc}$ | 2.259 ± 0.2493$^{d}$ |
| (E & B) | 75 | 0.092 ± 0.0050$^{a}$ | 14.030 ± 4.6070$^{a}$ | 0.182 ± 0.2292$^{b}$ | 3.394 ± 0.2292$^{c}$ |
| (E & B) | 150 | 0.073 ± 0.0085$^{b}$ | 14.308 ± 2.7394$^{a}$ | 0.264 ± 0.5577$^{a}$ | 6.432 ± 0.5577$^{a}$ |

**The data are the average of 3 repetitions ± standard error (SE) and dissimilar letters indicate significant differences based on Duncan's test (P ≤ 0.05).**

from protein hydrolysis under stress. The production of osmolytes maintains the turgor potential, prevents oxidative damage, and protects the membrane structure by scavenging reactive oxygen species (Ilyas *et al.*, 2020). Salinity stress increases the amount of proline, and the accumulation of proline in plants exposed to biotic and abiotic stress is a documented and conserved response in most plant species (Signorelli *et al.*, 2015).



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Proline accumulates under water stress, salinity, low temperature, and heavy metals (Chul Chun *et al.,* 2018). Researchers have reported that the amount of proline increased under salinity stress in the rice and lettuce (Kibria *et al.,* 2017; Ahmed *et al.,* 2019). In this study, the combined treatment of rhizospheric bacteria and salinity stress decreased proline compared to the control. Co-inoculation of bacteria by creating good nutritional conditions such as phosphate dissolution, auxin production, increasing the level of absorption of water and nutrients by the roots, and reducing stress to absorb elements, which may reduce the amount of stress and proline. The oxidative reduction stress and proline production decreased by increasing the enzymatic and non-enzymatic antioxidant activity by rhizosphere bacteria under salinity stress. In a research, the reduction of proline in plant inoculation by mycorrhiza under salt stress was considered to be due to the reduction of stress on the plant as a result of inoculation (Lee *et al.,* 2015). Malondialdehyde increased with increasing salinity stress, and plants stimulated the production of reactive oxygen species (ROS) under abiotic stresses, which can damage the production of biomolecules such as lipids, proteins, and nucleic acids. Membrane lipid peroxidation is one of the most significant damages to ROS. Membrane lipid peroxidation in plants is detected by measuring malondialdehyde (MAD), which is a widely used indicator of oxidative lipid damage caused by environmental stress (Kong *et al.,* 2016). Researchers investigated the amount of malondialdehyde in tomato and safflower cultivars. The combined treatment of rhizosphere bacteria and salt stress decreased the malondialdehyde compared to the control because the rhizosphere bacteria in this part increased the plant's defense system by stimulating the expression of enzymatic antioxidants and other enzymes, decreasing ROS and stabilizing the cell membrane (Attia *et al.,* 2021; Motamedi *et al.,* 2010). Erdogan found that inoculation of strawberries with *Paenibacillus polymyxa* RC05, *P. polymyxa* RC35, *Pseudomonas fluorescens* RC77, *P. fluorescens* RC86, *Pseudomonas putida* RC06, *P. putida* 2/2, and *Rhodococcus erythropolis* RC9 reduces the content of malondialdehyde under water stress, which was consistent with the present study (Erdogan *et al.,* 2016). In this study, antioxidant enzymes superoxide, including superoxides dismutase, catalase, peroxidase, and non-enzymatic antioxidant phenol increased with increasing salinity stress and produced reactive oxygen species enhanced in response to salinity stress. ROS production damages various cellular components such as proteins, lipids, DNA, and RNA, and the plant expands various biochemical and physiological mechanisms to survive under salinity stress (Sadak *et al.,* 2020; Kumar *et al.,* 2020). Other researchers found that catalase, peroxidase, and superoxide dismutase enzymes increased in the leaves of the Sanjjo variety rice plant with increasing salinity stress, which was aligned with this research (Yaghubi *et al.,* 2014). Similar to this study, researchers have stated that salinity increased the content of phenolic compounds in the *Salvia officinalis* plant (Valifard *et al.,* 2014). The combination of rhizosphere bacteria and salt stress increased the activity of superoxide dismutase, peroxidase, and catalase enzymes compared to the control. The reason for the increase in the activity of the superoxide dismutase enzyme (except for rhizosphere bacteria B and salt stress of 75 mM sodium chloride), peroxidase and catalase enzymes in the combined treatment of rhizosphere bacteria and salt stress is due to the ability of rhizosphere bacteria so that plant growth-promoting rhizobacteria (PGPR) uses several mechanisms to protect plant growth under abiotic stresses. Rhizobacteria protect plants against salt toxicity by activating the antioxidant defense system by increasing the expression and activity of key enzymes such as superoxide dismutase, peroxidase, and catalase by scavenging excessively produced reactive oxygen species (Sharma *et al.,* 2017). The decrease in the superoxide dismutase enzyme may be due to reducing the stress of this bacterium with another mechanism and the decrease in producing reactive oxygen species and this enzyme. Studies have observed that the inoculation of *Pisum sativum* with Bradyrhizobium and growth-promoting bacteria (*Actinomadura* sp. (183-EL strain), *Paenibacillus graminis* (MC 04.21 strain), *Bacillus* sp. (IPACC11 strain), *Streptomyces* sp. (212 strain) increased the activity of the superoxide dismutase enzyme in Nodules exposed to salinity stress (Santosa *et al.,* 2018). Inoculation of wheat seeds with *Enterobacter cloacae* (BT) and *Bacillus cereus* (BW) increased peroxidase activity under salt stress (Nasibi and Ahmadi Mousavi, 2020). Research reported that several potential bacterial strains, such as *Klebsiella, Pseudomonas, Agrobacterium*, and *Ochrobactrum* showed a high growth effect on peanuts under control as well as saline conditions (Chakraborty *et al.,* 2011). Inoculation of *Pisum sativum* with *Bacillus subtilis* RhStr_71, *Bacillus safensis* RhStr_223, and *Bacillus cereus* RhStr_JH5 under salt stress increased the antioxidant enzymes superoxide dismutase, catalase, and peroxidase (Gupta *et al.,* 2021). Inoculation of *rice* (*Oryza sativa* L.) with three strains of *Bacillus* spp., ALT11, ALT12, and ALT30, under salt stress (70 and 140 mM NaCl) increased the activities of antioxidants POD, CAT, and SOD (Khan *et al.,* 2021). In another study, inoculation with two salt-tolerant strains, YL07 (*Bacillus atrophaeus*) and YL10 (*Planococcus soli*) caused a significant increase in the amount of antioxidant enzymes SOD, CAT, and POD compared to the control (Hou *et al.,* 2022). Phenolic compounds are a group of secondary metabolites with different biological activities, the most important of which is their antioxidant properties (Minh *et al.,* 2016). The treatment of glycol grown in sodium soils with *Bacillus subtilis* (CSR-G-1), *Bacillus pumilus* (CSR-B-2) (CSR-B-3), *Bacillus thuringensis* strains increased the phenol compared to the control (Damodaran *et al.,* 2014).



[ DOI: DOI: 10.22034/13.64.37 ]

Inoculation of *Pisum sativum* with *Bacillus subtilis* RhStr_71, *Bacillus safensis* RhStr_223, and *Bacillus cereus* RhStr_JH5 under salinity stress increased the amount of phenol (Gupta *et al.,* 2021). Inoculation of cucumber plants with *Bacillus* and *Pseudomonas* strains under salinity stress has increased total phenolic compounds compared to the control (Saberi-Riseh *et al.,* 2020).

**Conclusion**

In this study, the combined treatment with rhizosphere bacteria and salinity stress increased the amount of total phenol and antioxidant enzymes (superoxide dismutase, catalase and peroxidase) compared to the control, and also it caused a decrease in the amount of malondialdehyde and proline content. These changes indicate that rhizospheric bacteria protect the plant against destructive effects of reactive oxygen species caused by salt stress by increasing the amount of total phenol and antioxidant enzymes in the plant and this effect may cause a decrease the production of malondialdehyde and proline in the plant. As a conclusion, inoculation of *A. absinthium* with *Bacillus* bacteria improved the plant tolerance against salinity.

**Conflict of interest**

The authors declare that there is no conflict of interest.

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