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ORIGINAL RESEARCH

Biological amelioration of water stress in rapeseed (*Brassica napus* L.) by exopolysaccharides-producing *Pseudomonas protegens* ML15

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Abstract

Rapeseed (*Brassica napus* L.) is a globally significant oilseed crop with high economic value. However, water deficit significantly limits its growth and productivity. Exopolysaccharides (EPS)-producing bacteria offer a promising strategy to counteract drought stress, leveraging their high water retention capabilities and plant growth-promoting (PGP) properties. This study was conducted to characterize the PGP traits of selected EPS-producing bacteria strains and evaluate its efficacy in enhancing rapeseed resilience under drought conditions. Among five EPS-producing bacteria evaluated, *Pseudomonas protegens* ML15 was selected for its best performance. This strain demonstrated a range of plant growth-promoting traits, such as the solubilization of phosphate, potassium, and zinc, alongside the production of ammonia, siderophores, and proline. It also exhibited antioxidant activity and the ability to form biofilms, even under water-stressed conditions. Inoculation of rapeseed with strain ML15 increased germination percentages and seedling length. Notably, whether rapeseed plants were subjected to drought-induced stress or maintained under normal conditions, treatment with *P. protegens* ML15 inoculation consistently improved plant length and overall biomass. Under drought-stressed conditions, inoculated plants exhibited reduced malondialdehyde levels and increased vegetation indices, chlorophyll, protein, proline, and phenolic content. They also showed enhanced activity of antioxidant enzymes, such as catalase and peroxidase, compared to uninoculated rapeseed plants. These findings underscore the potential of EPS-producing bacteria like *P. protegens* ML15 to mitigate water stress in plants, providing ecological and economic benefits that support agricultural sustainability.

1 | INTRODUCTION

Rapeseed (*Brassica napus* L.) oil is the third most-produced vegetable oil worldwide, following palm and soybean oil (Banaś et al., 2023). Moreover, rapeseed serves as a source of animal feed and also plays a crucial role as a biodiesel source (Batool et al., 2022; Yan et al., 2023). The growing significance of oil crops has driven projections for a 24% rise in rapeseed production from 2019 to 2050 to meet the demands of an expanding population (Ma et al., 2024). However, this issue is a challenge in the face of global climate change, which is exacerbating drought stress, thus heavily affecting crop yields and quality (Niu et al., 2018; Fan et al., 2023). As a result of drought conditions,

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plants undergo various morphological, biochemical, and physiological alterations both as a consequence and as part of their natural defense mechanisms (Gowtham et al., 2020; Abdelaal et al., 2021; Poudel et al., 2021). The root system immediately detects water deficiency, which initiates signal transmission through the xylem to the leaves, where the abscisic acid (ABA) is produced as a major signal for drought stress (Cruz De Carvalho 2008; Li et al., 2023). Moreover, ABA stimulates the generation of reactive oxygen species (ROS), which promote stomatal closure to regulate water loss (Li et al., 2022). Unluckily this leads to lower uptake and fixation of carbon dioxide (CO_2), consequently decreasing the intensity of photosynthesis (Gowtham et al., 2020). ROS at high concentrations can reach phytotoxic levels during prolonged drought stress, inducing oxidative stress and cellular damage that can lead to death of the cell (Cruz De Carvalho 2008; Rezayian et al., 2018; Hasanuzzaman et al., 2020). A multifaceted antioxidant defense system is utilized by plants, involving both non-enzymatic (ascorbic acid, phenolic compounds, α-tocopherol, glutathione) and enzymatic components (superoxide dismutase, catalase, peroxidase) to eliminate or detoxify excess ROS (Hasanuzzaman et al., 2020; Li et al., 2022). Furthermore, under conditions of water stress, plants exhibit elevated levels of osmoprotectants including proline, trehalose, mannitol, and glycine betaine to sustain internal physiological functions (Seleiman et al., 2021). Additionally, in response to drought stress, plants undergo significant morphological adaptations, such as a reduction in leaf size, area, and number, along with adjustments in shoot and root length (Bogati & Walczak, 2022).

Soil microbes interact with plant roots to selectively shape the microbial community in the rhizosphere, thereby enhancing the drought resistance and supporting plant growth and development (Fan et al., 2023; Zboralski and Filion 2023). According to Prudent et al., (2020), soils with high microbial diversity can assist plants in responding to drought stress by stimulating lateral root branching, increasing soil water availability to the root system, and improving soil structure. However, the widespread use of chemical fertilizers is known to negatively impact soil quality, disrupt microbial community dynamics, and reduce diversity, which in turn increases plant susceptibility to stress (Wu et al., 2020). Utilizing plant growth-promoting bacteria (PGPB) as bio-fertilizers emerges as a promising strategy to avoid the adverse effects of chemical fertilizer applications. These bio-fertilizers not only ameliorate soil conditions and bolster plants' resilience to drought but also offer ecological and economic advantages that foster agricultural sustainability (Delshadi et al., 2017; Rosa et al., 2023).

Pseudomonas spp. are renowned for their dual capabilities in plant growth promotion (PGP) and biocontrol, even under drought stress. They achieve this through various mechanisms, including enhancing root nutrient uptake and synthesizing various secondary metabolites such as plant hormones, osmolytes, antioxidant enzymes, exopolysaccharides, and forming biofilms (Ashry et al., 2022; Oskuei et al., 2023; Zboralski and Filion 2023). Exopolysaccharides (EPS), which are high molecular weight polymers, establish a hydrated microenvironment around bacterial cells, thereby reducing water loss and enhancing bacterial survival during drought stress (Morcillo and Manzanera 2021; Rosa et al., 2023; Ma et al., 2024). The presence of EPS-producing PGPB in the soil can contribute to alleviating drought stress in plants by enhancing soil structure and water retention, facilitating bacterial colonization, and activating various antioxidant mechanisms in plants. These processes collectively extend the timeframe for plants to adapt metabolically to drought conditions (Bhagat et al., 2021; Morcillo and Manzanera 2021). Naseem and Bano (2014) demonstrated that priming maize seedlings with EPS-producing bacteria like *Pseudomonas aeruginosa* Pa2, *Proteus penneri* Pp1, and *Alcaligenes faecalis* AF3, enhances plant survival and growth under drought stress. This is achieved by improving soil moisture, increasing relative water content, promoting proline accumulation, and boosting antioxidant enzyme activity.

The current study aimed to identify EPS-producing bacteria capable of thriving under water-stressed conditions through screening. Subsequently, selected isolates were characterized for key PGP traits to improve plant growth and stress resilience under both normal and drought-stressed conditions. The study assessed the effect of bacterial inoculation on rapeseed germination and evaluated the effectiveness of a selected isolate with growth-promoting activity in protecting rapeseed plants from drought stress. Key morphological parameters such as biomass, root length, and shoot length were also measured. Furthermore, biochemical markers including chlorophyll content, proline accumulation, phenolic compounds, protein content, and activities of antioxidant enzymes like catalase and peroxidase, along with malondialdehyde (MDA) levels in leaves, were analyzed to assess plant responses to drought stress. The presence of EPS-producing PGPB represents a significant factor influencing plant growth, making them promising biofertilizers for managing stress. Therefore, we hypothesized that employing EPS-producing PGPB with multiple growth-promoting properties can consistently enhance plant resilience under drought stress. This approach holds the potential to boost crop productivity, promote environmental conservation, and foster agricultural sustainability.

2 | MATERIALS AND METHODS

2.1 | Plant material

The seeds of winter oil rapeseed (*Brassica napus* L. variety Gemini) were sourced from Plant Breeding Strzelce sp. z o.o, IHAR group, Kuyavian-Pomeranian Voivodeship, Poland. The crop was cultivated in individual pots (16 cm in diameter and 13.5 cm in height) filled with a soil-perlite mixture in a 6:1 ratio. The soil collected from a garden, had a pH of 6.5. All pots were placed in a greenhouse maintained at 25 ± 4°C with a continuous 24-hour photoperiod.

2.2 | Microbial culture

A total of five bacterial isolates from the previous study (Fiodor et al., 2023) were chosen to be tested. Four isolates (*Pseudomonas*

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putida AF1I1, *Pseudomonas fluorescens* AF8I4, *Burkholderia ambifaria* AF8II10, *Bacillus cereus* AF8II13) were previously isolated from rhizosphere soil of cereal crops in Hołodolina, Poland, and one isolate (*Pseudomonas protegens* ML15) from Błędowska Desert, Poland. According to the data, those isolates were enabled to grow in Luria Bertani (LB) broth medium under drought conditions. Polyethylene glycol 6000 (PEG) at 30% (w/v) was added to the medium to mimic drought conditions. For this reason, these isolates will be referred to as drought-tolerant isolates hereinafter. Each bacterial strain was cultured on LB agar medium, incubated at 30°C for 24 h, and then stored at 4°C for subsequent analysis.

2.3 | Exopolysaccharides-producing bacteria

Exopolysaccharide (EPS) production in drought-tolerant isolates was evaluated to identify EPS-producing bacteria. An overnight liquid culture of each isolate (2%, v/v) was inoculated into LB broth medium supplemented with and without the addition of PEG-6000 (ThermoFisher; 24% w/v) to induce drought stress, with the medium without PEG-6000 serving as the control. All cultures were incubated at 30°C with shaking at 120 rpm for five days. EPS were extracted by centrifugation at 12 857 *g* for 30 min at 4°C. Subsequently, an equal volume of chilled absolute ethanol was added to the supernatant, and the mixture was stored overnight at 4°C. The precipitated material was collected by centrifugation at 12 857 *g* for 45 min at 4°C and then dried overnight at 50°C. The dry weight of the EPS was then calculated (Song et al., 2019; Banerjee et al., 2020).

2.4 | Evaluation of selected EPS-producing bacteria for plant growth promotion traits

A drought-tolerant isolate was chosen based on both EPS production ability and quantity. This isolate was subsequently assessed for various plant growth-promoting (PGP) traits under both drought-stressed conditions and control conditions. The drought-stressed environment was simulated by supplementing the medium with 24% (w/v) PEG-6000, whereas the control conditions were maintained in a medium without the addition of PEG-6000.

2.4.1 | Quantitative estimation of phosphate (P) solubilization

Phosphate solubilization by the selected isolate was quantitatively estimated using 40 mL of National Botanical Research Institute's phosphate growth medium (NBRIP) liquid medium containing the following components per 1 liter: 10 g glucose, 5 g $MgCl_2{\cdot}6H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$:, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$ and 5 g $Ca_3(PO_4)_2$ with a pH 7.0. The NBRIP medium was inoculated with 2% (v/v) of liquid bacterial culture at a concentration of 10^9 CFU ml^{-1}. The cultures were incubated at 30°C with shaking at 150 rpm. Supernatants from the bacterial cultures were collected on days 5, 10, and 15. These samples were centrifuged at 12 857 *g* for 10 min at 4°C (Elhaissoufi et al., 2020). Phosphate availability in the supernatants was measured spectrophotometrically using the molybdenum blue method with KH_2PO_4 standards as a reference. This analysis was conducted on a spectrophotometer (Evolution 201, Thermo Scientific) at a wavelength of 880 nm (U.S. Environmental Protection Agency, 1978).

2.4.2 | Quantitative estimation of potassium (K) solubilization

The potassium solubilization activity of the selected isolate was determined quantitatively using atomic absorption spectrophotometry (AAS). The isolate was cultured in 40 mL of Alexandrov medium, which contained gram per liter: 5 g glucose, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCO_3$, 2 g K-feldspar, 0.005 g $FeCl_3$, 2 g.

Ca_3PO_4, and adjusted to pH 7.0 (Boubekri et al., 2021). The culture was incubated at 30°C with shaking at 150 rpm. Samples were withdrawn at different time intervals, 5, 10, and 15 days, and centrifuged at 12 857 *g* for 10 min at 4°C to measure the available potassium concentration. The supernatants were analyzed using AAS (iCE 3000 series, Thermo Fisher) by aspirating each sample directly into the instrument, with absorbance readings recorded at 766.5 nm. Potassium concentration (mg l^{-1}) in each sample was then calculated by comparing absorbance values against a previously established potassium standard curve.

2.4.3 | Quantitative estimation of zinc (Zn) solubilization

Zinc solubilization activity was evaluated using mineral salts medium comprising per liter: 10 g glucose, 1 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g K_2HPO_4, 0.2 g $MgSO_4$, and adjusted to pH 7.0 (Dinesh et al., 2015). The medium was supplemented with 0.15% (18.5 mM) ZnO as a sole source of zinc. The overnight-grown culture of the selected isolate was inoculated at 2% (v/v) into 40 mL of the prepared mineral salt medium. The cultures were incubated at 30°C in a shaking incubator at 150 rpm. Samples were collected at 5, 10, and 15 days post-inoculation to monitor zinc solubilization over time. After collection, samples were centrifuged at 12 857 *g* for 10 min at 4°C. Zinc concentration in the supernatant was determined using AAS. Each sample's supernatant was aspirated into the instrument with absorbance readings recorded at 213.9 nm. Zinc concentration (mg l^{-1}) in each sample was then calculated from the absorbance values using a zinc standard curve.

2.4.4 | Quantitative estimation of indole acetic acid (IAA) production

IAA production was quantified using a colorimetric method adapted from Hartmann et al., (1983) with modifications by

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Pranaw et al., (2020). Briefly, a bacterial liquid culture (2%, v/v) was inoculated into 40 mL of Jensen's broth supplemented with 5 mM tryptophan (PanReac) and incubated at 30°C with agitation at 150 rpm for five days. Samples were collected on days 1, 3, and 5, and centrifuged at 12 857 *g* for 10 min. The supernatant was mixed with Salkowski reagent in a 4:1 ratio and allowed to react in the dark for 30 min. The appearance of the color pink was an indicator of a positive of IAA production. Absorbance was read using a spectrophotometer at 530 nm. IAA content was calculated according to the calibration curve of IAA (Sigma Aldrich).

2.4.5 | Quantitative estimation of ammonia production

The production of ammonia was quantified following the Mukherjee et al., (2019) with some modifications. A fresh bacterial culture (2%, v/v) was inoculated in 40 mL of peptone broth and incubated at 30°C with shaking at 150 rpm. After incubation, the bacterial culture was centrifuged for 5 min at 12 857 *g*. Then, 2 mL of supernatant was mixed with 40 μL of potassium-sodium tartrate (0.177 M) and 40 μL of Nessler's reagent (VWR). The resulting color change from yellow to dark brown indicated the presence of ammonia, which was measured spectrophotometrically at 435 nm. The ammonia content was calculated using the standard curve of ammonium sulfate ($(NH_4)_2SO_4$).

2.4.6 | Quantitative estimation of siderophores production

Bacterial cultures grown in LB medium were collected on days 5 and 10 and subsequently centrifuged at 12 857 *g* for 5 min to obtain the supernatant. An equal volume of CAS assay solution was then added to the culture supernatant, thoroughly mixed, and incubated for 20 min at room temperature in the dark. Siderophores in the supernatant chelated iron from the dye complex, reducing the intensity of the blue color. The change in color was measured spectrophotometrically at 630 nm, following the method described by Mukherjee et al., (2019). LB broth medium served as the blank for these measurements. The percentage of siderophore units was calculated using the following equation:

$$\%\,siderophore\ unit = \left[\frac{Ar - As}{Ar}\right] x\,100 \quad (1)$$

where Ar is the absorbance of reference (uninoculated growth media mixed with CAS assay solution) and As is the absorbance of the sample.

2.4.7 | Biofilm formation assay

Biofilm formation of the selected isolate was evaluated using a microtiter plate method, following the protocol of Oleńska et al., (2021) and Kostakioti et al., (2013) with slight modifications. Liquid cultures (200 μL) were transferred into six replicate wells of a sterile 96-well microtiter plate and incubated for 1 and 3 d at 30°C under static conditions. After the incubation period, the medium was carefully aspirated and discarded. The biofilm deposits were gently rinsed three times with 100 μL of sterile dH_2O. The wells containing biofilms were then treated with 100 μL of 0.1% (v/v) crystal violet solution for 30 min to stain the biofilm. After staining, the dye was discarded, and the wells were rinsed three times with sterile dH_2O to remove the excess stain. The microtitre plates were then air-dried at room temperature. Following drying, the stained biofilms were solubilized by adding 100 μL of ethanol and acetone mixture (4:1, v/v) to each well. The optical density (OD) of the resulting solution was measured at 575 nm using a microplate reader. Biofilm formation was considered positive when the OD_{575} was greater than 0.250.

2.4.8 | Quantitative estimation of biosurfactant production

The production of biosurfactants by the selected isolate was assessed using the emulsification index method. A cell-free supernatant (CFS) was obtained from bacterial cultures grown in a minimal medium and collected on days 1 and 3. Briefly, equal volumes of hexadecane and CFS were mixed in a clear, flat-bottom test tube by vortexing for 2 min. The mixture was then allowed to stand at room temperature for 24 h to allow for phase separation. The emulsification index (E24) was calculated based on the height of the emulsion layer formed relative to the total height of the liquid column, as described by Sharma and Pandey (2020).

$$E_{24} = \frac{height\ of\ the\ formed\ emulsion}{height\ of\ the\ total\ solution}\, x\,100 \quad (2)$$

2.4.9 | Quantitative estimation of proline production

Proline production by the selected isolate was quantified following the method described by Ashry et al., (2022). The procedure involved mixing 2.0 mL of bacterial supernatant with 2.0 mL of glacial acetic acid and 2.0 mL of acid ninhydrin solution (prepared by dissolving 2.5 g of ninhydrin in 60 mL of glacial acetic acid and 40 mL of 6 M phosphoric acid) in a glass tube. The mixture was placed in a boiling water bath for 1 h, followed by immediate cooling in an ice bath. After cooling, 4.0 mL of toluene was added and mixed vigorously for 20 s, resulting in two distinct layers. The upper toluene layer containing the chromophore was carefully separated from the aqueous phase. The absorbance of the toluene phase was measured at 520 nm using a spectrophotometer, with toluene serving as a blank. The concentration of proline in the samples was determined using a standard curve generated with known concentrations of proline (MP Biomedicals).

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2.4.10 | Antioxidant activity evaluation using DPPH

The antioxidant activity of the selected isolate was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, as outlined by Hosseini et al., (2018). The bacterial culture was grown in LB broth medium, and the supernatant was collected by centrifugation at 12 857 *g* for 10 min. For the assay, 50 μL of the bacterial supernatant was added to 250 μL of 0.025 g l^{-1} DPPH (free radical, 95%; Sigma-Aldrich) in methanol. The mixture was thoroughly vortexed and incubated in the dark for 30 min. Following incubation, the absorbance of the solution was measured at 515 nm using a microplate spectrophotometer (Infinite 200 Proc, Tucan), with methanol serving as the blank. The reduction in absorbance compared to the control indicates the antioxidant activity of the bacterial supernatant.

2.5 | In vivo evaluation of seed treatment with EPS-producing bacteria

Seed germination tests were performed under control and drought-stressed conditions to assess the impacts of EPS-producing bacteria on seed germination. Rapeseed (*Brassica napus* L.) was used for the experiments. Initially, seeds were surface sterilized following the protocol of Sauer and Burroughs (1986) with minor modifications. The seeds were immersed in 2% (v/v) sodium hypochlorite for 1 min, followed by a 1 min immersion in 70% (v/v) ethanol. Subsequently, the seeds were rinsed three times with sterile dH_2O.

Following sterilization, the seeds were soaked for two hours in one of the two treatments: either with sterile dH_2O or a suspension of EPS-producing bacterial cells in dH_2O. The treated seeds were then placed on Petri dishes lined with two sterile filter paper, which were moistened with 5 mL of either sterile dH_2O or 19% (v/v) PEG-6000 solution in dH_2O, simulating control and drought-stressed conditions, respectively. The dishes were kept at room temperature for five days. Each treatment was replicated five times. Germinated seeds were counted for each Petri dish and the total length of the seedlings was measured. The germination percentage was calculated following the method described by Ashry et al., (2022).

$$\text{Germination percentage}\,(\%) = \frac{\text{number of germinated seeds}}{\text{total number of seeds}} \times 100 \quad (3)$$

2.6 | Impact of EPS-producing bacteria on drought stress mitigation in rapeseed plants under greenhouse conditions

2.6.1 | Inoculum preparation

A single loop of selected EPS-producing bacterial isolate was transferred from the LB agar plate into 50 mL of LB broth medium in 100 mL of the flask. The culture was incubated in a shaking incubator at 30°C, 150 rpm for 48 h resulting in a bacterial suspension with a concentration of 10^9 CFU ml^{-1}.

2.6.2 | Seed coating preparation

Suspension of bacterial cells was obtained by centrifugation at 12 857 *g* for 5 min, 4°C. Following this, the bacterial cell suspensions were mixed with a gum arabic solution (MP Biomedicals; 40%, w/v). In addition, gum arabic solution (40%, w/v) without bacterial suspension served as a control (Arvin et al., 2012). The seeds were surface sterilized as described in Section 2.5. Subsequently, the sterilized seeds were immersed in a mixture of gum arabic with or without the addition of bacterial cells.

2.6.3 | Pot experiments

Three coated rapeseed seeds were sown in each pot (16 × 10 cm) containing a non-sterile soil and vermiculite mixture at a 6:1 ratio, respectively. The experiment comprised four treatments (Table 1) with five replicates each. Initially, the seeds of rapeseed plants were divided into two groups: one was coated with a solution of gum arabic, and the other with bacterial cells in a gum arabic solution. All plants were cultivated for 21 days with daily watering. For half of the treatments drought stress was induced for seven days by withholding water followed by two recovery days with irrigation (Ullah et al., 2012). Subsequently, the rapeseed plants were harvested.

2.6.4 | Growth parameter assessments

Various growth parameters were measured, including both morphological parameters like shoot height, root length, and biomass, and biochemical parameters like vegetation indices, photosynthesis pigments content, MDA, proline, total soluble protein content, and antioxidant enzymes. Shoot length was recorded from the base of the shoot to the tip of the tallest leaf, while root length was measured from the base of the shoot to the end of the main root. The fresh weight of the stem and roots was determined immediately after sample collection (Fan et al., 2023).

Non-destructive measurements of vegetation indices, including NDVI (Normalized Difference Vegetation Index), G (Greenness Index), CNDVI (Canopy Normalized Difference Vegetation Index), chlorophyll *a*, and chlorophyll *b*, were performed using leaf spectroscopy

TABLE 1 Experimental design for evaluating the efficacy of selected EPS-producing bacteria in mitigating drought stress in rapeseed plants.

Treatment code	Conditions	Seed coating elements
C1	Normal	Gum arabic
T1	Normal	Bacterial cells with gum arabic
C2	Drought-stressed	Gum arabic
T2	Drought-stressed	Bacterial cells with gum arabic

(CI-710 s SpectraVue Leaf, Ave Camas) on the final day before harvesting, between 9 a.m. and 12 p.m.

For biochemical analyses, fresh leaves were collected, frozen in liquid nitrogen, weighed to 100 mg, and placed in 2 mL Eppendorf tubes. Samples were ground using a plastic pestle. For MDA measurement, samples were extracted with 1.2 mL of 80% cold ethanol. For other analyses (total soluble protein, total phenolic content, proline, and antioxidant enzymes), samples were extracted with 1.5 mL of phosphate buffer (50 mM, pH 7). The homogenized samples were centrifuged at 12 857 *g* for 5 min at 4°C to obtain the supernatant for further analysis.

MDA content was determined using a modified thiobarbituric acid (TBA) method (Du & Bramlage, 1992). Supernatant aliquots (0.25 mL) were mixed with 0.25 mL of 0.65% TBA in 20% trichloroacetic acid (TCA) and 0.01% butylated hydroxytoluene (BHT). A second set of 0.25 mL supernatant was mixed with 0.25 mL of 20% TCA and 0.01% BHT. Samples were incubated at 95°C for 20 min, cooled on ice, and centrifuged at 12 857 *g* for 5 min at 4°C. Absorbance was measured at 440 nm, 532 nm, and 600 nm using a microplate spectrophotometer (Tucan, Infinite 200 Pro). MDA concentration (nmol g^{-1} FW) was calculated using an extinction coefficient of 157 mM cm^{-1}.

Total soluble protein content was quantified using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). Total phenolic content was measured using the Folin–Ciocalteu method (Vallverdú-Queralt et al., 2011). Each supernatant (0.2 mL) was mixed with 1.8 mL dH_2O, 0.12 mL Folin reagent, and 0.3 mL Na_2CO_3 (20%). After 1 hour of incubation in the dark, the optical density was measured at 760 nm. Gallic acid was used as the standard, and total phenolic content was expressed as gallic acid equivalents (μg gallic acid mg^{-1} FW). Proline content was determined as described in Section 2.4.9.

Catalase (CAT) activity was measured by mixing 0.1 mL of supernatant, 1.9 mL of water, and 1 mL of H_2O_2 (2 μL ml^{-1}), followed by a 5 min incubation at 35°C. CAT activity was assessed spectrophotometrically at 240 nm, with one unit of CAT activity defined as a 0.01 change in absorbance per minute (Lalay et al., 2022). Guaiacol peroxidase (POD) activity was measured by mixing 0.05 mL of supernatant, 2 mL of water, 1 mL of guaiacol (3 μL ml^{-1}), and 1 mL of H_2O_2 (3 μL ml^{-1}), followed by a 5 min incubation at 35°C. POD activity was measured at 470 nm, with one unit of POD activity defined as a 0.1 change in absorbance per minute (Mei et al., 2009).

2.7 | Data analysis

Data were statistically analyzed using IBM Statistic SPSS 22 software. EPS production by drought-tolerant bacteria, PGP traits of selected EPS-producing bacteria, and the effect of these bacteria on the rapeseed seedlings and plants under drought-stressed conditions were examined using one-way ANOVA. Differences between means were evaluated using Duncan's multiple range test at a significance level of $p < 0.05$.

3 | RESULTS

3.1 | Screening of EPS-producing bacteria

Five drought-tolerant bacterial isolates were screened based on their EPS production under both drought-stressed and control conditions. All tested isolates exhibited the ability to produce EPS under both conditions. Drought conditions resulted in a two- to six-fold increase in EPS production by all tested bacteria, relative to control conditions. *Pseudomonas protegens* ML15 (*P. protegens* ML15) yielded the highest amount at 570 μg ml^{-1} under control conditions and 1190 μg ml^{-1} under drought conditions (Figure 1). A statistically significant

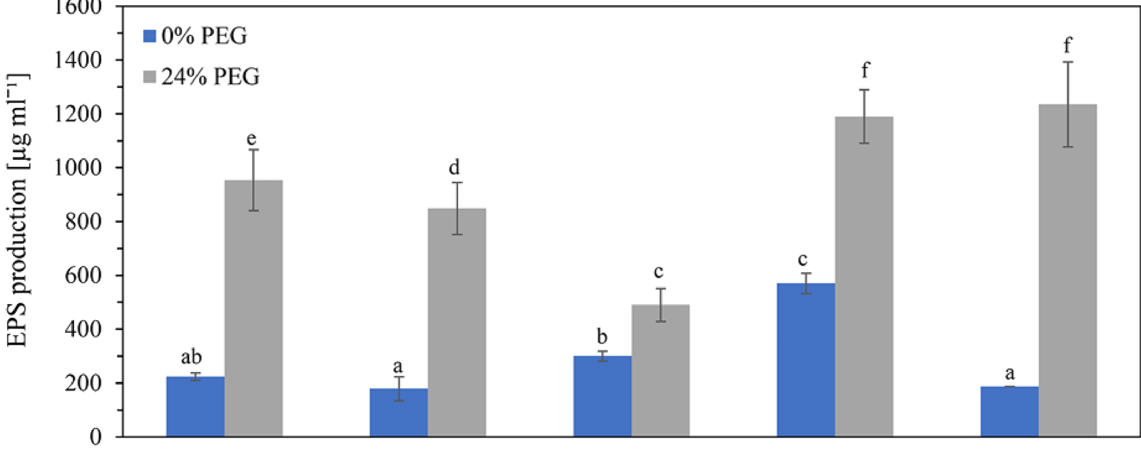

FIGURE 1 Assessment of EPS production by drought-tolerant bacteria under control and drought-stressed conditions. Each data represents the mean ± standard deviation (SD) of three replicates. Values with the different superscript letters are significantly different at the test level of 5% based on Duncan's t-test.

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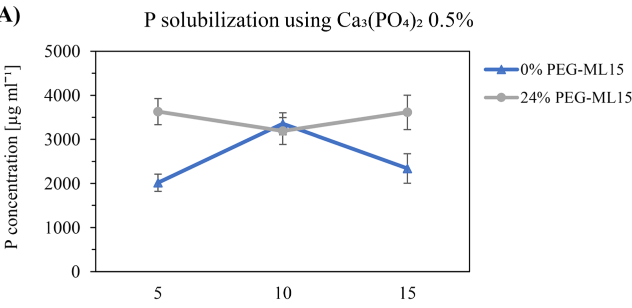
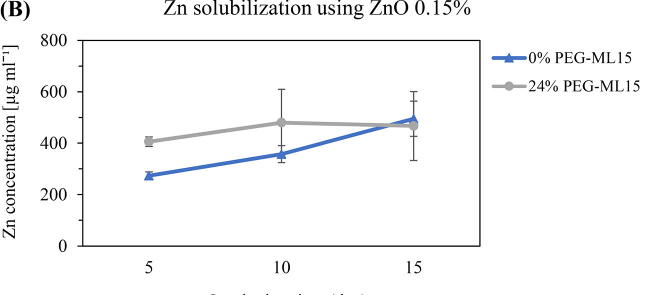
difference in EPS production was observed among all tested drought-tolerant bacteria under both control and drought conditions. The ML15 strain was selected for further experiments due to its demonstrated capability to produce a higher amount of EPS under both control and drought-stressed conditions compared to other tested isolates.

3.2 | Characterization of *P. protegens* ML15 for plant growth promotion traits under control and drought-stressed conditions

The plant growth promotion (PGP) traits of *P. protegens* ML15 were thoroughly evaluated, including its ability to solubilize insoluble sources of P, K, and Zn, as well as the production of IAA, ammonia, and siderophores. These assessments were conducted in a liquid medium under both drought-stressed and control conditions. During the incubation period, we observed that *P. protegens* ML15 solubilized P in a medium with tricalcium phosphate as the sole P source, with concentrations ranging from 2020 to 3356 μg ml^{-1} under control conditions (Figure 2). In contrast, *P. protegens* ML15 released higher amounts of P, showing a 35–44% increase under drought conditions. Additionally, *P. protegens* ML15 released soluble zinc at approximately 18.2% to 33.0% under control conditions and 27.1% to 32.0% under drought conditions in the medium supplemented with ZnO. Furthermore, the K concentration in the medium exhibited an upward trend over time, both in control and drought-stressed conditions, upon the addition of *P. protegens* ML15. These results indicated that *P. protegens* ML15 effectively solubilized K from K-feldspar, which served as the sole source of K in the medium. Specifically, the K concentration increased from 9.18 μg ml^{-1} on day 5 to 10.15 μg ml^{-1} on day 15 under control conditions, and from 8.11 μg ml^{-1} on day 5 to 10.33 μg ml^{-1} on day 15 under drought conditions. It is noteworthy that in the absence of bacterial addition, the medium containing 24% PEG-6000 exhibited a significantly higher concentration of K. This finding suggests that the presence of PEG influenced the solubilization of K from the feldspar source. Overall, the concentrations of P, Zn, and K for all treatments showed significant differences ($p < 0.05$), indicating the substantial impact of *P. protegens* ML15 on nutrient solubilization under both control and drought-stressed conditions.

In the next step, we confirmed that *P. protegens* ML15 did not show IAA production under control conditions, which is consistent with a previous report (Fiodor et al., 2023). Additionally, we excluded the production of this hormone under drought-stressed conditions. Incubation of the strain under drought conditions also revealed changes in other tested traits. We detected a five-fold increase in ammonia production under drought conditions, especially on day 10, compared to the control (Figure 3). Conversely, under drought conditions, the siderophore production decreased from 28% to 22% siderophore units over the same period from day 5 to 10. While the siderophore production increased from 11% to 71% siderophore units from day 5 to 10 under control conditions.





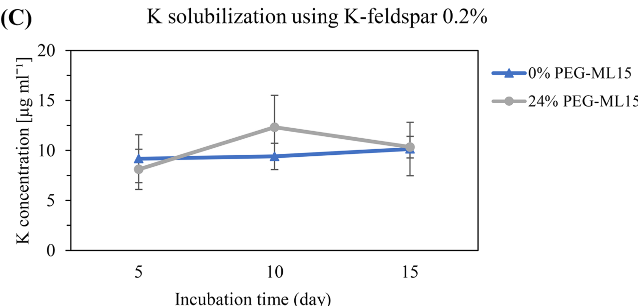


FIGURE 2 Evaluation of the nutrient solubilization ability of *P. protegens* ML15 under control and drought-stressed conditions. (A) Phosphate solubilization, (B) Zinc solubilization, (C) Potassium solubilization. Each data represents the mean ± SD of three replicates.

Furthermore, this study evaluated several mechanisms employed by *P. protegens* ML15 to mitigate adverse stress environments, including, biofilm formation, biosurfactant production, proline production, and antioxidant activity. Initial observations revealed negligible biofilm formation on day 1 under both conditions, as indicated by dye absorbance ($OD_{575} < 0.25$). However, after just three days, a statistically significant increase in biofilm formation by 16.1% was observed compared to the control group. The emulsification index, a measure of biosurfactant production, revealed that ML15 produces biosurfactants only under control conditions on day 3 of culture incubation. Proline production was only observed after three days, with drought conditions causing a 22.9% increase compared to control. DPPH radical scavenging activity was recorded in both conditions, without statistically significant differences. These findings highlight the varied responses of ML15 to stress conditions, with notable differences in

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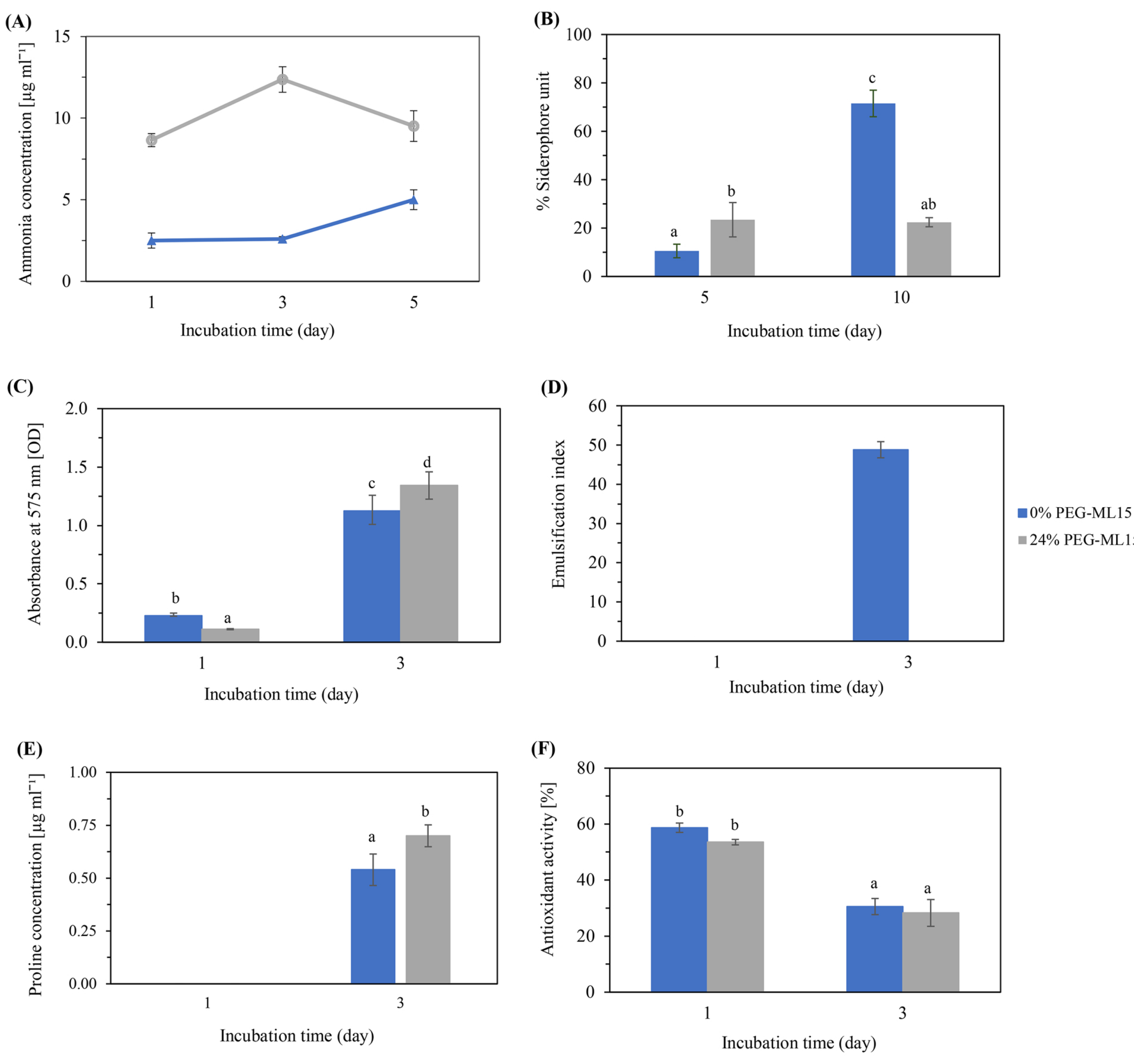

FIGURE 3 Evaluation of different PGP traits of *P. protegens* ML15 under control and drought-stressed conditions. (A) Ammonia production, (B) Siderophores production, (C) Biofilm formation, (D) Biosurfactant production, (E) Proline production, (F) Antioxidant activity. Each data represents the mean ± SD of three replicates. Values with the different superscript letters are significantly different at the test level of 5% based on Duncan's t-test.

metabolic activities and stress tolerance mechanisms in response to varying environmental conditions.

3.3 | *In vivo* evaluation of seed treatment with EPS-producing *P. protegens* ML15

We investigated the effect of *P. protegens* ML15 inoculation on rapeseed germination and total seedling length under both, drought-stressed (19% PEG-6000 addition) conditions and control conditions, as illustrated in Figure 4. Seeds inoculated with bacterial cells of *P. protegens* ML15 exhibited a germinated percentage of 96%, representing a 9.1% increase compared to uninoculated seeds under drought-stressed conditions. Additionally, inoculation of seeds with bacterial cell suspension significantly increased the total length of seedlings under both, control and drought-stressed conditions, compared to the seedlings from uninoculated seeds.

3.4 | The effect of *P. protegens* ML15 in mitigating drought stress in rapeseed under greenhouse conditions

An *in vivo* experiment was conducted to evaluate the effectiveness of *P. protegens* ML15 application in protecting rapeseed plants from drought stress. Various treatment combinations were implemented, wherein seeds of rapeseed were coated with gum arabic with or without *P. protegens* ML15 cells addition, were grown under both water-stressed and normal (control) conditions. Without bacterial treatment, drought-stressed conditions (C2) led to reductions in the fresh weight and stem length of rapeseed plants by 44% and 1.9%, respectively, compared to plants grown under control conditions without bacterial treatment (C1; Table 2). Notably, the application of *P. protegens* ML15 cells under both control (T1) and water-stressed (T2) conditions significantly improved plant growth metrics. Stem fresh weight increased by 26.0% and 35.6%, roots fresh weight by 59.3% and 64.3%, and

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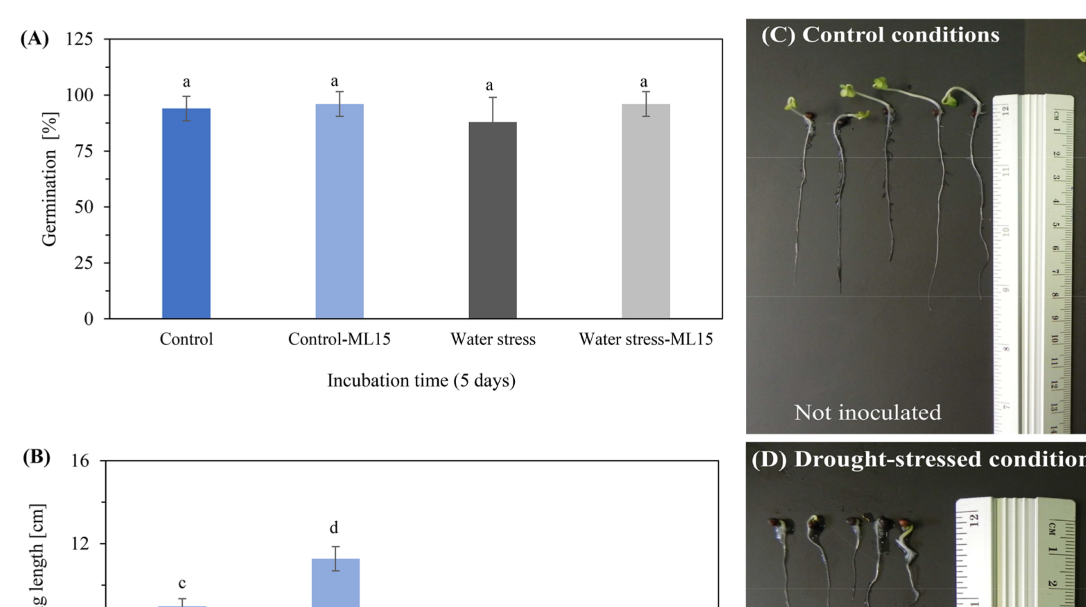

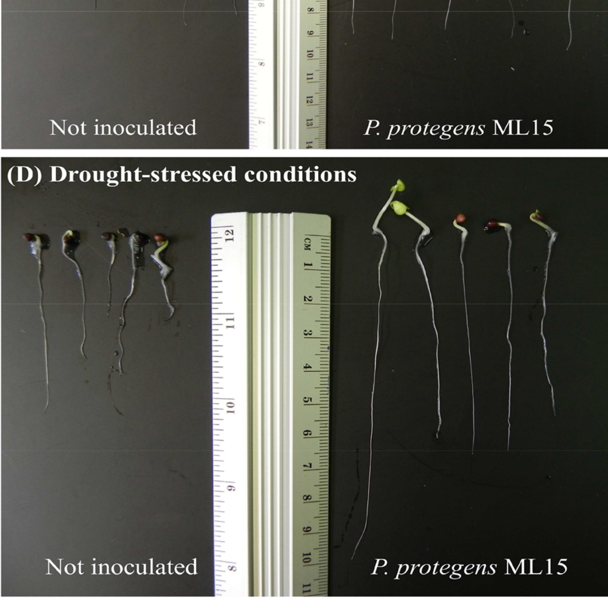

FIGURE 4 Effect of *P. protegens* ML15 inoculation on germination percentage and rapeseed seedling length under control and drought-stressed conditions. (A) Germination percentage, (B) Total seedling length, (C) Visual comparison of seedling growth. Each data represents the mean ± SD of five replicates. Values with the different superscript letters are significantly different at the test level of 5% based on Duncan's t-test.

TABLE 2 The effect of rapeseed seeds coated with *P. protegens* ML15 cells on morphology parameters of rapeseed plants under control and drought-stressed conditions.

	Fresh weight (g)		Plant length (cm)	
Treatment	Stem	Roots	Stem	Roots
C1	2.38 ± 0.5^{b}	0.16 ± 0.04^{a}	21.4 ± 2.7^{a}	9.8 ± 2.1^{a}
T1	3.22 ± 0.3^{c}	0.40 ± 0.09^{b}	25.0 ± 2.4^{b}	11.4 ± 1.5^{a}
C2	1.30 ± 0.2^{a}	0.13 ± 0.04^{a}	21.0 ± 2.4^{a}	16.5 ± 2.8^{b}
T2	2.02 ± 0.3^{b}	0.35 ± 0.07^{b}	21.6 ± 1.3^{a}	20.6 ± 2.1^{c}

Different uppercase letters indicate significant differences (p < 0.05). The treatments are defined as follows: (C1) rapeseed seeds coated with gum arabic, grown under control conditions; (T1) rapeseed seeds coated with gum arabic and *P. protegens* ML15 cells, grown under control conditions; (C2) rapeseed seeds coated with gum arabic, grown under drought-stressed conditions; (T2) rapeseed seeds coated with gum arabic and *P. protegens* ML15 cells, grown under drought-stressed conditions.

root length by 14.0% and 20.0% compared to C1 and C2 treatments, respectively. Additionally, the stem length of plants in the T1 treatment increased significantly by 14.4% compared to the C1 treatment.

In this study, we also investigated the impact of bacterial application on the biochemical response of rapeseed plants under water stress by assessing the vegetation index (NDVI, G, CNDVI), and pigment levels of chlorophyll *a* and chlorophyll *b* in rapeseed leaves using leaf spectroscopy. Under both control and drought-stressed conditions, treatments with bacterial cells (T1 and T2) consistently displayed higher values for all tested parameters compared to those without bacterial cell treatments (C1 and C2; Table 3). However, it is noteworthy that drought stress further increased the levels of all tested parameters.

Total protein levels exhibited significant variation in rapeseed leaves under the tested conditions. Notably, in rapeseed leaves with bacterial application (T1 and T2), total protein significantly increased by approximately 25.3% to 26.3% compared to rapeseed leaves without bacterial application (C1 and C2).

MDA content was measured to evaluate oxidative stress induced by drought stress. Under drought-stressed conditions, rapeseed leaves without bacterial coating treatment (C2) showed a significant increase in MDA content by approximately 29.1% (from 23.4 to 33.0 nmol g^{-1}) compared to control conditions (C1; Table 4). However, inoculation of *P. protegens* ML15 cells exhibited lower MDA values by 4.3% under control conditions and 8.1% under drought-stressed conditions compared to uninoculated treatments.

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TABLE 3 The effect of rapeseed seeds coated with *P. protegens* ML15 cells on vegetation indices and chlorophyll content of rapeseed leaves under control and drought-stressed conditions.

Treatment	NDVI (index)	G (index)	CNDVI (index)	Chlorophyll *a* ($\mu g\ cm^{-3}$)	Chlorophyll *b* ($\mu g\ cm^{-3}$)
C1	0.54 ± 0.02^{a}	1.93 ± 0.095^{a}	0.23 ± 0.029^{a}	7.7 ± 0.60^{a}	11.8 ± 1.04^{a}
T1	0.55 ± 0.04^{ab}	1.99 ± 0.016^{ab}	0.23 ± 0.023^{a}	8.0 ± 0.61^{a}	12.2 ± 1.02^{ab}
C2	0.56 ± 0.02^{ab}	2.03 ± 0.063^{b}	0.23 ± 0.021^{a}	8.1 ± 0.35^{ab}	12.3 ± 0.77^{ab}
T2	0.57 ± 0.02^{c}	2.03 ± 0.060^{b}	0.24 ± 0.030^{a}	8.6 ± 0.71^{b}	13.1 ± 1.34^{b}

Different uppercase letters indicate significant differences ($p < 0.05$).

TABLE 4 The effect of rapeseed seeds coated with *P. protegens* ML15 cells on biochemical parameters of rapeseed leaves under control and drought-stressed conditions.

Treatment	Total protein ($\mu g\ mg^{-1}$ FW)	MDA content (nmol g FW^{-1})	Phenolic content ($\mu g\ mg^{-1}$ FW)	Proline contents ($\mu g\ mg^{-1}$ FW)	Catalase activity (U g^{-1} FW)	Peroxidase activity (U g^{-1} FW)
C1	1.25 ± 0.08^{b}	23.4 ± 3.2^{a}	3.7 ± 0.25^{a}	0.024 ± 0.00^{a}	17.55 ± 0.59^{a}	0.09 ± 0.02^{a}
T1	1.68 ± 0.20^{d}	22.4 ± 0.5^{a}	3.8 ± 0.32^{a}	0.023 ± 0.00^{a}	17.61 ± 0.21^{a}	0.08 ± 0.01^{a}
C2	1.14 ± 0.04^{a}	33.0 ± 7.3^{b}	4.3 ± 0.10^{b}	0.027 ± 0.00^{ab}	17.56 ± 0.65^{a}	0.11 ± 0.01^{b}
T2	1.55 ± 0.03^{c}	30.3 ± 4.5^{ab}	4.6 ± 0.06^{b}	0.030 ± 0.00^{b}	17.72 ± 0.44^{a}	0.14 ± 0.01^{c}

Different uppercase letters indicate significant differences ($p < 0.05$).

The proline content increased significantly under drought conditions in both treatments with and without bacterial applications (T2 and C2; Table 4). Interestingly, treatment T2 exhibited higher proline content (10.5%) compared to the treatment C2. Nevertheless, under control conditions, there were no significant differences in proline content between treatments C1 and T1. This suggests that the presence of bacteria may have influenced proline accumulation under drought stress. Similarly, phenolic content increased significantly under drought conditions for both treatments, with and without bacterial coating. However, bacterial coating did not result in a significant difference in the phenolic content of rapeseed under both control and drought conditions. This indicated that while drought stress induced phenolic accumulation, the presence of bacteria did not further enhance this response. Regarding enzymatic activity, catalase activity did not show significant differences among treatments under both stressed and control conditions. In contrast to control circumstances, peroxidase activity was higher during drought-stressed. Treatment C2 exhibited lower peroxidase activity compared to treatment T2 under drought-stressed conditions, suggesting a potential modulation of antioxidant activity by bacterial application. Overall, these findings suggest complex interactions between bacterial application, drought stress, and plant responses.

4 | DISCUSSION

The interaction between plants and PGPB can play a significant role in acclimating and adapting to drought stress (Zboralski & Filion, 2023). The present study highlights the beneficial contributions of the promising drought-tolerant bacterium *P. protegens* ML15 in improving the resilience and productivity of rapeseed plants under water-deficit conditions. In this study, we employed five drought-tolerant bacterial strains, previously identified for their remarkable ability to endure drought stress, demonstrated by their survival under conditions induced by the addition of PEG-6000 at concentrations up to 30% (w/v) (Fiodor et al., 2023). However, the 24% (w/v) PEG-6000 concentration was selected to create moderate drought conditions, which supports an effective assessment of PGP effects. This level of stress is representative of typical agricultural water deficits, providing an environment that sustains both bacterial and plant viability. Among these, we selected the most promising isolate based on its EPS production under both control and drought-stressed conditions. EPS production is recognized as a form of physiological adaptation that enables microbes to survive adverse conditions (Morcillo and Manzanera 2021; Latif et al., 2022). EPS are mixtures of high molecular weight polymers that possess unique water-retention and cementing properties (Naseem et al., 2018; Ilyas et al., 2020; Ma et al., 2024). Notably, our study demonstrated that all five drought-tolerant strains exhibited the ability to produce EPS under both control and stressed conditions. Furthermore, our findings revealed that under drought-stressed conditions, all tested bacteria produced higher amounts of EPS compared to control conditions, suggesting that stress conditions stimulate an increase in EPS production. Among the tested strains, *P. protegens* ML15 was selected for its significant capacity to produce the highest concentration of EPS under both control and stressed conditions. This observation is consistent with previous studies which demonstrated that *Bacillus subtilis* and *Pseudomonas putida* GAP-P45 increased EPS production under water-stressed conditions (Vurukonda et al., 2016b; Ilyas et al., 2020). Additionally, Naseem et al., (2018) highlighted that PGPB produced more EPS during drought-stressed than under normal conditions, with EPS production increasing as drought severity rises.

Further, we emphasized the potential of *P. protegens* ML15 for other PGP traits under both control and drought-stressed conditions,

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establishing it as a promising candidate capable of alleviating water deficit stress and promoting plant growth. Our study revealed that *P. protegens* ML15 can provide soluble P, K, Zn from $Ca_3(PO_4)_2$, K-feldspar, and ZnO, respectively for the plants even under stressed conditions. Rigi et al., (2023), reported that the presence of *Pseudomonas* strain enhanced the availability and absorptions of P, K, and Zn in plants under drought stress by lowering pH, enhancing chelating substances, and facilitating acidolysis of the surrounding area of microorganisms. Moreover, the inoculation of *P. protegens* ML15 in the medium supplemented with PEG-6000 exhibited a higher concentration of P, Zn, and K compared to the uninoculated medium, further underscoring the enhanced solubilization capacity facilitated by the bacterial strain.

Under drought conditions, *P. protegens* ML15 increased the production of ammonia, proline, and biofilm formation indicating stress-induced responses. Ammonia-producing bacteria play roles in nitrogen cycling thus, give impact to plant growth (Wang et al., 2023). While as osmolytes, proline accumulation serves as an osmotic regulator, aiding in drought tolerance (Ilyas et al., 2020). Biofilm formation is considered a protective strategy against adverse conditions, consistent with research showing increased biofilm formation of *Pseudomonas putida* FBKV2 under drought stress (Vurukonda et al., 2016a). Furthermore, our study revealed a decrease in siderophore and biosurfactant production, as well as antioxidant activity under drought-stressed conditions. This finding aligns with previous studies showing reduced siderophore production by *Providencia* sp. under drought stress (Vishnupradeep et al., 2022). Despite the decrease in these activities, siderophore-producing bacteria can still alleviate Fe deficiency and enhance plant physiological processes under stress conditions (Singh et al., 2022). Biosurfactants efficiently solubilize and increase the supply of micronutrients and trace metals to plants by reducing interfacial tension and increasing the solubility and mobility of ionic nutrients (Silva et al., 2024). The antioxidant activity of *P. protegens* ML15 was observed to be lower under drought conditions than control conditions. This finding aligns with a study conducted by Ashry et al., (2022), where the antioxidant activity of *Bacillus cereus* DS4 and *Bacillus albus* DS9 decreased with increasing PEG concentrations until −1.2 MPa. However, PGPB notably increases the activity of ROS-scavenging antioxidant enzymes, thereby decreasing the excess ROS accumulation in drought stress (Gowtham et al., 2020). Unfortunately, *P. protegens* ML15 did not produce IAA, which could have helped alleviate drought stress by promoting root growth and enhancing water and nutrient uptake from the soil (Poudel et al., 2021). Overall, these findings suggest that *P. protegens* ML15 exhibits different physiological responses and activities under normal and drought-stressed conditions. This adaptability highlights its potential role in mitigating the effects of drought stress on plants and soil ecosystems.

Seed germination and seedling growth are stages that are extremely sensitive to drought stress (Rezayian et al., 2018). The present study demonstrated that the application of *P. protegens* ML15 on rapeseeds improved germination percentage and seedling length under both control and drought-stressed conditions. Furthermore, we conducted greenhouse experiments to assess the efficacy of *P. protegens* ML15 in aiding rapeseed plants tolerate to drought stress. This investigation aimed to elucidate how the application of *P. protegens* ML15 could potentially enhance the resilience of rapeseed plants to drought stress by modulating their morphological, physiological, and biochemical responses. The results showed that drought stress reduced the biomass of rapeseed plants. This was expected since drought stress can be attributed to impaired photosynthesis due to dehydration and reduction in the turgor, which results in a decrease in growth and cell development in the aerial part of plants (Chiappero et al., 2019; Delsh adi et al., 2017). Furthermore, our study found that rapeseed coated with *P. protegens* ML15 had a more stimulating effect on plant growth, resulting in increased biomass and plant length (both shoot and roots) compared to uninoculated treatments, even under drought-stressed conditions. This improvement indicated enhanced tolerance to abiotic stress. Similar results were previously reported by Oskuei et al., (2023), *Pseudomonas fluorescens* FY32 inoculation increased the dry weights and root length of rapeseed plants under moderate drought stress. Notably, PGPB alters the host root system architecture, leading to enhancements in root surface area, root branching, root length, and an increase in the number of root tips, particularly in response to drought stress (Gowtham et al., 2020). Our findings corroborate this, as the fresh weight and length of the roots of rapeseed plants treated with *P. protegens* strain ML15 showed an increase compared to untreated plants.

Bacterial inoculation and stress conditions can induce changes in the biochemical response of plants. Our study observed that drought-stressed led to an increase in the vegetation indices and chlorophyll content, including NDVI, G, CNDVI, chlorophyll *a*, and chlorophyll *b*, in rapeseed leaves compared to control conditions. Similar results were reported by Alizade et al., (2020) and Rosa et al., (2023), who noted increased chlorophyll content and vegetation indices under drought stress in winter wild oat and tomato plants, respectively. This contrasts with the general expectation of decreased leaf chlorophyll content under drought stress (Liu et al., 2022; Hu et al., 2023). However, our study showed that rapeseed treated with *P. protegens* ML15 exhibited higher vegetation indices and chlorophyll content in rapeseed leaves compared to those without inoculation. This enhancement in chlorophyll synthesis may be attributed to the ability of *P. protegens* ML15 to supply additional iron through the production of siderophores. Excess ROS production causes increased membrane lipid peroxidation and consequent damage to proteins, nucleic acids, and lipids in drought conditions (Gowtham et al., 2020). Our study revealed that drought conditions decreased the total protein content in rapeseed leaves. However, inoculation with *P. protegens* ML15 resulted in higher total protein content compared to uninoculated treatments under both conditions. Furthermore, we observed lower levels of MDA in rapeseeds inoculated with *P. protegens* ML15 under both control and drought-stressed conditions. MDA, as a result of lipid peroxidation, is used as a biomarker of membrane damage caused by various abiotic stresses (Vishnupradeep et al., 2022). Additionally, we found that under drought-stressed conditions, *P. protegens* ML15-inoculated plants showed increased accumulation of proline, phenolic content, and antioxidant enzymes. Phenols play a role in

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scavenging ROS-mediated free radicals, while enhanced proline accumulation and peroxidase activity help mitigate stress effects by regulating membrane stability, osmotic adjustment, and ROS scavenging processes (Kavian et al., 2023). These findings suggest that *P. protegens* ML15 may protect plants from oxidative damage by modulating the levels of proline, phenolics, and antioxidant enzymes in rapeseed plants. Our results are consistent with a study by Kazemi Oskuei et al., (2023), which demonstrated that inoculation with *Pseudomonas fluorescens* FY32 led to significantly higher activities of antioxidant enzymes, thereby alleviating the adverse effects of drought stress on rapeseed.

The drought-tolerant properties of *P. protegens* ML15 can be attributed to the enhancement of rapeseed plant growth under both control and drought-stressed conditions. This bacterium demonstrated the ability to produce EPS and other PGP traits, including the solubilization of nutrients, production of ammonia and siderophores, biosurfactant, osmoregulation, antioxidant activities, and biofilm formation capabilities. These various traits enable *P. protegens* ML15 to adapt to and shield the plants from stress-induced damage caused by drought.

Future research should prioritize developing effective and stable formulations for EPS-producing bacterial bioinoculants to maintain their viability and performance under the diverse conditions encountered in field applications. Unlike controlled laboratory settings, field environments vary significantly, and bioinoculant formulations must withstand fluctuations in temperature, soil moisture, and soil type.

5 | CONCLUSIONS

Our study identified *P. protegens* ML15 as a highly effective EPS-producing bacterium with multiple PGP traits. Specifically, under drought-stressed conditions, *P. protegens* ML15 demonstrated enhanced abilities to solubilize phosphate, potassium, and zinc. Additionally, it exhibited significant production of EPS, ammonia, siderophores, proline, and biofilm formation, along with antioxidant activity. Inoculation of rapeseed with *P. protegens* ML15 significantly enhanced its resilience to drought stress. This was evidenced by improved germination percentages, seedling length, overall biomass of plants, protein content, proline content, phenolic content, and antioxidant enzymes accompanied by reduced levels of MDA. The results highlight *P. protegens* ML15 as a promising bioinoculant for improving rapeseed growth and resilience under drought conditions, offering both ecological and economic benefits for sustainable agriculture.

AUTHOR CONTRIBUTIONS

The manuscript was written through the contributions of all authors. N.A. contributed to the conceptualization, investigation, formal analysis, and wrote the original draft. A.F. was responsible for the review and editing of the manuscript. L.D. also contributed to the review and editing. K.P. was involved in the conceptualization, supervision, methodology, project administration, and acquisition of funding. All authors have approved the final version of the manuscript.

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