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Phosphate-Solubilizing *Enterobacter ludwigii* AFFR02 and *Bacillus megaterium* Mj1212 Rescues Alfalfa's Growth under Post-Drought Stress

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Abstract: Drought stress is a prevalent environmental stress that adversely affects agricultural industries worldwide. In this study, bacterial isolates, AFFR02 and Mj1212, showed tolerance to polyethylene glycol-induced (PEG) drought stress (approximately 15%) and possess strong phosphate-solubilizing capacity. Moreover, we investigated the plant growth attributes, chlorophyll content, and ion uptake in alfalfa plants (*Medicago sativa* L) inoculated with isolates AFFR02 and Mj1212 under drought stress. We observed that drought stress drastically affects alfalfa's growth attributes: shoot length: SL (24.88%), root length: RL (29.62%), shoot fresh weight: SFW (49.62%), root fresh weight: RFW (45.09%), stalk diameter: SD (52.84%), and chlorophyll content: CC (19.2%). However, in bacterial-inoculated alfalfa plants, the growth attributes significantly recovered were SL (12.42%), RL (21.30%), SFW (50.74%), RFW (46.42%), SD (76.72%), and CC (17.98%). In drought-stressed alfalfa plants, we observed a significant decrease in the relative water content (7.45%), whereas there was an increase in electrical conductivity (68.87%) and abscisic acid contents (164.42%). Antioxidant analysis showed a significant increase in total phenolic content (46.08%), DPPH-scavenging activity (39.66%), total flavonoid (13.68%), and superoxide dismutase (28.51%) in alfalfa treated with drought stress and bacterial isolates AFFR02 and Mj1212 simultaneously. Moreover, an increase in inductively coupled plasma (ICP) analysis of potassium (17.98%), phosphorous (11.14%), calcium (3.07%), and magnesium (6.71%) was recorded for bacteria-inoculated alfalfa plants under drought stress. In conclusion, bacterial isolates AFFR02 and Mj1212 enhance alfalfa growth under drought stress. Therefore, the isolates could be used as potential candidates in smart-climate agricultural practices in drought-stricken areas worldwide.

Keywords: phosphate solubilization; bacterial isolates; drought stress; growth attributes; antioxidants

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1. Background

Environmental factors, such as drought, salinity, temperature, heavy metals, and freezing stress, are important agricultural problems that impose stress on crop plants and cause widespread crop losses worldwide [1–9]. The continuous increase in temperature and water scarcity is increasing the frequency of severe drought conditions [10]. Drought stress is defined as the scarcity of water available to plants, and 50% of the world's arid or semiarid land is subjected to drought stress [11]. Drought stress intervenes in the normal biochemical, physiological, and morphological processes of plants by reducing leaf size,

stem extension, root proliferation, photosynthesis, nutrient, and water relation [10,12–14]. Mineral nutrients are essential chemical elements for plant growth and development [15]. Drought stress causes nutrient deficiencies and interrupts the supply of essential trace elements because the physicochemical properties of soil reduce the mobility and absorbance of individual nutrients [15,16]. A decrease in the availability of nutrients leads to poor growth, development, and productivity of crop plants under drought stress [17]. Among essential nutrients, phosphorous is a vital macronutrient least available to plants and plays a significant role in growth and metabolic activities [18].

It is widely recognized that the perception of abiotic stress triggers the interaction of signal transduction cascades with the pathways transduced by phytohormones and defense antioxidants [1]. The fluctuation of stress-responsive plant hormones plays a central role in regulating growth response under stress conditions [19–22]. Among various phytohormones, abscisic acid (ABA) is called the stress hormone [23,24], and the level of ABA increases via ABA biosynthesis in harsh environments, including drought stress [12,25,26]. ABA is a primary phytohormone regulating various physiological processes, such as stomatal closure and favoring the maintenance of root growth by optimizing water uptake during drought stress [1,25]. Drought stress also causes membrane damage and stomatal closure by enhancing metabolite flux and generating reactive oxygen species, leading to increased oxidative load and severely damaging biological macromolecules [1,27]. To maintain cellular redox potential, plants tend to accumulate more antioxidants to mitigate cellular damage and stress conditions [28].

So far, different strategies have been developed to improve drought stress tolerance in crop plants, including legumes [1,29,30]. An alternative strategy to improve drought tolerance may be introducing drought-tolerant microbes that enhance crop growth and development under drought stress [11,13,29–33]. Numerous studies have shown that plant growth-promoting drought-tolerant bacteria is a relatively simple low-cost alternative strategy, and beneficially impacts growth attributes, biomass, and chlorophyll content (CC) under drought stress [11–13,32,34–36]. Plants can actively engage microorganisms in the surrounding environment by exerting positive effects on plant performance and stress resilience, including drought stress [12,34,37]. Previously-reported drought-tolerant isolates solubilized more phosphorus under stress conditions compared with normal conditions [18,38]. P-solubilizing bacteria have attracted attention because of their agroeconomic environmental-friendly approach, safer to use as P-fertilizer instead of using expensive P-chemical fertilizers [38].

Alfalfa (*Medicago sativa* L.) is a perennial legume that is mostly grown on grassland, pastures, and many places globally [5,39]. Alfalfa has a great agronomic importance and is grown on 30 million hectares of land worldwide [40]. Alfalfa is known as the queen of forage because of its high yield capacity, abundant forage for animals, and its ability to improve soil fertility [5,41]. It is also used as biofuel, and in pharmaceutical factories, it is used to produce monoclonal antibodies, industrial proteins, and enzymes, such as alpha–amylase, cellulose, and lignin peroxidase [42]. Alfalfa is also consumed by humans in sprout because of its highly nutritious low fiber, crude protein, important minerals, and amino acids [43]. Drought stress is a significant abiotic stress affecting the productivity and annual yield of alfalfa [44]. Bouizgaren et al. [45] reported a 49% decrease in biomass and photosynthesis in alfalfa due to water stress. Various researchers previously reported drought stress tolerance through various organic fertilizers and chemicals [46–48]. However, chemical fertilizer use leads to a marked deterioration of agricultural soil and underground water quality [49,50]. Using biofertilizers instead of fertilizers and chemicals improves plant growth by sustaining environmental health and soil productivity [49,51,52]. This study examines the growth attributes of alfalfa plants inoculated with drought stress-tolerant AFFR02 and MJ122 under control and post-drought stress. Furthermore, we have also determined the endogenous ABA, and different antioxidant and mineral uptake regulation in inoculated and non-inoculated alfalfa under control and post-drought stress.

2. Materials and Methods

2.1. Isolation, Screening, and Identification

Rhizospheric bacteria were isolated from Seosan, Chungcheongnam-do Province, and Geongbuk-do Province in the Republic of Korea, according to the detailed method of [53,54]. All isolates were screened for different PEG (0%, 5%, 10%, and 15%) concentrations and phosphate solubilization. To elevate the drought tolerance of isolated rhizospheric bacteria, different PEG (0%, 5%, 10%, and 15%) concentrations were added in Luria broth (LB) media and kept in a shaking incubator (120 rpm; 28 °C) for 4 day. The optical density (OD) (600 nm) was taken at a regular interval of 12 h for 4 d by using a spectrophotometer (Shimadzu, Kyoto, Japan). The National Botanical Institute Phosphate media (0.5%) was used to inoculate isolated microbes, and they were incubated at 30 °C [55,56]. The bacterial isolate formed a cleared halo on plates, indicating phosphate solubilization. For the phosphate-solubilizing ability, bacterial isolates were inoculated into 100 mL of optimum medium containing tricalcium phosphate at 35 °C. Quantitative spectrophotometric analysis of soluble phosphate was performed according to the described method of Kang et al. [57]. Alternatively, the pH was measured daily; the pH of the medium was recorded using pH meter equipped with a glass electrode. Furthermore, for bacterial identification, the 16S rRNA gene was amplified using general bacterial primers 27F and 1492R. The nucleotide sequences of PCR products were compared using the BLAST NCBI and EzTaxon program. A phylogenetic tree was constructed following the neighbor-joining method using MEGA v.7 and was sent to the GenBank database for accession number. Isolate Mj1212 had phosphate-solubilizing activity and promotes mustard plant growth under normal conditions [58].

2.2. Plant Growth-Promoting Effect of Isolate AFFR02 and MJ1212 on Alfalfa under Normal and Post-drought Stress

Alfalfa seeds were purchased from Nature & Kids Korea Ltd. and sown in trays filled with autoclaved horticultural soil (which contained 51.5% coco peat, 10% peat moss, 13% vermiculite, 15% perlite, 10% zeolite, humic acid, 0.1% fertilizer, and 0.4% fungus-free bio-soil; Shinsung Mineral Co., Ltd., Goesan, Korea). After 2 weeks of germination, one seedling was transferred to each pot (100 mm diameter × 90 mm depth) and grown in a greenhouse. The experiment was conducted in the Kyungpook National University greenhouse, at Daegu, under natural light with controlled environmental conditions: day/night temperature: 28 ± 3 °C/25 ± 3 °C, with relative humidity of 60%–70%. The experiment was conducted in a completely randomized design, with each treatment replicated 10 times throughout the study. The experimental design included (a) no stress: (200 mL water per week), (b) 200 mL of cell suspension of isolate MJ1212 (2.75×10^7 CFU/mL) and AFFR02 (2.08×10^7 CFU/mL)/week), (c) drought stress: (50 mL water per week), and (d) bacterial-treated (50 mL cell suspension of isolate MJ1212 and AFFR02/week). After 2 weeks of drought stress, alfalfa plants were treated with normal water for 3 weeks (post-drought stress). The shoot/root length (SL/RL), shoot/root fresh weight (SFW/RFW), stalk diameter (SD), and CC were measured using a portable chlorophyll meter (SPAD-502, Konica, Japan). For chlorophyll, a and b and total carotenoids, the detailed method of Khan et al. [59] was followed using 80% acetone. Their content was measured spectrophotometrically at wavelengths of 663 nm, 465 nm, and 480 nm, respectively.

2.3. Determination of Leaf Water Potential and Electrolyte Leakage in Alfalfa Plants under Normal and Post-Drought-Stressed Conditions

Leaf relative water content (RWC) was measured according to a previously described method of Lubna et al. [60]. Individual leaves were collected and weighed (fresh weight; FW) and immersed in distilled water overnight. At the end, turgid weight (TW) was measured and kept in a preheated oven (75 °C for 48 h) to obtain dry weight (DW). The RWC was calculated using the formula: RWC % = ([FW − DW]/[TW − DW]) × 100. For electrolytic leakage (EL), 500 mg leaves were cut (5 mm), kept in 10 mL deionized water in a tube, and placed

in a water bath at 32 °C. After 2 h, initial EC was measured using an EC1 m. Further plant samples were autoclaved (121 °C for 15 min), cooled (25 °C), and final EC2 was measured. EL was estimated using the following formula: EL = EC1 − EC2 × 100.

2.4. Endogenous Abscisic Acid Quantification in Alfalfa Plants under Normal and Post-Drought-Stressed Conditions

Endogenous ABA was quantified and extracted according to a method by Khan et al. [61] and Asaf et al. [62]. ABA was extracted from the aerial parts (freeze-dried plant samples, 0.3 g) with 30 mL extraction solution (95% isopropanol and 5% glacial acetic acid), and a chromatograph was run using 10 ng of Me-[2H6]-ABA standard. The suspension was filtered, and the filtrate was concentrated using a rotary evaporator. The residue was suspended in 4 mL of 1 N NaOH solution and rinsed three times with 3 mL of methylene chloride to eliminate traces of lipophilic materials. After decreasing the pH of the aqueous phase to 3.5 by adding 6N HCL, it was extracted by solvent extraction with ethyl acetate three times. The ethyl acetate extract was then evaporated, and dry residue was resuspended in a phosphate buffer solution (pH 8) and passed through the polyvinylpolypyrrolidone (PVPP) column. The eluted phosphate buffer solution was partitioned thrice with ethyl acetate (EtOAc) after adjusting the pH to 3.5 with 6N HCL. All three aliquots extracted were pooled and evaporated using a rotary evaporator. Furthermore, the fraction was methylated with diazomethane for detection, and ABA was quantified using gas chromatography–mass spectrometry (GC–MS) (6890N network gas chromatograph, Agilent Technologies). Software from ThermoQuest Corp., Manchester, UK, was used to monitor signal ions (m/z 162 and 190 for Me-ABA, and m/z 166 and 194 for Me-[2H6]-ABA).

2.5. Antioxidant Enzyme and Nonenzymatic Activities in Alfalfa Plants under Normal and Post-Drought-Stressed Conditions

The detailed method of Adhikari et al. [55] was followed to determine polyphenol content. Briefly, samples were extracted with 100% methanol and measured using a spectrophotometer (Shimadzu, Kyoto, Japan) at 750 nm. For flavonoid content, sample extracts were mixed with double distilled water and then $NaNO_2$ was added. After 5 min, 10% of 60 µL $AlCl_3$ and 1M NaOH was added and vortexed. The absorbance reading was taken at 500 nm using a spectrophotometer, as reported by Adhikari et al. [63]. Superoxide dismutase (SOD) was measured according to the detailed method of Lubna et al. [60]. SOD activity was expressed as enzyme unit (EU) nmol/g. For the DPPH-scavenging activity, a detailed method of Blois [64] was used with some modification. Absorption was measured at 517 nm using a spectrophotometer and calculated using the following equation: scavenging effect (%) = $1 - (Abs_{sample} - Abs_{control}) \times 100$.

2.6. Determination of Mineral Uptake in Alfalfa Plants under Normal and Post-Drought -Stressed Conditions

Potassium (K), phosphorous (P), calcium (Ca), and magnesium (Mg) content in the shoots of bacterial-inoculated and non-inoculated post-drought alfalfa plants were investigated according to the detailed method of Sahile et al. [8] and Kang et al. [65], using an inductively coupled plasma mass spectrometer (ICP-MS; Optima 7900DV, Perkin-Elmer, Waltham, MA, USA).

2.7. Statistical Analysis

The results of this study were subjected to statistical analysis. The difference in the mean values was compared using Duncan's multiple range test using statistical analysis system (SAS) v.9.3. For graphical presentation, Graph Pad Prism was used. All experiments were conducted in triplicate.

3. Results

3.1. Isolation and Screening

Alfalfa plants were collected from Seosan, Chungcheongnam-do Province, Korea. Sixteen rhizospheric bacterial strains were isolated from the roots of alfalfa plants and screened for phosphate-solubilizing activity and PEG stress tolerance. We used isolate Mj1212 with phosphate-solubilizing activity to promote mustard plant growth under normal conditions [58].

3.2. Screening for Polyethylene Glycol (PEG) Tolerance

All isolates, including Mj1212, were examined for their ability to grow in different PEG (0%, 5%, 10%, and 15%) concentration stress on LB media. The results from this investigation showed that under approximately 5% PEG, the growth of all isolates was normal, whereas at 10% PEG stress, bacterial growth declined. Only three isolates (AFFR02, AFFR07, and Mj1212) showed growth in 15% PEG LB media (Figure S1).

3.3. Phosphate-Solubilizing Ability of Isolate AFFR02 and Mj1212

All isolates were screened for phosphate solubilization. The results showed that on National Botanical Research Institute's phosphate (NBRIP) media plates, the formation of clear halos indicates tricalcium phosphate solubilization capacity. Nine isolates were positive for phosphate solubilization (Figure 1A). Therefore, the phosphate solubilization potential of selected isolates was cross-checked by monitoring the pH of bacterial-inoculated NBRIP media every 24 h (Figure 1B). The pH results showed a decrease in response to phosphate-solubilizing activity of isolate AFFR02 and Mj1212 from an initial pH of 7.0 to 4.2 after 96 h. In contrast, the P-solubilizing curve showed an increase that confirms the phosphate-solubilizing activity of AFFR02 and Mj1212 in inoculated NBRIP liquid media (Figure 1C).

3.4. Identification of Effective Rhizospheric Isolate

Based on their tolerance to PEG and having the highest potential of P-solubilizing characteristic, isolate AFFR02 was selected and identified. To find sequences similar to 16S rRNA gene sequence of isolate AFFR02, we checked the database of GenBank, NCBI, and EzTaxon. Identification results of 16S rRNA gene sequence of isolate AFFR01 showed close similarity to *Enterobacter ludwigii* (accession no: Kt261055). Furthermore, the selected sequence nucleotides were sent to the GenBank database and registered with accession number MW345827 for isolate AFFR02 (Figure 2).

3.5. Beneficial Effect of Isolate AFFR02 and Mj1212 on Alfalfa Plants under Normal and Post-Drought-Stressed Conditions

Growth attribute, biomass, and CC was investigated in alfalfa plants inoculated with isolate AFFR02 and Mj1212 under normal and post-drought stress. Under normal conditions, an increase in SL (11.24%; for percentage, mean/average value of AFFR02 and Mj1212 was used), RL (13.2%), SFW (33.08%), RFW (43.13%), SD (15.85%), and CC (5.20%) was observed in bacterial-inoculated (AFFR02 and Mj1212) alfalfa plants compared with control plants (only water treated). Alternatively, drought stress inhibited plant growth parameters and a decrease was observed in the growth attributes of post-drought-stressed alfalfa plants (SL (24.88%), RL (29.62%), SFW (49.62%), RFW (45.09%), SD (52.84%), and CC (19.2%)) compared with the control (unstressed/only water-treated plants) (Table 1; Figure 3). However, an increase in the growth attributes, SL (12.42%), RL (21.30%), SFW (50.74%), RFW (46.42%), SD (76.72%), and CC (17.98%), of post-drought-stressed alfalfa plants inoculated with AFFR02 and Mj1212 was observed compared with post-drought-stressed control alfalfa plants (Table 1; Figure 3). Furthermore, a decrease in *Chl a* (45.09%), *Chl b* (45.11%), and total carotenoid content (51.32%) was observed in post-drought-stressed alfalfa plants compared with the control (unstressed/only water-treated plants). However, alfalfa plants treated with isolate AFFR02 and Mj1212 mitigated drought stress, and an

increase in *Chl a* (23.04%), *Chl b* (25.89%), and total carotenoid content (54.51%) were observed in post-drought-stressed control alfalfa plants (Table 2).

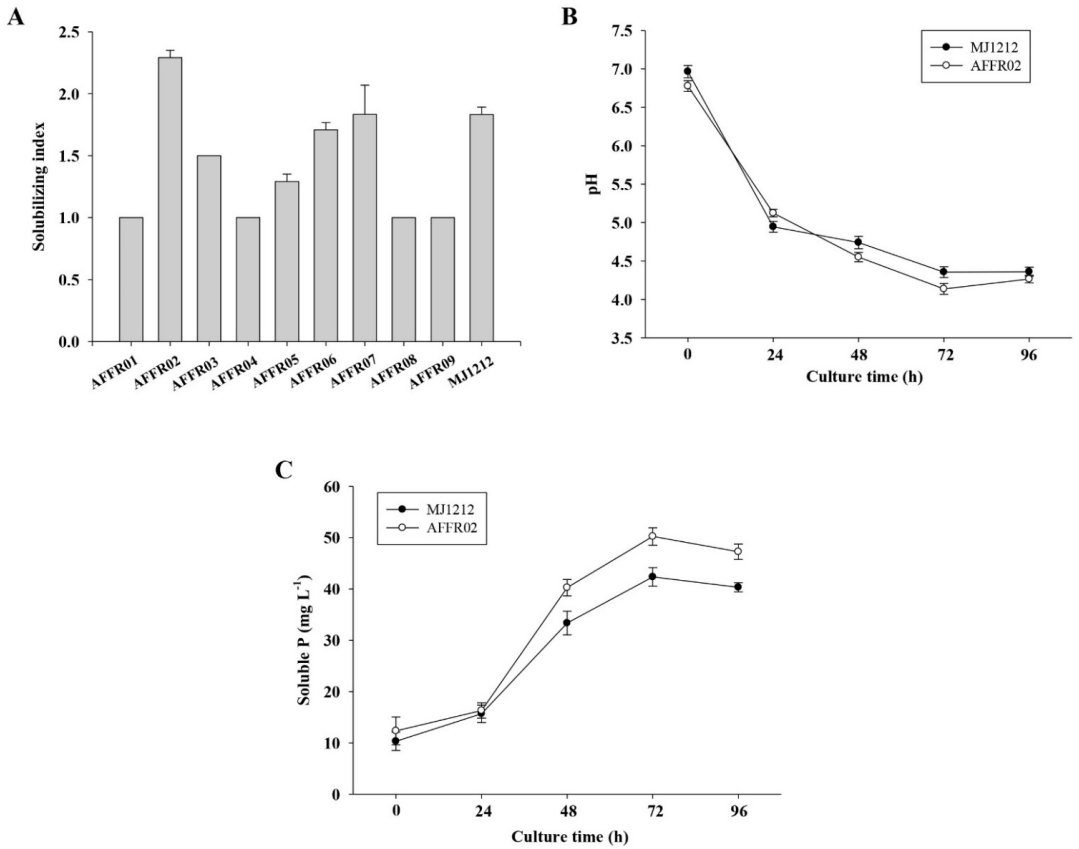

Figure 1. (**A**) Phosphate solubilization ability of isolated bacteria, (**B**) pH value of liquid National Botanical Research Institute's phosphate (NBRIP) medium, and (**C**) the rate of the halo formation of isolate AFFR02 and MJ1212 in the NBRIP medium. The values given are the mean of three replicates, and error bars indicate standard deviation.

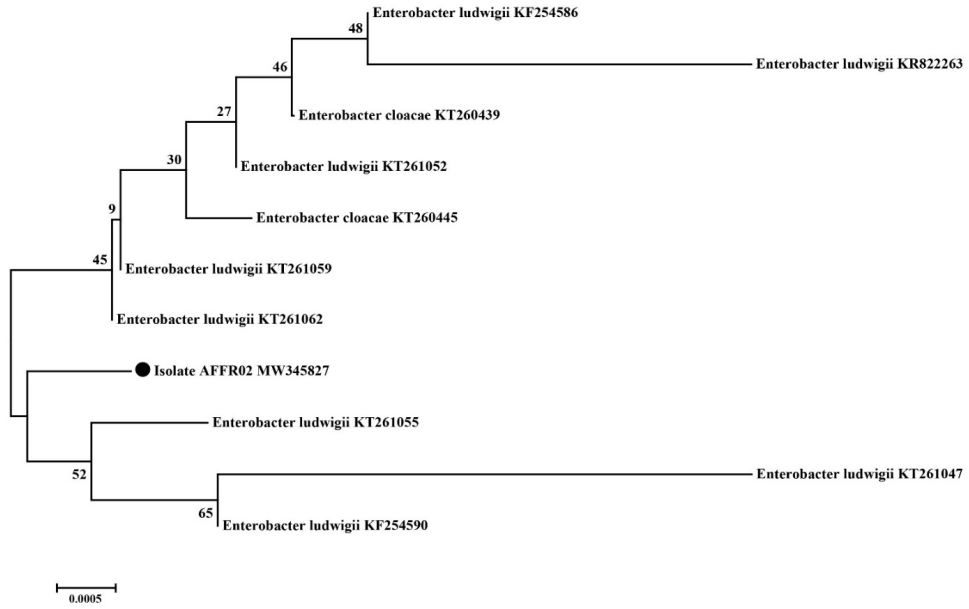

Figure 2. Identification of bacterial isolate AFFR02 using MEGA v.6. Phylogenetic analysis was performed by constructing neighbor-joining trees using a 16S rRNA gene sequence.

Table 1. Effect of isolate AFFR02 and MJ1212 on the growth attributes of alfalfa plants under normal and post-drought stress conditions. Each value in the table is the mean ± standard deviation of three replicates. Mean in columns followed by different letters describes significant difference by using Duncan's multiple range test at $p > 0.05$.

	Shoot Length (cm)	Root Length (cm)	Shoot Weight (g)	Root Weight (g)	Stalk Diameter (mm)
Control					
Control	20.9 ± 1.64a	16.46 ± 0.53b	1.33 ± 0.04b	0.51 ± 0.025b	2.46 ± 0.029b
MJ1212	23.6 ± 0.61a	18.08 ± 0.61b	1.81 ± 0.06b	0.80 ± 0.026b	2.88 ± 0.024b
AFFR02	22.9 ± 1.12a	18.42 ± 0.37a	1.73 ± 0.18a	0.67 ± 0.028a	2.78 ± 0.037a
Post-drought Stress					
Control	15.7 ± 0.33b	10.75 ± 0.39b	0.67 ± 0.032c	0.25 ± 0.03b	1.16 ± 0.024c
MJ1212	17.92 ± 0.86a	13.4 ± 0.25b	1.03 ± 0.018b	0.43 ± 0.01b	2.08 ± 0.029b
AFFR02	17.82 ± 0.82a	14.5 ± 0.21a	1.08 ± 0.016a	0. 37 ± 0.01a	2.52 ± 0.028a

Figure 3. Effect of bacterial isolates AFFR02 and Mj1212 on the growth of alfalfa plants under normal and post-drought stress conditions.

3.6. Effect of Relative Water Content, Electrolytic Leakage, and Endogenous Abscisic Acid Content on Alfalfa Plants under Normal and Post-Drought-Stressed Conditions

Water potential content of drought alfalfa showed a great influence in AFFR02- and Mj1212-inoculated plants. Under normal conditions, no significant differences were observed in bacterial-inoculated and control plants. However, in post-drought-stressed alfalfa plants, a significant decrease in RWC was observed (27.45%). In contrast, an increase in RWC in alfalfa post-drought-stressed plants was observed (24.06%) compared with post-drought-stressed alfalfa plants (Figure 4A). Similarly, EC results showed a higher level in post-drought-stressed plants (68.87%) than in control plants. However, a decrease in EC in AFFR02- and Mj1212-inoculated alfalfa post-drought-stressed plants (19.82%) was observed compared with post-drought-stressed alfalfa plants (Figure 4B). Furthermore, endogenous ABA regulation was observed in post-drought alfalfa plants in AFFR02- and Mj1212-inoculated and non-inoculated plants. ABA results showed no difference under normal conditions; however, a significant increase in ABA content (164.42%) was observed in post-drought alfalfa plants. A significant decrease in ABA content (24.41%)

in bacterial-inoculated plants was observed compared with control post-drought-stressed alfalfa plants (Figure 4C).

Table 2. Effect of isolate AFFR02 and MJ1212 on chlorophyll content (SPAD), chlorophyll A (*Chl a*), chlorophyll B (*Chl b*), and total carotenoid (CART) of alfalfa plants under normal and post-drought stress. Each value in the table is the mean ± standard deviation of three replicates. Mean in columns followed by different letters describes significant difference by using Duncan's multiple range test at $p > 0.05$.

	Chlorophyll (SPAD)	*Chl a*	*Chl b*	CART
Control				
Control	37.5 ± 1.43b	26.1 ± 0.96b	22.04 ± 0.90b	1.1 ± 0.06b
MJ1212	39.1 ± 1.16a	31.1 ± 0.54a	27.06 ± 0.54a	1.18 ± 0.02b
AFFR02	39.8 ± 1.76a	31.7 ± 0.96a	27.7 ± 0.35a	1.3 ± 0.04a
Post-Drought Stress				
Control	30.3 ± 0.61c	14.08 ± 0.88b	12.08 ± 0.51b	0.51 ± 0.07b
MJ1212	35.4 ± 1.71b	17.1 ± 0.63a	15.7 ± 0.57a	0.89 ± 0.08a
AFFR02	37.1 ± 1.72a	17.8 ± 0.37a	15.86 ± 0.06a	0.82 ± 0.06a

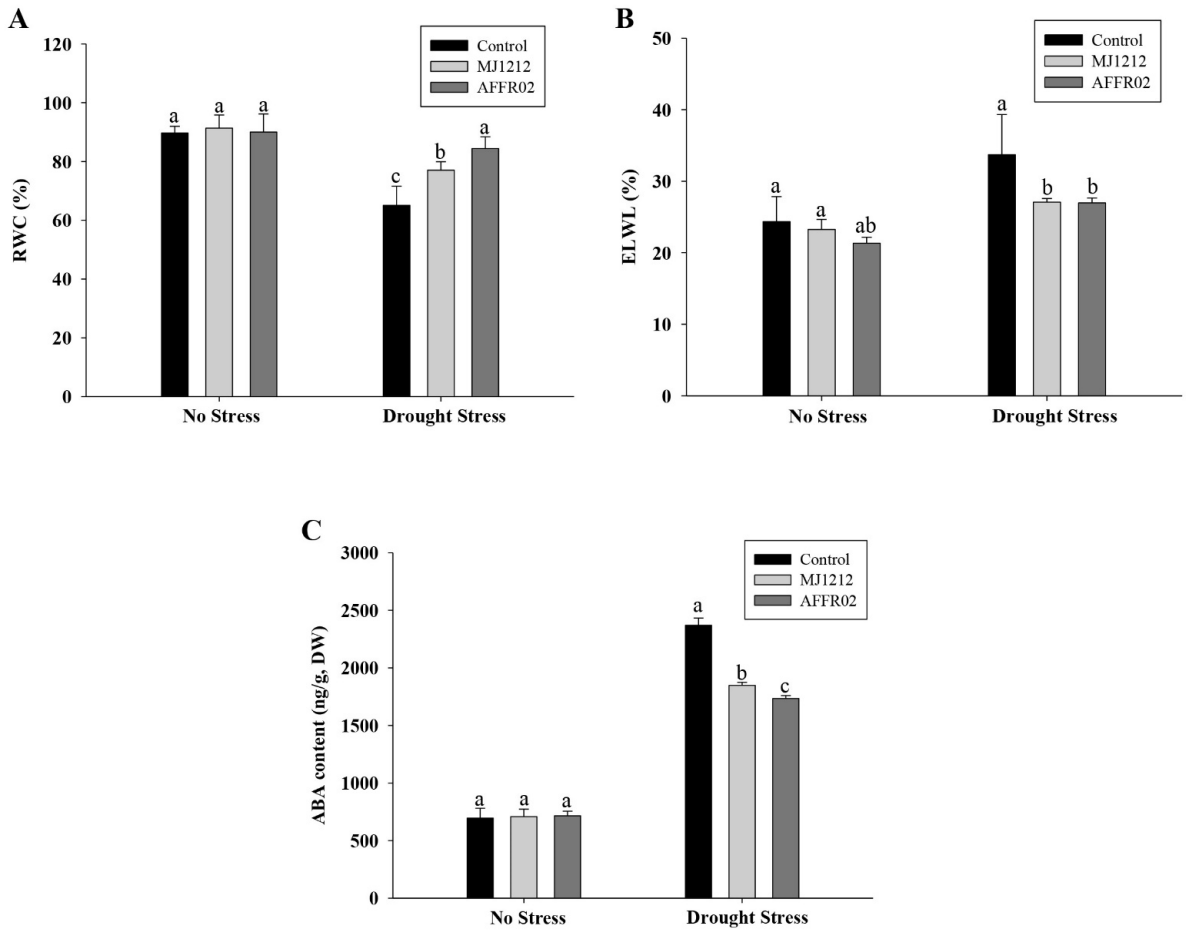

Figure 4. Effect of isolate AFFR02 and MJ1212 on (**A**) relative water content (RWC), (**B**) electrolyte leakage (EC), and (**C**) abscisic acid content (ABA) of alfalfa plants under normal and post-drought stress. The values given are the mean of three replicates and error bars indicate the standard deviation. The bars presented with different letters are significantly different from each other by using Duncan's multiple range test at $p > 0.05$.

3.7. Regulation of Antioxidants in Alfalfa Plants Inoculated with Isolate AFFR02 and Mj1212 under Normal and Post-Drought-Stressed Conditions

Different antioxidants, such as total phenolic content (TPC), DPPH, superoxide dismutase (SOD), and total flavonoid content (TFC) were observed in post-drought-stressed alfalfa, inoculated, and non-inoculated AFFR02 and Mj1212 plants (Figure 5). TPC content was significantly higher in alfalfa post-drought-stressed AFFR02- and Mj1212-inoculated plants (46.08%) than the sole post-drought-stressed and control plants (Figure 5A). DPPH results showed a significant enhancement in DPPH-scavenging activity in post-drought-stressed plants (68.70%). However, in AFFR02- and Mj1212-inoculated plants, significantly higher scavenging DPPH activity was observed (39.66%) compared with post-drought-stressed alfalfa plants (Figure 5B). Similarly, SOD (63.72%) and TFC (77.30%) content showed no significant difference under normal conditions, whereas an increase in TFC (13.68%) and SOD (28.51%) content in post-drought-stressed alfalfa plants was observed (Figure 5C,D).

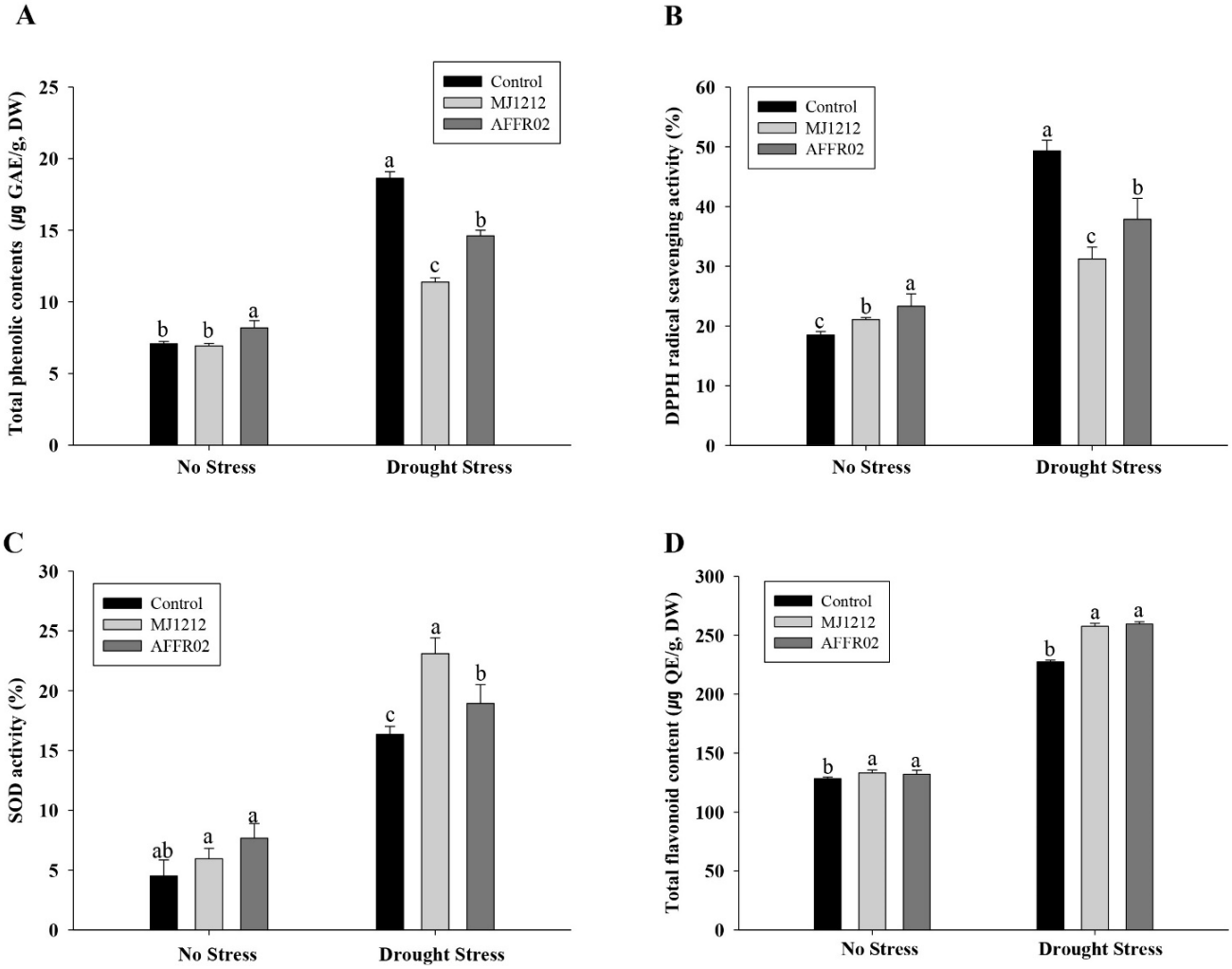

Figure 5. Effect of isolate AFFR02 and MJ1212 on (**A**) total phenolic content (TPC), (**B**) DPPH relative scavenging activity, (**C**) superoxide dismutase (SOD), and (**D**) total flavonoid content (TFC) of alfalfa plants under normal and post-drought stress. The values given are the mean of three replicates and error bars indicate the standard deviation. The bars presented with different letters are significantly different from each other by using Duncan's multiple range test at $p > 0.05$.

3.8. Ion Uptake in Alfalfa Plants Inoculated with Isolate AFFR02 and Mj1212 under Normal and Post-Drought-Stressed Conditions

In this study, ICP analysis of potassium (K), phosphorous (P), calcium (Ca), and magnesium (Mg) was investigated in post-drought alfalfa AFFR02- and Mj1212-inoculated plants (Figure 6). ICP results showed that a decrease in ion uptake; K (31.30%), P (24.85%), Ca (8.75%), and Mg (21.18%) content was observed in post-drought-stressed alfalfa plants (Figure 5). However, alfalfa inoculated with AFFR02 and Mj1212 alleviated drought stress and enhanced nutrient uptake; K (17.98%), P (11.14%), Ca (3.07%), and Mg (6.71%) content were observed in post-drought-stressed inoculated plants (Figure 6).

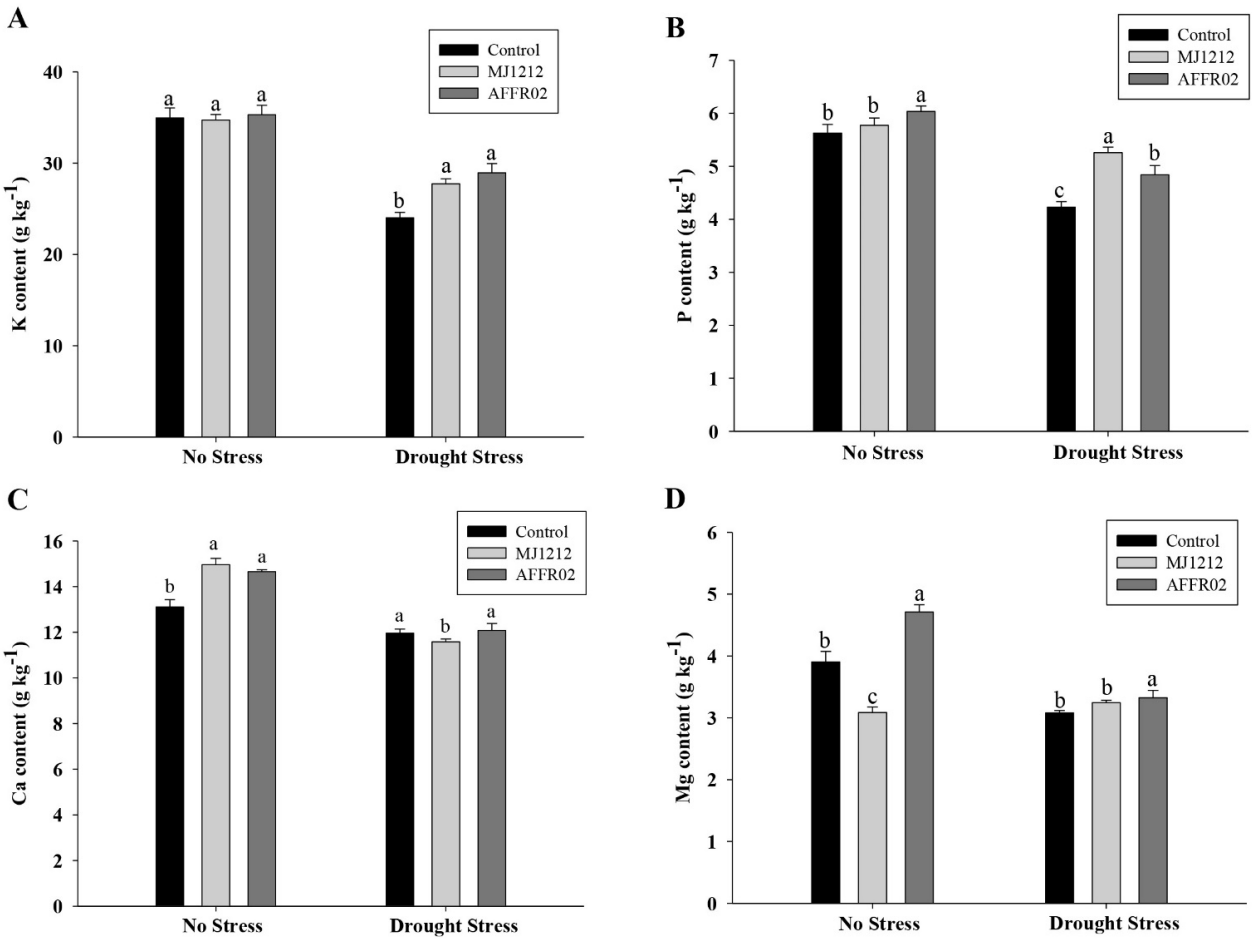

Figure 6. Effect of isolate AFFR02 and MJ1212 on (**A**) potassium content (K), (**B**) phosphorous (P), (**C**) calcium (Ca), and (**D**) magnesium content (Mg) of alfalfa plants under normal and post-drought stress. The values given are the mean of three replicates and error bars indicate the standard deviation. The bars presented with different letters are significantly different from each other by using Duncan's multiple range test at $p > 0.05$.

4. Discussion

As previously reported in many studies, drought stress has been recognized as a critical environmental factor that limits plant development and metabolism [10,39,66]. The results of this study showed that plant biomass and growth attributes were significantly reduced under drought stress. These results are similar to the findings of Kusaka et al. [67], Chimenti et al. [68], and Erice et al. [69], indicating that drought stress causes a significant reduction in the quality of dry biomass of alfalfa, pearl millet, corn, and broccoli. This reduction in plant biomass may allow these cultivars to maintain RWC by decreasing the size of transpiring organs during drought stress [39]. The mutualistic interaction between plant–microbe interactions can enhance drought stress tolerance by enhancing growth performance. In this experiment, isolate AFFR02 and MJ1212 moderated the adverse

effect of drought stress and improved plant growth and biomass content in post-drought alfalfa plants (Table 1; Figure 3). Enhancement in the growth attribute's (root/shoot length) biomass (fresh/DW) has been reported in several plants, such as wheat (*Azospirillum sp*. and *Azospirillum brasilense*), common bean (*Paenibacillus polymyxa* and *Rhizobium tropici*), Arabidopsis (*Pirifomospora indica*), and broccoli (*Variovorax* sp.), inoculated with microbes under drought stress [11–13,32,35]. Similarly, CCs are also sensitive to drought stress, and a reduction in CC under drought stress has been reported in several plants, such as broccoli, cucumber, and tomato. In these experiments, CC decreased under drought stress (Table 2). However, enhancement in CC, *Chl a*, *Chl b*, and total carotenoid content were observed in post-drought alfalfa plants inoculated with isolate AFFR02 and MJ1212 (Table 2). Enhancement in the photosynthetic rate and induced change in different ROS-scavenging enzymes have been reported in plants inoculated with drought-tolerant isolates, such as *Variovorax* sp. (broccoli), *Bacillus cereus*, *Bacillus subtilis*, *Serratia* sp. (cucumber), *Achromobacter piechaudii* (tomato), and *Bacillus* sp. (corn), which helps the plants cope with drought stress [12,70–72].

Drought stress affects nutrient uptake by impairing the translocation of some nutrients. This investigation of ICP analysis showed that drought stress negatively affects K, P, Ca, and Mg content in alfalfa (Figure 6). These results agree with others, indicating that drought stress decreases Mg, P, and other nutrient content in alfalfa [39]. Enhancing nutrient availability through solubilization and chelation of minerals increased nutrient uptake efficiency [33]. P-solubilizing bacteria have attracted attention because of their agroeconomic biotechnological approach and can solubilize more phosphorus under stress [18,38]. Phosphorous (P) is a significant macronutrient required for various metabolic processes, such as photosynthesis and respiration. However, less P is available, and P-solubilizing bacteria can beneficially affect P uptake [18,38]. Results of P uptake showed that an increase in AFFR02- and Mj1212-inoculated alfalfa plants might be due to its P-solubilizing activity (Figure 6B).

Similarly, potassium (K) plays an important role in the biochemical and physiological processes of plant growth, and survival of plants under stress [73]. Under drought stress, the diffusion rate of K in soil toward the roots decreases, depressing plant resistance to drought stress [73]. It was reported by Erdogan et al. [74] that strawberry plants inoculated with three PGPR (*Paenibacillus polymyxa, Rhodococcus erythropois, and Pesudomonas fluorescens*) mitigate drought stress and enhance nutrient uptake, including K. Furthermore, an increase in Ca and Mg content was observed in AFFR02- and Mj1212-inoculated alfalfa plants (Figure 6C,D). It was reported that Ca and Mg playing a vital role in membrane protection, modulation of ions in the chloroplast, and postponing oxidative damage caused by drought stress [75,76]. In plants, 6%–25% of the total chlorophyll plays a vital role in photosynthesis and activation of many enzymes [77]. The decrease in chlorophyll content in post-drought-stressed alfalfa plants might be due to a decrease in Mg content (Figure 6D).

Various environmental stresses, including drought stress, lead to decreased water availability and osmotic stress that promote the synthesis of endogenous ABA phytohormonal regulation and different antioxidant systems [23,78,79]. It was also observed that alfalfa plants inoculated with AFFR02 and MJ1212 induced drought stress tolerance by reducing water loss (Figure 4A). This investigation showed that bacterial-inoculated plants had a higher RWC and lower EL than control post-drought-stressed alfalfa plants (Figure 4). A decrease in RWC and an increase in EL indicate a loss of turgor under drought stress, which results in less availability of water to plants [80,81]. In this investigation, lower EL and higher RWC was observed in bacterial-inoculated alfalfa post-drought-stressed plants. Our finding is also supported by other studies that reported that microbial-inoculated plants decrease stress, help fetch higher water content for plants, and decrease stomatal aperture [81–84]. Similarly, the decrease in the EL of post-drought alfalfa-inoculated plants mainly attribute to the integrity and stability of cellular tissue compared with drought-stressed plants. This increase in RWC and decrease in EL was also correlated with stomatal openings regulated by a complex hormonal network, such

as ABA [35,84]. Since the discovery of ABA, several efforts have been devoted to understanding the synthesis of ABA under stress conditions [85–87]. Endogenous ABA level is significantly increased during drought stress by stimulating stomatal closure and adaptive physiological responses [12].

To further elucidate the role of microbes under post-drought stress in alfalfa plants, we investigate different antioxidant effects. Under stress conditions, an increase in damaging levels of ROS is a common factor [88,89]; however, to cope with ROS damage, plants induce enzymatic and nonenzymatic components [90]. SOD acts as a main enzymatic scavenger that scavenges superoxide (O_2^-) and hydrogen peroxide (H_2O_2) to H_2O and O_2 [3,4,91]. This study showed that bacterial-inoculated alfalfa plants have higher SOD activities, suggesting inoculated microbe-induced drought tolerance in alfalfa plants (Figure 5C). Furthermore, higher phenolic content, DPPH radicals, and total flavonoids were observed in bacterial-inoculated alfalfa post-drought-stressed plants compared with post-drought-stressed (untreated) plants (Figure 5). This higher accumulation of total phenolic content is essential for maintaining the osmotic potential of plants, increasing physiological activities during drought stress [92]. Our results agree with the report of [92–94], who noted that PGPR induction increases phenolic content in wheat and pulse crops. Similarly, Singh et al. [95] and Asghari et al. [96] reported higher antioxidants, such as DPPH in inoculated rice and pennyroyal plants. Alternatively, other antioxidants' total flavonoids play a vital role by eliminating singlet oxygen and alleviating stress [93]. In bacterial-inoculated post-drought-stressed alfalfa plants, higher TFC content was observed (Figure 5D). These results agree with the report of [93,97], who reported an increase in TFC content in *Cariniana estrellensis* and *Cymbopogon citratus* bacterial-inoculated drought-stressed plants.

5. Conclusions

This investigation showed that the local strain is closely related and identified as *Enterobacter ludwigii*. It was also observed that drought stress reduced the growth attributes of alfalfa. However, alfalfa plants inoculated with isolate AFFR02 and Mj1212 improved plant capacity to mitigate drought stress. Therefore, based on these results, further studies using genomic approaches to identify field trails of drought-stressed affected areas for their practical applications are needed.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11060485/s1, Figure S1: Growth of bacterial isolates in LB media supplemented with different concentration of polyethylene glycol (PEG) stress (0%, 5%, 10% and 15%) for 4 days.

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