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ENDOPHYTIC BACTERIAL EFFECTS ON SEED GERMINATION AND MOBILIZATION OF RESERVES IN *AMMODENDRON BIOFOLIUM*

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Abstract

The main aim of this study was to analyze the mobilization of storage reserves during seed germination of *Ammodendron bifolium* by host plant-endophytic bacteria interaction and to determine the contribution of endophytic bacteria in plant establishment. The seeds were inoculated with three different endophytic bacteria from *A. bifolium*, *Staphylococcus* sp. AY3, *Kocuria* sp. AY9 and *Bacillus* sp. AG18, and they were germinated in the dark. Fresh weight changes and early seedling growth were assessed, and the content of storage compounds was quantified using biochemical assays in all germinated and non-germinated seeds. To understand the mechanism promoting seed germination, the activities of extracellular enzymes of bacterial isolates were also analyzed by the plate assay method. The results showed that treatment with endophytic bacteria accelerated seed germination; promoted further water absorption and radicle growth; and also promoted degradation of sucrose, protein and lipids during the germination process. At the same time, our results also showed that strain AG18 was able to produce protease and amylase, strain AY9 had only amylase activity, and strain AY3 had no extracellular enzyme activity. In summary, our current study showed that (i) endophytic bacteria improved seed germination and post-germination seedling growth of *A. bifolium*; (ii) inoculation with endophytic bacteria could promote storage reserve mobilization during or following germination; (iii) the degradation of protein, lipids and sucrose could provide essential energy for post-germination growth; and (iv) three bacterial isolates might have different action mechanisms on seed germination.

Key words: Endophytic bacteria; *Ammodendron bifolium*; Seed germination; Reserve mobilization; Radicle growth

Introduction

The study of plant-endophytic bacteria interaction is quite significant for understanding the ecological and environmental impacts of this symbiotic association. Understanding the process better could provide researchers with new insights into biological control (Araújo *et al*., 2002; Senthilkumar *et al*., 2009), environmental remediation (Barac *et al*., 2004; Chen *et al*., 2010; Egamberdieva *et al*., 2015) and plant stress resistance (Ding *et al*., 2011; Saravanakumar *et al*., 2011; Yaish *et al*., 2015). Plant colonization by endophytic bacteria is also believed to help plants adapt better to the environment and then accelerate seedling emergence and plant establishment, especially in arid and semi-arid systems (Puente & Bashan 1993; Lima *et al*., 2015; Soussi *et al*., 2015). There is an increasing body of evidence that suggests plant survival in arid ecosystems depends on specific bacterial communities that are capable of facilitating plant adaptation, improving plant functionalities and protecting plants from environmental stresses (Marasco *et al*., 2012; Shelef *et al*., 2013; Mapelli *et al*., 2013; Ferjani *et al*., 2015). For example, interactions with endophytic bacteria help desert plants to establish themselves and grow on rocks without soil (Puente *et al*., 2004; Puente *et al*., 2009).

Due to these beneficial effects, endophytic bacteria have been isolated from a variety of plant species grown in harsh environmental conditions (Sgroy *et al*., 2009; Lopez *et al*., 2011; Xia *et al*., 2013), and these bacteria could be potential tools used for rehabilitation of degraded soils and vegetation. The bacterial communities in these plants are predominantly *Proteobacteria* and *Firmicutes* with a low abundance of *Actinobacteria*. The most studied endophytic plant growth-promoting bacteria were *Bacillus* and *Pseudomonas* strains, which were discovered through inoculation experiments. However, much less is known about the impacts of endophytic bacteria isolated from *Ammodendron bifolium* on plant growth and other plant performance parameters.

A. bifolium (Pall.) Yakovl. is a perennial deciduous shrub distributed in the Takeermohuer Desert located in Northwestern Xinjiang and it is the only species of the *Ammodendron* genus in China (Xinjiang Flora Editorial Committee, 2011). Although *A. bifolium* is an excellent sand-fixing plant, it is an endangered plant species due to anthropogenic activities. Therefore, measures have been taken to protect it. Seed germination is a critical stage for plant survival and timely seedling establishment especially in stressful environments (Puente & Bashan 1993; Gutterman, 2000; Wang *et al*., 2017). Degradation and utilization of storage compounds during or following germination provide essential energy for early seedling growth prior to the development of autotrophy (Ichie *et al*., 2001; Pritchard *et al*., 2002; Eckstein *et al*., 2016). Given the crucial performance of plant-associated microbiomes in soil restoration, seed germination and plant establishment in arid lands, endophytic bacteria from *A. bifolium* may also have potential value for studying their adaptation mechanisms in terms of plant-microbe interaction. The three endophytic bacteria in this study were isolated from healthy tissues of wild *A. bifolium* plants, and they showed a promoting effect on germination and early seedling growth. Subsequently, we analyzed the physiological and biochemical processes underlying germination and early seedling growth in *A.*

bifolium by host plant-endophytic bacteria interaction and explored their adaptation strategies in arid and semi-arid desert areas as well as their potential for the restoration of degraded ecosystems.

Materials and Methods

Bacterial strains and inocula preparation: Three endophytic bacteria used in this study (AY3, AY9 and AG18) were isolated in our laboratory from different plant tissues of *A. bifolium* and stored at –80 °C in 20% (v/v) glycerol. They were identified as belonging to *Staphylococcus* sp. AY3 (accession number KR045836), *Kocuria* sp. AY9 (accession number KR045840), and *Bacillus* sp. AG18 (accession number KR045821) by 16S rRNA gene sequence analysis.

To prepare inocula, 1 ml aliquots of a bacterial culture grown overnight (approximately OD600=1.0) were inoculated in 20 ml nutrient broth (3 g beef extract, 10 g proteose peptone, 5 g NaCl in one liter of dH_2O, pH 7.2) at 30 °C for 24 h (early stationary phase) with 150 rpm shaking. Subsequently, the accumulated biomass was harvested by centrifugation at 10000 *g* for 10 min at 4 °C, and the cell pellets were washed twice with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.0) and finally suspended in PBS (approximately 10^9 Colony Forming Units (CFU) ml^{-1}).

Seed bacterization and germination assay: *A. bifolium* seeds were surface sterilized by immersion in 5% sodium hypochlorite for 15 min, followed by immersion in 75 % ethanol for 12 min. The seeds were then rinsed six times with sterilized distilled water. Detection of surface sterility was performed as described previously (Kuklinsky-sobral *et al*., 2004). Then, the surface-sterilized seeds were cut on seed coats with a sterile razor blade and soaked in PBS alone or with bacterial cells for 4 h. The seeds treated only with PBS were used as controls. After these steps, four replicates of 30 seedsfor each treatment were placed in petri dishes containing filter paper moistened with sterilized water and kept at 20 °C in the dark. Germination was scored as a radicle protrusion through the seed coat, and the germination percentage, fresh weight (FW) and dry weight (DW) were determined at 5 day intervals. The relative water content (RWC) was calculated as (FW-DW)/DW. At the same time, the radicle length was also measured and averaged to provide an index of early post-germination growth.

Sample collection and preparation: After seed bacterization, the samples containing all germinated and non-germinated seeds were collected at 5-day intervals for up to 15 days and homogenized in liquid N_2 into powders that were used for the estimation of carbohydrates, soluble protein and free amino acids. Other samples from the same treatments were dried to a constant weight in a hot air oven at 60 °C and then ground into powder, used for the extraction of lipids.

Determination of carbohydrates: Soluble carbohydrates were extracted and quantified using the colorimetric anthrone assay with minor modifications of the method of Hunt *et al*. (2005). Briefly, ground samples (100 mg) were extracted with 80% ethanol at 65 °C for 30 min and centrifuged (13000 *g*, 20 min). The supernatants were combined to provide a sample while the ethanol-insoluble residues were extracted with boiling water for 1 h. Subsequently, extracts from the supernatants of ethanol or water fractions were assayed for soluble sugar content using an anthrone reagent (37.5% ethanol (v/v), 62.5% concentrated sulfuric acid (v/v), and 0.1% anthrone (w/v)). Glucose, fructose and sucrose concentrations were determined in ethanol fractions using enzymatic methods (Rasmussen *et al*., 2008).Starch was extracted from the water-extracted residuesusing an acid hydrolysis method(McCready *et al*., 1950) and was measured usinganthrone colorimetry.

Measurement of protein and free amino acids: Protein content in powdered samples was determined using the Bradford method (1976). Free amino acids were quantified by an automatic amino acid analyzer (Sykam, S-433D) based on the reaction of various amino acids with ninhydrin solution, which were extracted as follows. Powdered samples (0.1 g) were weighed in 25 ml volumetric flasks, and 0.01 M HCl was added to the scale line. After 30 min of extraction, 2 ml filtrates were mixed with 2 ml of 8% sulfosalicylic acid and incubated at room temperature for 15 min. The samples were centrifuged, and the supernatant was used for determination of free amino acids. The chromatographic column used was an LCA K06/Na column with a sodium citrate buffer (A=0.12 N, pH 3.45; B=0.2 N, pH 10.85) mobile phase. The flow rates of the elution and derivative pumps were 0.45 ml min^{-1} and 0.25 ml min^{-1}, respectively. The detected wavelengths were 570 nm and 440 nm, and the volume of the injected solution was 25 µl.

Extraction of lipids: The lipids content in the samples was measured using the Soxhlet extraction method with minor modifications (Ramluckan *et al*., 2014). In other words, pre-dried, powdered samples (1 g) were weighed accurately and transferred to the extraction chamber in the Soxhlet apparatus. An aliquot of 100 ml of petroleum ether was added to the solvent cup, and the samples were refluxed with petroleum ether in a water bath. After extraction, the solvent was evaporated and the mass of the lipid extract remaining was measured.

Detection of extracellular enzyme activity of endophytic bacteria: Protease, amylase, and lipase activity were assayed with milk agar plates (containing 1% skim milk, v/v), starch agar plates (containing 0.2% starch, m/v) and peptone agar plates (containing 1% Tween 80, v/v), respectively, and the plates were incubated at 30 °C for 2 days (Saran *et al*., 2007; Akpan *et al*., 1999; Sierra, 1957). The formation of a clear halo around the bacterial colonies indicated a positive reaction.

Statistical analysis: Each treatment was repeated at least three times. The data are presented as the mean values ±SE. Analysis of variance (ANOVA) followed by the Least Significance Difference test was carried out to compare treatments using SPSS 13.0 software. Differences were considered to be significant at $p<0.05$.

Results and Discussion

Seed germination and post-germination growth: Germination of *A. bifolium* seeds was tested after inoculation with three different endophytic isolates. From Fig. 1a, it can be seen that seeds began to germinate after 5 days of imbibition, and then germination increased obviously 10 days after imbibition. Isolate AY3 significantly promoted seed germination at 10 days (43%) and 15 days (62%), followed by AY9 andAG18 (32% and 37% at 10 days and 50% and 53% at 15 days, respectively). In the control, the germination percentage was found to be 3% (at 10 days) and 13% (at 15 days). As shown in Fig. 1b and 1c, inoculation with the three isolates made seeds further absorbed more water and then promoted the growth of radicle length (Fig. 1d), which was increased by 380%, 190% and 270% for AY3, AY9 and AG18, respectively, after 15 days of treatment. It is well-known that plant growth-promoting bacteria are widely studied in agricultural crops but are rarely reported for wild plants, especially desert plants. Recently, a number of phosphate-solubilizing and diazotrophic bacteria from semi-arid and arid environments were selected for the study of plant growth promotion and were expected to be potential tools used for the reforestation and rehabilitation of degraded soil (Yu *et al*., 2011; Delgado *et al*., 2014; De Jesus Santos *et al*., 2014; Lima *et al*., 2015). Therefore, the three endophytic bacteria used in this study may also have potential roles for species conservation and their mechanisms for promoting plant growth may be diverse and deserve further study.

Carbohydrate content variation in bacterized seeds: The starch content was hardly changed 5 days after imbibition and decreased rapidly at 10 days in inoculated and non-inoculated seeds. Treatment with endophytic isolates significantly decreased starch content at 10 days, then the decrease became slower (Fig. 2a). While soluble sugar content began to decline rapidly at 5 days, especially in bacteria treated seeds, it changed as germination progressed (Fig. 2b). This result suggested that soluble sugars were degraded prior to starch during germination.

The values for sucrose, glucose and fructose content in all treatments are shown in Fig. 3. The most notable trend is that changes in sucrose content contrasted the changes for glucose and fructose. Sucrose is a major soluble carbohydrate and is stored in mature seeds, but glucose and fructose gradually disappear as the seed reaches maturity (Focks & Benning, 1998). Furthermore, our previous results showed that glucose and fructose were not detected in mature seeds (Zhu *et al*., 2016). Therefore, we made it clear that glucose and fructose were produced by sucrose degradation in this study.

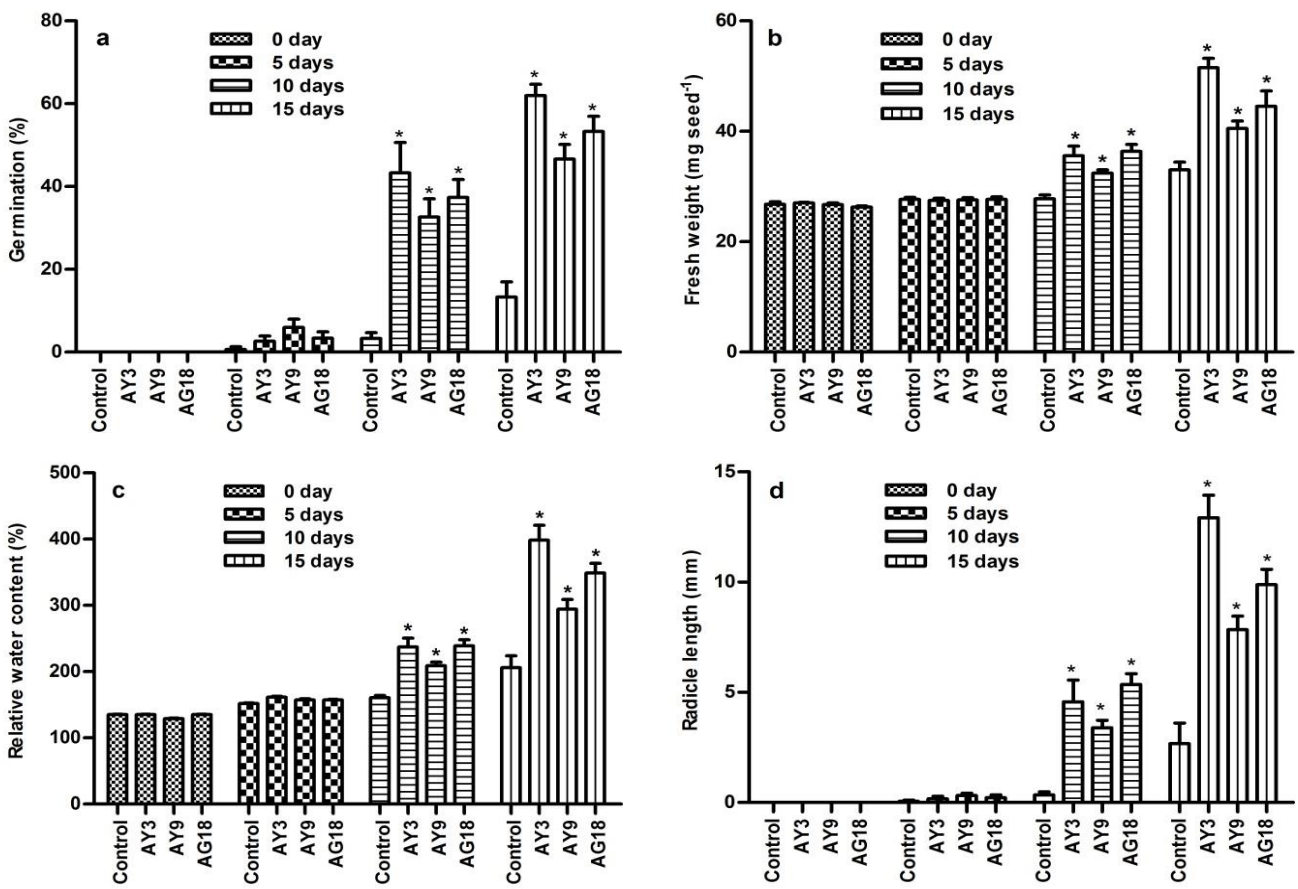

Fig. 1. Effects of endophytic bacterial inoculation on seed germination (a), fresh weight (b), relative water content (c) and radicle length (d) of *A. bifolium*. The seeds were soaked in the bacterial suspension for 4 h, then sown on filter paper and kept at 20°C in the dark for germination. Germination rate, fresh weight, water content and radicle length were recorded at different time intervals. Data are displayed as the means ±SE (n=4), and significant differences between the control and the endophytic bacterial treatments are indicated by an*asterisks* ($p<0.05$).

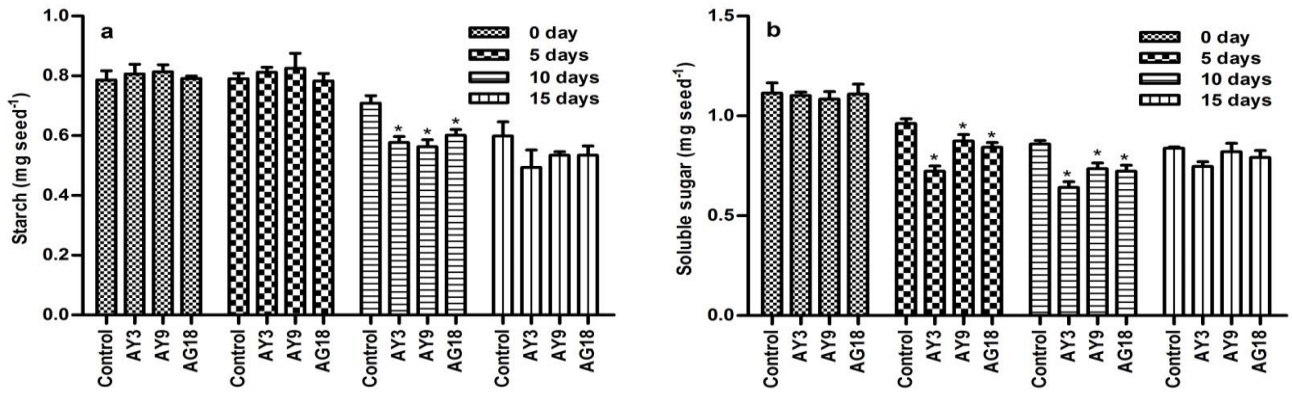

Fig. 2. Effects of endophytic bacterial treatment on starch (a) and soluble sugar (b). The bacterized seeds were sown on filter paper and kept at 20°C in the dark for germination. Seed samples were collected at various times and used for determination of starch and soluble sugar according to anthrone colorimetry. Data are displayed as the means ±SE (n=4), and significant differences between the control and the endophytic bacterial treatments are indicated by an*asterisks* ($p<0.05$).

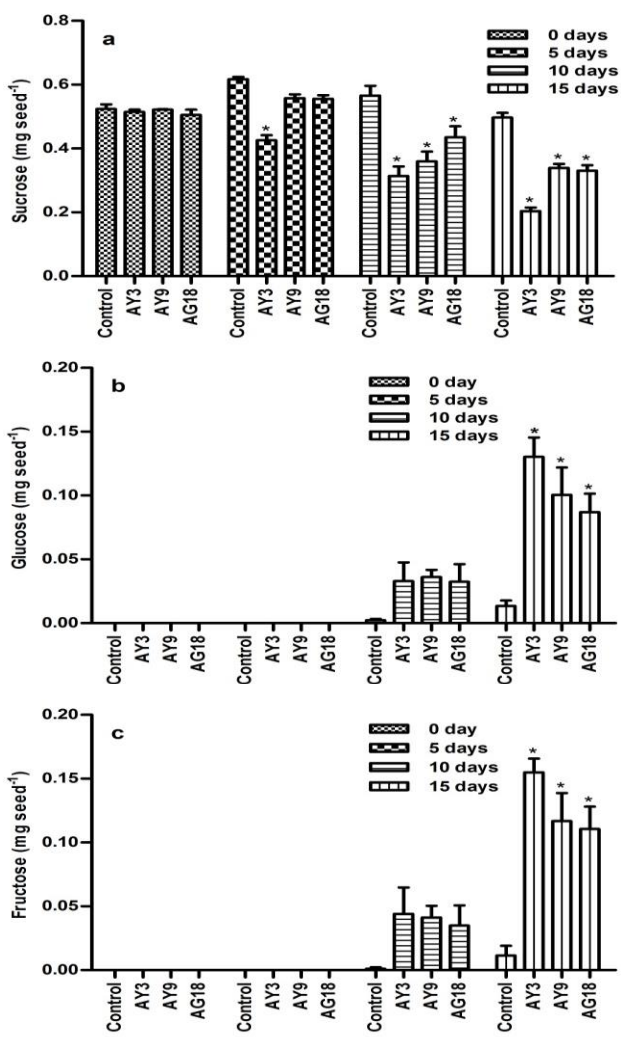

Fig. 3. Effects of endophytic bacterial treatment on sucrose (a), glucose (b), and fructose (c). The seeds were sown on filter paper after bacterization and kept at 20 °C in the dark. Seed samples were collected at different times to measure sucrose, glucose and fructose by enzymatic methods. Data are displayed as the means ±SE (n=4), and significant differences between the control and the endophytic bacterial treatments are indicated by an*asterisks* ($p<0.05$).

Protein and free amino acids in bacterized seeds: With the progress of germination, the amounts of protein in all treatments decreased gradually (Fig. 4a), which was accompanied by an increase in the content of the free amino acids (Fig. 4b). The protein content in seeds treated with three endophytic isolates were significantly reduced by 34%, 29% and 48%, respectively, while protein content in the control sample was only reduced by 13% at 15 days after imbibition. This result suggested that degradation of proteins started 5 days after imbibition (specifically at the beginning of germination). Pritchard *et al*. (2002) reported that germination and storage reserve mobilization are two independent processes in Arabidopsis. However, our results showed that the two events may not be regulated independently in *A. Bifolium*, which is similar to previous reports (Li *et al*., 2017).

Lipid content variation in bacterized seeds: The changes in lipid content are shown in Fig. 5. Lipid content in all treatments was almost unchanged within 5 days after imbibition, and its degradation was initiated afterward and continued through the whole germination process. Treatment with three endophytic isolates significantly promoted declines of 8%, 18%, and 14% at 10 days and 31%, 24%, and 32% at 15 days. Lipid content in the control sample was reduced by 3% at 10 days and 9% at 15 days. During early post-germination growth in oilseeds, lipids are mobilized and utilized to support seedling growth (Doman *et al*., 1982). In *A. bifolium* seeds, the major storage reserve is protein, followed by lipids (Zhu *et al*., 2016). By definition, germination begins with imbibition and ends with radicle emergence through the seed coat (Nykiforuk & Johnson-Flanagan, 1999). Therefore, we ascertained that lipids were mobilized after seed germination to support post-germination growth.

Extracellular enzyme production of endophytic bacteria: Previous studies showed that production of extracellular hydrolytic enzymes by endophytes may indirectly promote plant growth through the presence of cell wall hydrolytic enzymes that could assist in the plant

invasion process (Adriano-Anaya *et al*., 2006; Mostajeran *et al*., 2007). However, some researchers believed that production of hydrolytic enzymes might improve seedling growth (Deivanai *et al*., 2014). In this study, isolate AG18 was able to produce protease and amylase; AY9 only was able to produce amylase, while AY3 lacked any enzyme activity (Table 1). There is likely some other mechanism promoting plant growth. In any case, additional studies are needed to clarify the effects of this mechanism on plant growth.

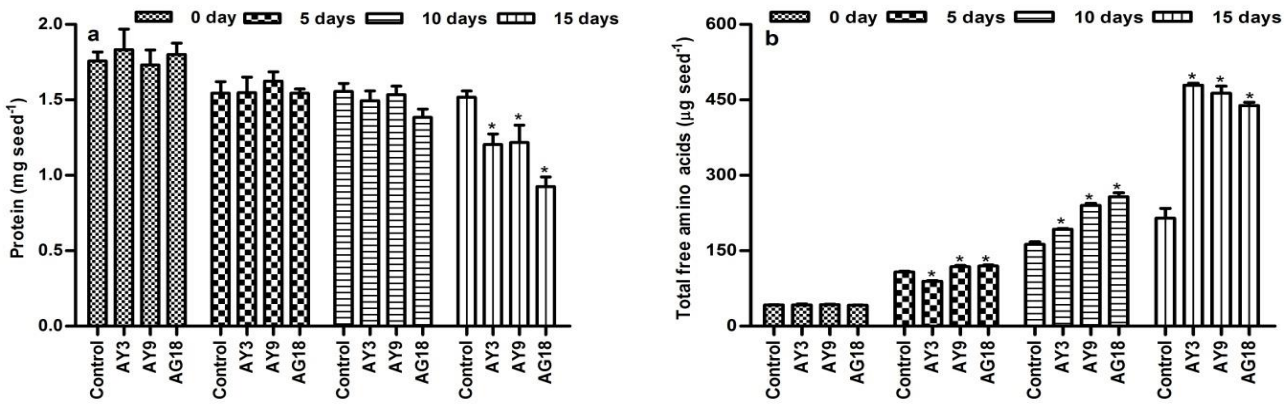

Fig. 4. Effects ofendophytic bacterial treatment on protein (a) and total free amino acids (b). The bacterized seeds were sown on filter paper and kept at 20 °C in the dark for germination. Seed samples were collected at different times for the protein assay utilizing the principle of protein-dye binding and for quantification of free amino acids by an automatic amino acid analyzer. Data are displayed as the means ±SE (n=4), and significant differences between the control and the endophytic bacterial treatments are indicated by an*asterisks* ($p<0.05$).

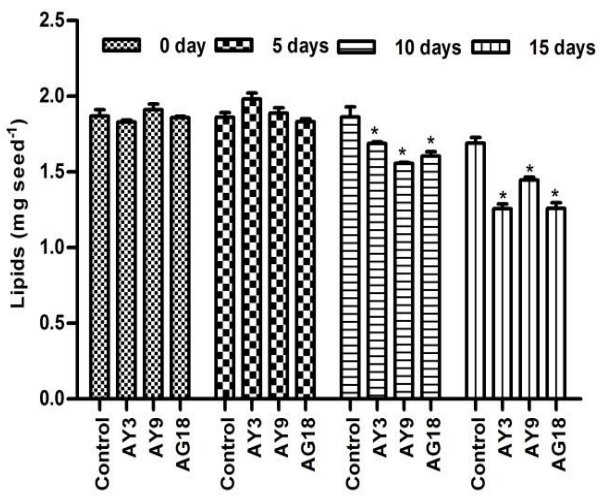

Fig. 5. Effects of endophytic bacterial treatment on lipid content. The bacterized seeds were sown on filter paper and kept at 20℃ in the dark for germination. Seed samples were collected at various times and used for extraction of lipids according to the Soxhlet extraction method. Data are displayed as the means ±SE (n= 3), and significant differences between the control and the endophytic bacterial treatments are indicated by an*asterisks* ($p<0.05$).

Table 1. Extracellular enzyme activity of endophytic bacterial isolates.

Isolate code	Amylase	Protease	Lipase
AY3	−	−	−
AY9	+	−	−
AG18	+	+	−

Note: "+" indicatesa positive result, and "–" indicates a negative result.

Conclusion

Three endophytic bacteria from *A. bifolium* were shown to have a promotion effect on host plant seed germination and post-germination radicle growth. At the same time, treatment with three endophytic isolates also promoted mobilization of storage reserves for providing essential energy to fuel growth. Therefore, endophytic bacteria from *A. bifolium* may have potential value for reforestation in arid and semi-arid ecosystems.

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