RESEARCH ARTICLE

Priming Apoplast-Associated *Bacillus Amyloliquefaciens* LAS10 Enhances Growth and Yield Attributes of Little Millet Under Drought Stress

Monisha Rajkumar, Shobana Narayanasamy and Sivakumar Uthandi *

Biocatalysts Laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore -641003.

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ABSTRACT

Little millet plant, in general, withstands various climatic changes and environmental stresses. The endophytic bacterial communities associated with these plants help the plant to develop enduring harsh conditions. Priming seeds with endophytic bacteria helps the host plant grow under adverse environments. The present study aimed to prime the seeds with apoplastic endophytic bacteria, *Bacillus amyloliquefaciens* LAS10 from little millet (var. ATL1) and to evaluate the efficiency to tolerate an osmotic potential of -10 bars (-1MPa). The seeds were primed with *B. amyloliquefaciens* LAS10 to investigate the plant growth attributes *in vitro* and *in vivo*. Induced drought stress was imposed using different concentrations of PEG6000 *in vitro* and by withholding the irrigation for ten days during panicle initiation *in vivo.* Biometric observations were recorded. The results showed significant differences both in vegetative growth parameters as well as yield attributes compared with the control under moisture stress. During the panicle initiation stage (20 DAS), the shoot length (32.26, and 28.36 cm), root length (19.1, and 17.7 cm), total plant biomass (4.61, and 2.47 g), number of productive tillers plant^{-1}(5, and 3), the total number of panicles plant^{-1} (15.63, and 13.69), panicle length (9.9 cm, and 9.1 cm) and 1000 grain weight (12.91, and 11.92 g), grain yield plant^{-1} (16.92, and 12.24g), straw yield (21.65, and 19.31) registered more in *B. amyloliquefaciens* LAS10 primed plants compared to uninoculated control under both irrigated and drought stressed conditions, respectively. Thus, the present study confirmed that the inoculation of little millet with the apoplastic bacterium *B. amyloliquefaciens* LAS10 could promote plant growth and productivity under moisture stress. Further, the apoplastic bacterium *B. amyloliquefaciens* LAS10 can be recommended as a bio-inoculant for alleviating drought stress in little millet and enhancing crop fitness and sustainable little millet production.

Keywords: *Apoplast; Bacillus amyloliquefaciens; Little Millet; Drought; Yield.*

INTRODUCTION

Global agriculture has encountered challenges in achieving increased demands for food production due to the growing populations under limited water resources (Kang *et al*., 2009). The impact of the growing influence of human activities and industrial evolution ends up with global warming and rapid climate change (Anderson *et al*., 2020). This considerable climatic shift intensively results more often in drought, altered precipitation, and other abiotic stresses (Borell *et al*., 2020). Among them, drought is the most devasting stress that severely affects plant growth and productivity. Consequently, there is a new interest in finding solutions to various water-related issues, including drought and minimizing the impact on global food security (Alexanratos and Bruinsma, 2012). Sustainable agriculture is one of the better eco-friendly approaches to overcome the current water scarcity impacts (Arunthavasu *et al*., 2019). This can be accomplished by putting into practice several alternative and eco-friendly techniques, such as the implementation of plant growth-promoting bacteria (PGPB) and other beneficial endophytes to improve plant growth and fitness (Antoun and Prevost, 2005). Furthermore, bio-priming of beneficial bacteria in seeds promotes early germination, enhances plant growth, and protects the host plants from adverse environmental stresses and seed-borne diseases (Moeinzadeh *et al*., 2010; Reddy, 2013).

Apoplast represents the free diffusional space outside the plasma membrane, including plant cell walls, and enables the transport and interchange of solutes between cells and tissues (Krause *et al*., 2013). The plant apoplast is the main reservoir of bacterial endophytes (McCully, 2001) regarded as a site of interaction for pathogens and beneficial endophytes. Furthermore, the metabolites and proteins present in this region are known to play key

*Corresponding author's e-mail: usiva@tnau.ac.in

roles in plant growth and regulate the stress response against adverse environmental conditions (Huckelhoven, 2007). Hence, the isolation of beneficial bacterial strains adapted to this environment could be useful in developing natural biotechnological solutions for agriculture.

Indian little millet (*Panicum sumatrense*) belonging to the family *Poaceae* is suitable for arid and semiarid regions (Dubey *et al*., 2018). Little millet plants have a long history of cultivation under adverse climatic conditions (Dey *et al*., 2022). Besides, it is mostly cultivated under natural rain-fed irrigation, even during relatively dry seasons. It also grows well in adverse environments such as salty and dry soils as well at high temperatures. The high nutritional value and storage feasibility of little millet has led to considering this kernel as an important staple food by ancient and modern people (Sivakumar *et al*., 2006). To advance crop productivity in such drought-prone areas, it is necessary to understand the mechanism of plant responses with the ultimate goal of improving crop performance. Besides, it is necessary to improve the quality of external inputs by utilizing the best combination of beneficial microorganisms for seed priming to enhance germination, vigor and yield, and crop fitness to overcome abiotic stresses. In our earlier studies, potential plant growth promoting drought tolerant bacteria isolated from the apoplast of little millet (var. ATL1) was identified as *Bacillus amyloliquefaceins* LAS10 (Unpublished data). It can grow under the low osmotic potential of -10 bars (1 Mpa) and has higher PGP (Plant Growth Promoting) potential with ACCD (124.98 n moles α-ketobutyrate mg protein^{-1}h^{-1}), IAA (17.79 µg mL^{-1}) and siderophore (52.78% units) plays a significant role in plant growth and drought stress resilience. Furthermore, seed inoculation of *B. amyloliquefaciens* LAS10 improves seed germination and plant growth observed in little millets grown under *in vitro* drought-stressed conditions. With this background, the present study aimed to evaluate the potential drought-tolerant bacterium *B. amyloliquefaciens* LAS10 for plant growth, drought tolerance, and enhanced yield attributes under greenhouse conditions. The results of the present study would aid in the development of a newer bio-inoculum for the sustainable production of little millet plants under drought stress.

MATERIAL AND METHODS

Bacterial strain and culture conditions

Bacillus amyloliquefaceins LAS10 plant growth-promoting and drought-tolerant bacteria, isolated from the apoplast of little millet cultivars (var. ATL1) were grown under induced stress condition was used in this study obtained from Biocatalysts Laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore (Unpublished Data). The LAS10 strain was grown in nutrient broth, and incubated at 28 ± 2 ºC used for further experiments.

Seed priming and culture growth condition

The plant growth promotion ability of LAS10 was evaluated *in vitro* and carried over to a pot culture experiment. Little millet seeds were surface disinfected with 70% ethanol for 2 min and in 2% sodium hypochlorite for 2 min, followed by washing in sterile tap water (Miche *et al*., 2001). For this experiment, pure culture of *B. amyloliquefaciens* LAS10 was grown in nutrient broth for 24 h at 28 °C. The cells were harvested by centrifugation at 6000 rpm for 5 mins and diluted to a final concentration of 10^8 colony forming units (CFU) ml^{-1} in sterile 0.1 M Phosphate buffer (pH 7.2) (Cattelan *et al*., 1999). The surface-sterilized seeds were primed with bacterial suspension and buffer, respectively for 1 h, air-dried, and placed on sterile Whatman filter paper No.1 (Soumiya *et al*., 2021). The primed seeds were placed on a sterile filter paper in Petri dishes (9 cm) impregnated with sterile distilled water (No stress) and 10%, 20%, and 30% percentage of polyethylene glycol (PEG 6000) for inducing artificial drought. These concentrations correspond to osmotic potentials of -0.4MPa, -0.45MPa, and -1MPa, respectively). Three replicates were maintained with 15 seeds for each treatment. Seeds placed in sterile water maintained as mock (Narayanasamy *et al*., 2020). The seeds were incubated for five days in a plant growth chamber with a relative humidity of 60% at 28 °C for 12 h of light (200 moles m^{-2}s^{-1}). Morphological characteristics viz., root length, shoot length, and germination percentage of little millet due to inoculation were analyzed.

Pot culture experiment:

Little millet seeds (var. ATL1) obtained from the Department of Millets, Tamil Nadu Agricultural University, Coimbatore were used for this study. Seeds were grown and irrigated regularly with water under a 12 h photoperiod at temperatures ranging from 25 to 29 °C and a relative humidity of 70–75% in the glasshouse, Department of Genetics and Breeding, Tamil Nadu Agricultural University, Coimbatore. The pot culture experiment with four treatments in four replications was maintained under both non-stress and induced drought stress conditions. Yield and yield components were recorded during harvest. Moisture stress was imposed at the panicle initiation stage, by withholding water for 10 days. The control plants under non-stress conditions were watered daily to 100 % field capacity (FC). The experiments were repeated twice to check the integrity of the results obtained.

The treatment details are as follows:

Variety : ATL1

T1 : Control (Non-stress)

T2 : Seed priming with *B. amyloliquefaceins* LAS10 (Non-stress)

T3: Control (Drought stress)

T4 : Seed priming with *B. amyloliquefaceins* LAS10 (Drought-stress)

Replication: 4

Germination percentage

The germination was recorded at 3 days intervals up to 5 days. Seeds were considered as germinated when the radicle protrusion was minimum of 2 mm in length. The germination was recorded on day 5 and expressed as germination percentage.

$$\text{Germination percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Number of seeds kept for germination}} \times 100$$

Vigor index

The vigor index of the seedlings was calculated using the following formula proposed by Abdul-Baki and Anderson (1973).

Vigor Index = (Shoot length + Root length) x Germination percentage

Plant growth parameters

On the 7th day after sowing, seedlings from each replication were carefully removed at random. The length of the shoot was measured from the collar region to the tip of the longest leaf and expressed as cm. The plants were carefully removed with minimum root damage. The distance from the base of the shoot to the tip of the longest root was measured and the average was calculated for three plants in each replication and expressed in cm. Total plant biomass was recorded for 3 plants per pot and represented as DAS.

Yield components

In each treatment, three hills were selected at the time of panicle initiation, labeled and a number of panicle-producing tillers hill^{-1} was counted and the average value was recorded. The number of panicles per plant was counted in three plants and average values are expressed. Total grain and straw yield were recorded after harvest and expressed in grams. One hundred filled grains were sampled from each plant and weighed at 14 per cent moisture content and expressed in g.

Statistical analysis

The dataset was subjected to a two-way analysis of variance and means were separated by Duncan's multiple range test (DMRT) at 0.05 level of probability using statistical software SPSS version 20.0. Graph pad prism 8 was used for the construction of graphs. Mean values of the treatments were compared by Duncan's multiple range tests at $p \leq 0.05$.

RESULTS AND DISCUSSION

Plant growth promotion by B. amyloliquefaciens on little millet under in vitro

In recent years seed priming received increased attention to attain early seed germination and plant growth (Mahmood *et al*., 2016). Biopriming with bacterial endophytes has been reported earlier in crops such as carrots (Jensen *et al*. 2002), sweet corn (Callan, Mathre, and Miller 1990, 1991), and tomato (Warren and Bennett 1999). Biopriming has been practiced successfully and explained by different researchers (Callan, Mathre, and Miller 1991; Bennett, Mead, and Whipps 2009; Moeinzadeh *et al*., 2010; Chakraborty *et al*., 2011). Priming of seeds with beneficial bacterial inoculums is an attractive eco-friendly approach to improve germination rates under unfavorable environmental stress conditions, and to activate plant defense mechanisms at the early stages of plant growth due to induced tolerance (Kasim *et al*., 2013). In the present study, the drought-tolerant endophytic bacteria *Bacillus amyloliquefaciens* LAS10 was evaluated for its potential role in seed germination, plant growth promotion, and drought stress resilience under *in vitro* conditions. Priming of little millet seeds with potential strain enhanced the germination and seedling growth under induced moisture stress compared to the control. Treated seeds significantly promoted seed germination and seedling establishment under induced drought stress. Seed germination started after 72 h, irrespective of all treatments. However, an increase in PEG concentration (-0. 045, -0. 4, -0. 28, -0. 45, -0. 69 MPa of osmotic potential) considerably reduced the germination percentage compared to their respective controls. Among the treatments, un-inoculated seeds were exposed to moisture stress of -0. 45 MPa (20% PEG 6000) showed a reduction in seed germination, whereas control seeds registered 100 % seed germination after 5 DAS. A drastic reduction was observed in germination percentage on exposure to an osmotic potential of -0. 45 and -0.69 MPa (20 % and 25% PEG 6000). In general, the seeds primed with potential strains significantly improved the seed germination over control under induced moisture stress. Among the treatment, osmotic stress imposed seeds treated with LAS10 strain at -0. 45 MPa accounted for a 27.2 % increase in germination percent. LAS10 also increased the seed vigor index under maximum moisture stress of -0.45MPa (62.4%) and non-stress conditions (21.48%) over non-primed seeds (Table 1). The treated seedlings were able to withstand the induced drought over a period of 15 DAS whereas the untreated seedlings reduced after 7 DAS.

Plant morphology attributes in pot culture

Seed priming and different levels of stress treatment significantly influenced the root length and shoot length as observed on 7 DAS (Figure 1). Seeds biotized with LAS10 showed increased root length (13.5%) and shoot length (25.2%) over control seedlings on 10 DAS under induced moisture stress (20 % PEG 6000) (Figure 1a, Figure 1b). The positive response in morphological attributes in *B. amyloliquefaciens* inoculated plants, might be due to the IAA production from microbial origin, which is responsible for root elongation. Similar results were confirmed in a study by Kalarani *et al*., (2019) where *B. methylotrophicus* RABA6 registered higher plant height and root length when compared with control under both irrigated and induced moisture stress conditions. Inoculation with *Ochrobactrum* spp. strain NBRISH6 improved the root length, dry weight, and hairs in maize under abiotic stress conditions (Mishra *et al*., 2020). The mechanism of plant growth-promoting bacteria (PGPB) plays a key role in minimizing the damaging effects of drought on plants. The expression of the physiological effect of PGPB on crop plants involves both direct and indirect mechanisms (Lastochkina *et al*., 2019), stimulation of plant growth by improving the availability of essential elements (nitrogen fixation, phosphate solubilization, iron sequestration) to crop plants, the production of phytohormones and regulation in the host plant, and induction of systemic resistance and tolerance to stresses (Singh *et al*., 2015; Ishak *et al*., 2016). Timmusk *et al*., (2014) reported similar results where, wheat plants treated with *Bacillus thuringiensis* AZP2 had three times longer root hairs and longer, denser lateral roots. The seed vigor index was found to be increased in primed plants with LAS10 (31.2%) when compared to the control under induced stress conditions (-0.45MPa). Application of plant growth-promoting bacterial strain *B. subtilis* SF48 improved the plant growth and relative water content in tomato plants under drought stress conditions when compared to control plants (Gowtham *et al*., 2020).

Plant growth and yield attributes of primed little millet in pot culture

Number of productive tillers and panicle length

In general, moisture stress reduced the number of productive tillers (5.2, and 3.01) in both *B. amyloliquefaciens* LAS10 inoculated and uninoculated plants (Figure 2b). However, within the treatment under both irrigated and moisture-stressed conditions, *B. amyloliquefaciens* LAS10 inoculation registered an increase in the number of productive tillers compared to the control. Panicle length was found to be increased (22.2%) in primed seeds under drought conditions compared to irrigated conditions (Figure 2a).

Total biomass

The total biomass of primed little millet plants was found to be increased (47.7%) under normal conditions. Primed seeds increased (57.05%) the total biomass even under moisture deficit conditions compared to control (Figure 2d). Similar results were observed by Cakmakci *et al*., (2017), as wheat plants inoculated with *Azospirillum brasilense* Sp245 visualized an increase in the fresh weight, and an improvement in the indicators of the hydration status of seedlings in comparison with control samples under drought stress conditions. In another study, *A. brasilense* INTA Az-39 increased the yield, number of spikelets, and biomass accumulation of wheat plants grown in dry farming zones under the influence of the bacteria (Diaz zorita *et al*., 2009). Biopriming with PGPB improved the seed germination percent and growth development of radish and wheat under saline conditions (Lutts *et al*., 2016). The drought stress during vegetative growth, flowering, and terminal phase (Pantuwan *et al*., 2002), can cause a decline in floret initiation, causing slow grain filling, resulting in lower grain weight and ultimately poor yield (Kamoshita *et al*., 2004).

3.3.3. Grain and straw yield

Bioprimed little millet seeds visualized the increase in yield (35.2%), 1000-grain weight (49.7%), and straw yield (12.06%) of little millet compared to control (Figure 2c, Figure 2e, Figure 2f). In general, moisture stress reduces the total number of panicles and 1000-grain weight. Kalarani *et al*., (2019) reported that *B. methylotrophicus* RABA6 inoculated plants showed more panicle number and grain weight than uninoculated control. Another study also confirmed the same results in maize primed with different *Azotobacter* and *Azospirillum* strains showing that biopriming significantly increased the crop growth rate, dry matter accumulation, and grain yield (Sharifi, 2011). Another study of biopriming in safflower, with *Pseudomonas* strain 186 also depicted that biopriming increased the number of branches, heads per plant, diameter of the head, grain number per head, grains per plant, 1000 grain weight, and grain yield of the plants (Sharifi, 2012).

Table 1. Plant growth attributes of primed little millet seeds under *in vitro*

Drought stress PEG (%)	Germination percentage		Vigor index (%)		Fresh weight (mg/plant)	
	Control	LAS10	Control	LAS10	Control	LAS10
NS	100±0.91^{a}	100±0.85^{a}	114.91±1.09^{a}	1491.32±1.43^{a}	227.42±0.99^{b}	299.42±1.04^{a}
DS -0.4 Mpa	46.3±0.84^{b}	62.1±0.72^{a}	341.18±1.02^{b}	129.16±1.05^{a}	129.16±0.87^{bc}	143.9±1.55^{a}
DS -0.28 Mpa	0	33.56±0.43^{a}	135.87±2.12^{ab}	135.87±1.23^{a}	0	96.54±0.96^{ab}

Values are mean three replications (n = 3). Control – Absolute control without any treatments, LAS10- *B. amyloliquefaciens* LAS10. NS- Non-stress; DS- 0.14MPa- 10% PEG; DS 0.45Mpa- 20% PEG. Values are mean (±standard error) (n=5) and values followed by the same letter in each column are significantly different from each other on the observation day as determined by DMRT ($p \leq 0.05$).

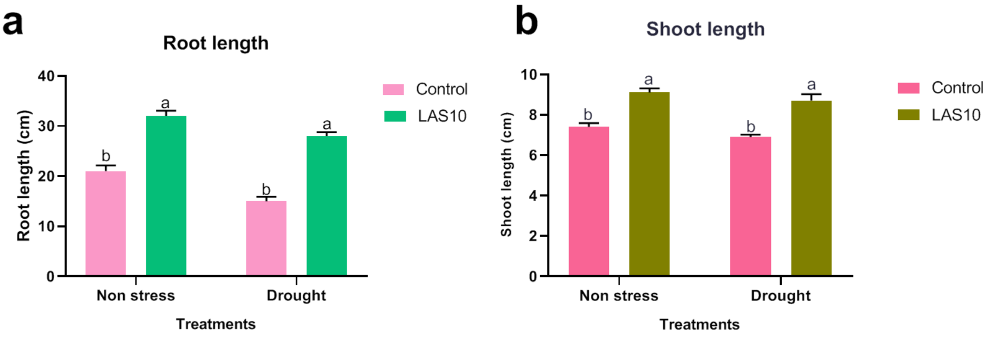

Figure 1. Plant growth promoting attributes of little millet plant primed with *B. amyloliquefaciens* LAS10 under induced drought stress. **a.** Root length. **b.** Shoot length. Values are mean (±standard error) (n=5) and values followed by the same letter in each column are significantly different from each other on the observation day as determined by DMRT ($p \leq 0.05$).

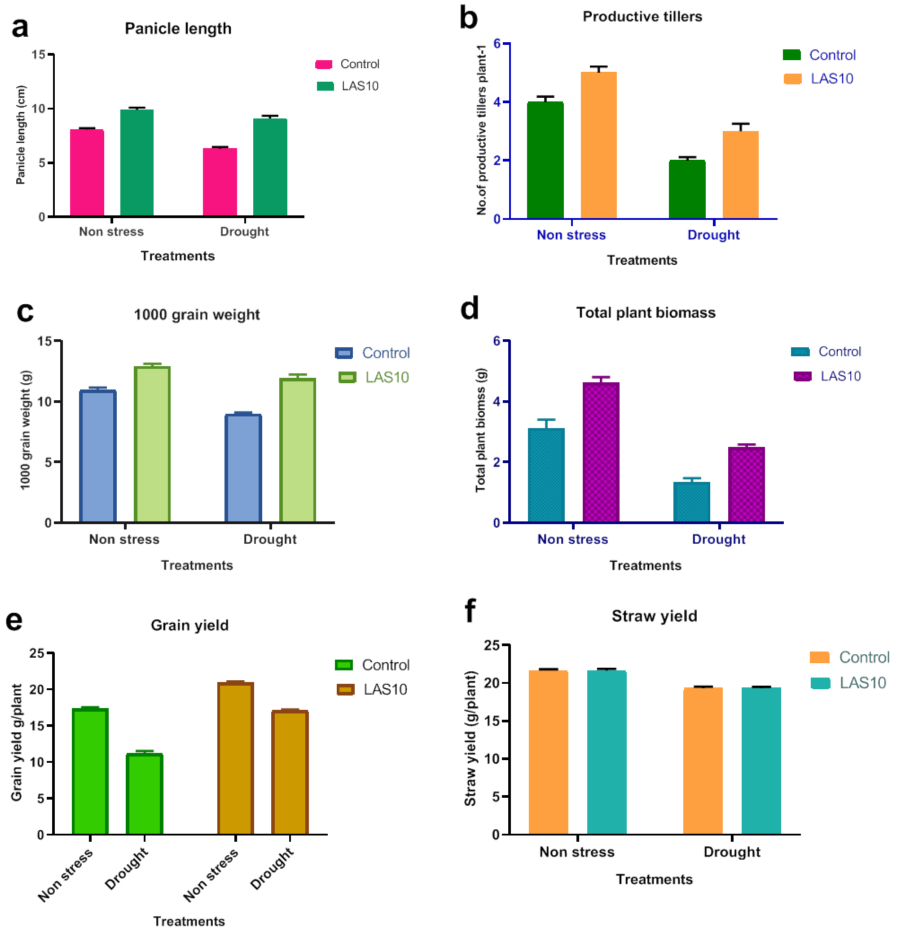

Figure 2. Crop yield attributes of little millet plant primed with *B. amyloliquefaciens* LAS10 under induced drought stress. **a.** Panicle length. **b.** Productive tillers. **c.** 1000-grain weight. **d.** Total biomass. e. Grain yield. **f.** Straw yield

Conclusion

The apoplast region of the plant is the primary site of endophyte invasion and serves as a niche for several beneficial microbe interactions. The apoplast-associated endophytic bacteria *B. amyloliquefaciens* LAS10 envisaged a prominent effect on plant growth performance viz., increased shoot length, root length, and yield attributes under drought-stressed conditions. The plant growth-promoting attributes of *B. amyloliquefaciens* LAS10 might be due to the auxin, phytohormone production, and nutrient uptake. Further studies may lead to the development of *B. amyloliquefaciens* LAS10 as a bioinoculant for alleviating drought stress and increasing little millet crop growth and fitness leading to enhanced crop productivity.

Ethics approval and consent to participate

Not applicable.

Originality and plagiarism

Authors ensured that the manuscript was written entirely as original works and that the work and/or words of others have been appropriately cited.

Consent for publication

All the authors agreed to publish the content.

Competing interests

There were no conflicts of interest in the publication of this content

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Author's contributions

Research grant and idea conceptualized by SU, All the experiments performed by MR. SU and SN have guided all analyses. The original draft was written by MR and the manuscript was reviewed and edited by SU, SN. All authors have read and approved the manuscript.

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