

Biostimulator effect of stress tolerant rhizobacteria on horticultural models

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Summary: The tolerant bacteria for abiotic stresses such salinity, drought, and different pH have been used as a good tool to improve plant growth in sustainable agriculture. A pot experiment was conducted to evaluate the potential of isolated stress-tolerant bacteria for red mud-polluted soil on growth performance of giant reed plants with increasing concentrations of NaCl 0.0; 0.1; 0.2; 0.3; 0.5; 1.0% under gnotobiotic conditions. At the same time biostimulator potential of isolated bacteria was observed in case of radish *in vitro* germination experiment under salt stress. It was found that the observed bacterial strain can tolerate the salt and pH moderately however it is resistant against hydrogen-peroxide caused oxidative stress in high concentration (up to 2640 mM). Molecular identification, basis on 16S rDNA showed 98% similarity to the *Bacillus aryabhata* bacterial strain. The isolated strain alleviated the negative effect of salt (0.05%) for the radish seed germination. However in higher salt concentration ($\geq 0.1\%$) the bacterial mitigating effect vanished. The inhibition of increasing salt concentration for giant reed plantlets was also alleviated by halotolerant bacteria treatment ($\geq 0.5\%$).

Keywords: halotolerant bacteria, salt tolerance, radish, *Arundo donax* L., stress alleviation

1. Introduction

Due to the unique genetic characteristics that prokaryotes including bacteria possess, they are able to easily adapt and survive under extreme environmental conditions such as drought, salinity and wide ranges of pH. It is the vitality in the agro-ecosystems that researchers are investigating for opportunities to enhance agricultural inputs in the form of Plant Growth Promoting Rhizobacteria (PGPR). The products of PGPR can be a significant component of management practices to achieve the attainable yield in degraded soil. The success lies in their aggressive root colonization potential around the rhizosphere. The stress adaptability of microbes is due to sophisticated signalling system that microbes have for eliciting an adaptive response to stresses. The most dramatic of these behaviours are the purposeful migration or movement of the cells toward favourable conditions. Also, such bacteria can be exploited as a successful strategy for protecting the plants against the deleterious effects caused by soil- and seed-borne deleterious plant pathogens. Thus, the PGPR biotechnologies can be exploited as a low input, sustainable, and environmentally friendly technology for stress management in plants (Maheshwari, 2012).

For decades, agriculture, in areas that suffer from water scarcity such arid and semiarid environments, has faced a serious problem with increasing soil salinity. Salt

is a necessary element for all life, but too much can be a problem. Saline soils will have a relatively high number of sodium ions compared to healthy soils. Excessive salt not only destroys the soil structure, but it also attracts water and blocks its absorption to plant roots. As a result, plants may exhibit signs of drought even when the soil is wet or waterlogged. Soil salinity is a major abiotic stress in plant agriculture strongly, influencing plant productivity worldwide. Just as the results of high soil salinity on plants are seen in leaf and stem burns, it is hard on earthworms and microorganisms as well (Khan and Panda 2008). The total salt-affected land worldwide is estimated to be 900 million ha, 6% of the total global land mass (Flowers 2004). According to the Food and Agricultural Organization (FAO), if corrective measures are not taken, salinization of arable land will result in 30% land loss in the next 25 years and up to 50% by the year 2050 (Munns 2002). Salinity prevents plants from taking up water, exposing them to drought stress. These stresses have an adverse effect on plants, hampering their growth and finally production. Soil salinity is defined as the concentration of dissolvable salts extracted from soil by water (Richards 1954). Natural boundaries imposed by soil salinity also limit the caloric and the nutritional potential of agricultural production. These constraints are most acute in the areas devoted to agriculture; therefore, the urgent need of biological agents (bio-preparations) is accepted worldwide. Interest in the use

of such bio-preparations that replenish the soil, add value, and enhance production and yield in saline conditions is the primary recommendation.

Giant reed (*Arundo donax* L.) is a perennial rhizomatous grass (Poaceae family), native to the freshwater regions of Eastern Asia, but nowadays considered as a sub-cosmopolitan species given its worldwide distribution (Bell, 1997; Alshaal et al. 2013a). It is a hydrophyte, growing along lakes, streams, drains and other wet sites. The genus *Arundo* is able to reach the height of 14 m and is among the fastest-growing terrestrial plants. It can produce more than 50 t ha⁻¹ aboveground dry biomass (Tucker, 1990; Sharma et al., 1998; Günes and Saygin, 1996). As a consequence of its high and fast biological productivity, giant reed is widely cultivated to yield non-food crop that can meet requirements for energy, paper pulp production, bio-fuels and construction of build materials, but it has other different uses such as music tools with stem, medicine with roots and soil erosion control through re-vegetation.

Arundo displays unique physiological features whereby it readily absorbs and concentrates toxic chemicals from contaminated soil with no appreciable harm to its own growth and development (Perdue, 1958; Alshaal et al. 2013b). It is one of the mostly used plants as a trace element bio-accumulator, especially via phytoremediation processes, due to its capacity of absorbing contaminants such as metals that cannot be easily biodegraded. Giant reed can grow in different environments with spacious ranges of pH, salinity, drought and trace metals without any symptoms of stresses and can easily adapt to different ecological conditions and grow in all types of soils. However, because of its great adaptability to different ecological conditions, giant reed is considered noxious invasive weeds in riparian habitats throughout the world (Coffman et al., 2010).

The present study was conducted in an attempt to identify and characterize salt and pH tolerant bacteria that is isolated from naturally red mud-polluted soil for its numerous PGP traits. Isolated strain was then checked for its ability to ameliorate saline stress under gnotobiotic conditions using *Arundo* plants and it. Alleviating effect of isolated strain was also observed in radish *in vitro* germination experiment under salt stress.

2. Materials and methods

2.1. Soil sampling and isolation of bacteria after steaming process

On October 4, 2010, the western dam of the red mud reservoir of an alumina plant in Ajka (Hungary) collapsed. Hungarian Aluminium Company (MAL) reported that About 700.000 m³ of the red mud/water mixture with a pH of about 12 was released from the reservoir. Two weeks after the Alumina factory catastrophe, a composite red mud-polluted soil sample (0-25 cm) was randomly collected from a red mud polluted field in Ajka, Hungary

(47° 5' 3.97" N, 17° 28' 30.08" E). After sampling, a portion of soil sample was sieved through 2 mm in order to separate plant debris and visible fauna, and then stored at 4°C. Red mud polluted soil was autoclaved at 121.5 °C and 1.5 atm. for 1 h for three consecutive days with incubation at 28 °C between autoclaving. After steaming process ten-fold serial dilutions of the samples were made by mixing the soil with sterile water shaking for 15 min at 150 rpm, and then plating on sterile sucrose-minimal salts low-phosphate (SLP) medium (sucrose 1%; (NH₄)₂SO₄ 0.1%; K₂HPO₄ 0.05%; MgSO₄ 0.05%; NaCl 0.01%; yeast extract 0.05%; CaCO₃ 0.05%; pH 7.2) (Sheng et al., 2008). The plates were incubated at 28 °C for 3-4 days waiting if any microbe growing. Only colony was grown (A4). Hereinafter this strain was observed in detail.

2.2. Salt tolerance

NaCl (0,01%, 2%, 5%, 7%, 10%) were added to liquid sucrose-minimal salts low-phosphate (SLP) medium. The medium was inoculated with bacteria in Log phase. Incubation 27 °C on orbital shaker (80 rpm) was for 24 hour. OD was measured on 600 nm by spectrophotometer (Amersham Biosciences Ultrospec 2100 Pro UV/Visible).

2.3. pH tolerance

The pH of liquid sucrose-minimal salts low-phosphate (SLP) medium was set on wide range of pH as 3.2, 4.2, 7.2, 11.2, 12.2, 13.2. The medium was inoculated with bacteria in Log phase. Incubation 27 °C on orbital shaker (80 rpm) was for 24 hours. OD was measured on 600 nm by spectrophotometer (Amersham Biosciences Ultrospec 2100 Pro UV/Visible).

2.4. Oxidative stress

Increasing concentrations of hydrogen-peroxide (880, 1760, 2640, 4400, 6600, 8800 mM H₂O₂) were added to liquid SLP medium. The medium were inoculated with bacteria in Log phase. Incubation 27 °C on orbital shaker (80 rpm) in dark. Sampling was hourly and the OD was measured.

2.5. Characterization Based on 16S rDNS Gene Sequencing and Phylogenetic Analyses

DNA isolation from A4 bacterial strain was performed by Fermentas genomic DNA purification kit. The concentration of DNA was measured by spectrophotometric method (Amersham Biosciences Ultrospec Nanodrop). The 16S rDNS region were amplified by PCR reaction using 27f and 1492r universal primers. Sequence of 27f primer: 5'-GAGTTTGATCACTGGCTCAG-3' and sequence of 1492r primer: 5'- TACGGCTACCTTGTTACGACTT-3'.

The PCR reaction program was the next: 95 °C 3 min, 95 °C 0,5 min., 54 °C 0,5 min., 75 °C 1 min-, 72 °C 10 min., 4 C ∞. MJ Research Minicycler machine was used. The PCR product were purified by Thermo scientific GeneJet PCR Purification kit. The qualitative and quantitative checking of PCR product were fulfilled on 1% agarose gel. Partial sequencing was performed. The consensus gene sequences were used to identify the bacterial isolates with BLASTn analysis using NCBI GenBank nr database and obtained the homology. The phylogenetical analysis was done in MEGA5.2 program aligned using multiple alignment software, Clustal W. These alignment results were used to construct phylogenetic tree using MEGA 5.2 software tool.

2.6. Observation of halotolerant bacterium inoculation effects on Arundo growth under salt stress in gnotobiotic conditions

The experimental layout was a completely randomized design with three replications. To test the root elongation and growth promotion of Arundo plants by isolated bacterium under salt stress in gnotobiotic conditions, the following concentrations of NaCl 0.0; 0.1; 0.2; 0.3; 0.5; 1.0 % were to sterilize plastic pots containing 500 g of acid-washed and sterilize sand. The pots were irrigated and left for the next morning where three identical sterilize seedlings of Arundo were transplanted. Examined pots were divided into two portions; the first portion received 10 ml of inocula (10^8 CFU/ml), the second portion was left as control. All of tested pots were kept under greenhouse conditions. Four weeks later, the Arundo plants were evaluated, since plant length, number of shoots, number of leaves and length of new shoots were measured.

2.7. Plant Material

The plant material used for the current study was somatic embryo-derived plantlets of the Blossom ecotype of giant reed (*Arundo donax* L.) obtained from the University of South Carolina (Marton and Czako, 2004; 2007) and propagated in the Ottó Orsós Laboratory, Department of Plant Biotechnology, Debrecen University, Hungary.

2.8. Observation of halotolerant bacterium inoculation effects on radish seed germination under salt stress in vitro conditions

Sterile radish (*Raphanus sativus*) seed sowing were performed on half-strength MS medium (Murashige & Skoog, 1962) with added 0; 0,05; 0,1; 0,2% NaCl. The surface of the seeds were sterilized with 40% commercial bleach for 20 min thereafter rinsed with sterile distilled water for three times. Before sowing half part of seeds were inoculated in log phase bacteria suspension for 30 min and the other part was kept sterile. The germination time was one week.

3. Results and discussion

3.1. Salt tolerance of isolated bacterium

The salt tolerance of the isolate A4 was determined by measuring the optical density of bacterial growth on liquid SLP medium supplemented with 0.01–10% (w/v) NaCl. The results showed that the isolated bacterium was able to grow on SLP medium plus 0.01–7% (w/v) NaCl. The highest percentage of NaCl to 10% (w/v) suppressed the growth of the bacteria on SLP medium. Therefore, the population range of the bacterium A4 cultivated on SLP medium plus 5, 7, and 10% (w/v) NaCl was investigated to confirm its tolerance. Isolated bacterium was able to grow moderately in 7% (w/v) NaCl (Fig. 1). Therefore, this strain could be classified as halotolerant bacteria (Willey et al. 2009).

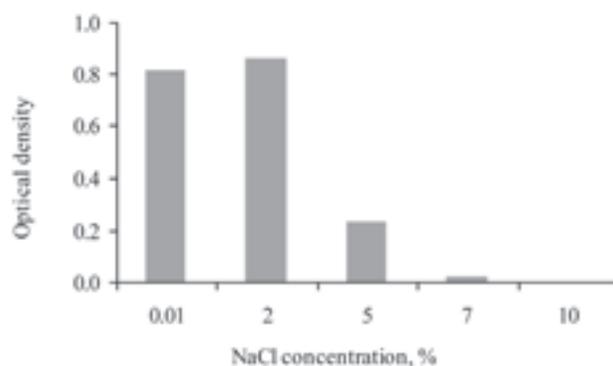


Fig. 1: The growth of isolated bacterium in liquid SLP medium supplemented with increasing NaCl concentrations

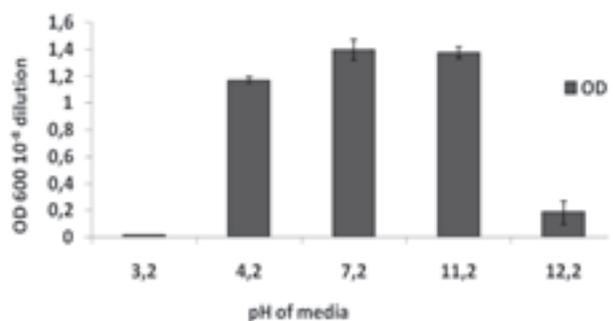


Fig. 2: The growth of tested bacterium A4 in liquid SLP medium treated with different pHs

3.2. Growth characteristics of Bacterium A4 under different pHs

The SLP liquid medium with different pH ranged from 3.2 to 12.2 was used to investigate the ability of isolated bacterial strain A4 to grow on different pH. Figure 2 shows that the tested bacterium grew well at pH 7.2, as well as its ability to survive at low and high pH, 3.2 and 12.2 respectively. pH is one of the critical growth factors of microbes beside temperature, dissolved gases, osmotic pressure and water availability. Most bacteria grow best around neutral pH values (6.5–7.0), but some thrive

in very acid conditions and some can even tolerate a pH as low as 1.0. Such acid loving microbes are called acidophiles. Even though they can live in very acid environments, their internal pH is much closer to neutral values.

3.3. Tolerance of Bacterium A4 to oxidative stress

In order to test the oxidative stress tolerance of isolated strain A4, different concentrations of H₂O₂ (1760, 2640, 4400, 6600, 8800 mM) were added to liquid SLP medium. The optical activity (OD) of liquid culture of bacterium at 600 nm increased during the first hour after adding H₂O₂ for all concentrations but this increasing was inversely proportional with the concentrations of H₂O₂ since high concentrations of H₂O₂ recorded low OD. The control treatment linearly increased with the time, on the other hand the treatments with H₂O₂ did not show any increase in the next 5 hours after the beginning of the test (Fig. 3).



Fig. 3: Oxidative stress tolerance of bacterium A4 in liquid SLP medium supplemented by increasing concentrations of H₂O₂

3.4. Bacterial identification

Based on the experiments of phenotypic characterization, the isolate is gram positive and rod-shaped. The antibiotic tolerance of isolate A4 was evaluated using 200 mg/L of Kanamycin, Streptomycin, Cefotaxin and Carbamycin. The results showed that the diameter of clear zone from isolate A4 was 1.13, 1.55, 4.96 and 0.5 cm, respectively for used antibiotics. According to these results, the isolate A4 is considered tolerant for Carbamycin and sensitive to Cefotaxin.

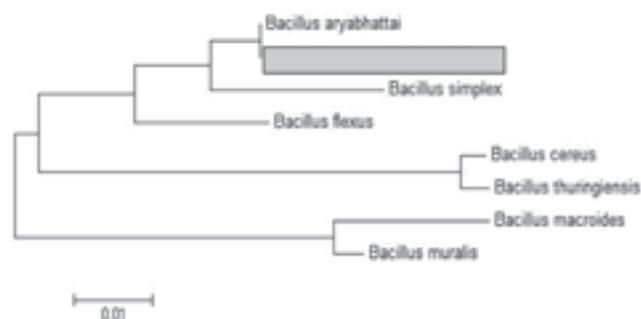


Fig. 4: The BLASTn analysis of Bacteria A4 basis on partial sequence showed 98% similarity to the Bacillus aryabhattai bacterial strain

In addition, the 16S rDNA region were amplified by PCR reaction using 27f and 1492r universal primers. The phylogenetical analysis of observed bacterial strain confirmed our morphological characterization hence it belongs to the *Bacillus* genus and 98% identification was shown with *Bacillus aryabhattai* (Fig. 4.)

3.5. Effect of bacterium inoculation on giant reed growth under salt stress

More specifically, plant growth promoting bacteria received paramount attention because of its importance and ability to induce the plant growth under stresses such salinity, as well as efficient root-colonizing ability and capacity to produce a wide range of enzymes and metabolites that help plants withstand varied biotic and abiotic stresses (Vessey 2003).

Ashraf et al. (2004) suggested that inoculated selected PGPR producing bacteria could serve as a useful tool for alleviating salinity stress in salt-sensitive plants. Owing to the PGPR properties and trend to be a biofertilizer of bacteria A4, greenhouse pot experiment was carried out in order to evaluate the effect of inoculation of it on growth of giant reed plants under increasing concentrations of NaCl. In general, increasing salt concentration decreased the plant length significantly with or without bacterial inoculation but in high concentration of NaCl the tested bacterium was able to keep giant reed plants survive, where this bacterium alleviated the stress of NaCl. All of number of shoots, leaves and length of new shoot also decreased with increasing doses of NaCl. Data in Table 1 showed that inoculation with A4 bacterial strain has alleviated the inhibiting effect of NaCl on growth of giant reed plants.

Table 1: Characteristics of giant reed plants inoculated by bacterium A4 under salt conditions

NaCl %	Plant length		No. of shoots		No. of leaves		Length of new shoot	
	with-out A4	with A4	with-out A4	with A4	with-out A4	with A4	with-out A4	with A4
0.0	12.4	12.8	1.7	0.5	3.1	4.5	3.5	3.6
0.1	13.9	6.3	0.6	0.5	5.7	2.4	4.4	3.2
0.2	6.5	6.8	0.0	0.7	2.3	2.6	0.0	1.3
0.3	5.7	2.8	0.0	0.0	2.3	0.7	0.0	0.0
0.5	0.0	0.3	0.0	0.1	0.0	0.1	0.0	0.1
1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

3.6. Effect of Bacillus aryabhattai inoculation of Radish germination

Besides the *Arundo donax* L. the presumed salt stress mitigating effect of bacterium A4 were studied in case of radish germination experiment. Basis on the results sodium-chloride was found that in 0.2 % concentration totally inhibited the germination of radish seeds (with and without

bacterial inoculation). However higher germination vigor was found in case of bacterial inoculated seeds on media completed with 0.05-0.1% NaCl compare to the inoculation free seeds (Fig. 5). These results Like the experiment with *Arundo*, salt stress alleviating effect of bacterium A4 could be seen in lower NaCl concentration of media. No positive effect of bacterial inoculation was found on control media.

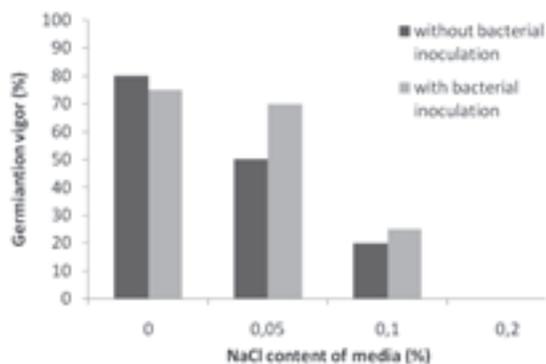


Fig. 5: Germination percent of Radish seeds on solid medium supplemented by different NaCl concentrations in presence of isolated bacterium A4

4. Summary

The present study is consider a pioneer report that highlights and studies the possible using of a stress tolerant bacterial strain as biofertilizer and PGPR with vegetable crop such radish and energetic crop such giant reed under salt stress observed. The data showed that the germination percent of radish seed under moderate concentration of NaCl was assisted by using the observed bacterial strain (A4). Giant reed growth characters were not so far for radish effect, where the vegetative parameters such plant length, number of shoots, leaves and length of new shoots were increased in salted pots with identified *Bacillus* strain compared to control (without bacterial inoculation). Considering the abiotic stress tolerance measurements this bacterial strain showed a great ability to grow and survive under abiotic stresses such high NaCl concentrations, different pH and oxidative stress with H₂O₂.

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