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Original Article

A Study On The Stress Tolerant Rhizobial Isolates From *Sesbania bispinosa*

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ABSTRACT: The present investigation was carried out to study the extremely diversified characteristics of rhizobial strains isolated from locally produced legume *Sesbania bispinosa*, a well-known green manure of Bangladesh, commonly known as Dhaincha. It was important to study the rhizobial isolate of this less studied plant of Bangladesh in order to learn about its association and to increase soil fertility. An attempt had been made to evaluate the effect of abiotic constraints (salt, pH and temperature) on the growth of rhizobia isolated from *Sesbania bispinosa* growing in different region of Bangladesh with a view to screen out stress tolerant isolates. Growth of pure rhizobial isolates on Yeast Mannitol Agar (YMA) medium having different concentration of NaCl (2%, 2.5%, 3%, 4%, 5%, 6% and 8%) and variable range of pH (4.5, 5.5, 6.5, 8.5, 9.5 and 10.5) were recorded after incubation at 30˚C for 24-48 hours. The growth of rhizobial isolates were also observed after incubation at 25˚C, 30˚C, 37˚C, 45˚C and 55˚C temperature, respectively. All the 46 isolates were able to tolerate up to 3% salt concentration. However it was observed that with increasing salt concentration isolates were found to be decreasing. It was further observed that, acidic and alkaline pH could not suppress the growth of these isolates. All isolates were found to grow upto 37˚C. However, 42 out of 46 isolates were able to tolerate 45˚C. The highest temperature (55˚C) was found to inhibit growth of about 43% isolates. The stress tolerant traits of these rhizobia are of potential value from the point of view for using *Sesbania* sp. as bio-fertilizer in different region of Bangladesh.

Key words: Sesbania, stress tolerant, bio-fertilizer

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INTRODUCTION

Nitrogen is the most limiting plant nutrient in agriculture (Singer and Munns, 1987). Despite its abundance, plants are unable to use N_2 directly. Many legumes, through symbiosis with rhizobia have the ability to reduce atmospheric N_2 through biological nitrogen fixation (BNF) into a form usable for growth. Increasing demand for agricultural products combined with a need to conserve the world's limited resources make increased use of BNF in agriculture and forestry an important global objective. Fast-growing N_2 fixing leguminous trees are being widely promoted as sources of renewable energy and biologically fixed N for associated crops (Brewbaker *et al.*, 1982; Dommergues 1987; Kang *et al.*, 1984). Biological Nitrogen Fixation can be considered as one of the most interesting microbial activity as it recycles nitrogen on earth possible and contributes to nitrogen homeostasis in the biosphere (Aquilanti *et al.*, 2004). Rhizobia are genetically diverse and physiologically heterogeneous group of symbiotic nitrogen fixing bacteria that form nodules on the roots or rarely on

the stem of legume hosts, within which the bacteria fix atmospheric nitrogen into ammonia. A fully functional symbiosis requires successful survival ability of bacteria even under adverse environmental conditions. Within the soil, rhizobia frequently encounter various stresses that affect their growth, their initial steps of symbiosis and the capability of nitrogen fixation (Zahran, 1999). Soil may lack the number of the specific rhizobia that is required for nitrogen fixation of the different legumes. S*esbania bispinosa* have generally been considered as an important legume for green manuring due to their ability to fix large quantities of N_2. Several environmental conditions are limiting factors to the growth and activity of the N_2-fixing plants. In the *Rhizobium*-legume symbiosis, which is a N_2-fixing system, the process of N_2 fixation is strongly related to the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors (e.g., salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture, inadequate photosynthesis, plant diseases, and grazing) impose limitations on the vigor of the host legume (Brockwe *et al*., 1995; Thies *et al*.,1995). Inoculation of stress tolerant strains of rhizobia may enhance the nodulation and nitrogen fixation ability of plants under stress conditions. The ability of legume hosts to grow and survive in saline conditions is improved when they are inoculated with salt tolerant stains of rhizobia (Zou *et al*., 1995; Hashem *et al*., 1998; Shamseldin and Wewrner, 2005). Rhzobial populations vary in their tolerance to major environmental factors (Urich and Zaspe, 2000; Wei *et al*., 2008; Biswas *et al*., 2008). Considering these, in the present study, rhizobia from *Sesbania bispinosa* were isolated and their *in-vitro* physiological stress tolerance (salt, pH and temperature) was evaluated.

MATERIALS AND METHODS

Experimental site

The host plant, *Sesbania bispinosa* was collected from seven different places of Bangladesh that included Dhaka, Feni, Shirajgonj, Kishorganj, Savar, Demra and Sylhet.

Collection of Nodules

Fresh and Healthy nodules were selected from each plant for this study. The selected nodules were usually light brown or reddish in color, which indicates that an active nitrogen fixation has been established between the nodule bacteria and the legume plant. Besides collection of *Rhizobium sp* from leguminous plant *Sesbania bispinosa*'s nodule, the rhizosphere around the plant nodule was also collected.

Surface Sterilization of the Nodules

Nodules were thoroughly washed under tap water and then carefully severed from the root with sterile forceps. Intact, undamaged nodules were immersed in 95% ethanol for 5-10 seconds to break the surface tension, and then they were transferred to a 3% solution of H_2O_2 and soaked for 2-3 minutes. Nodules were then rinsed in five changes of sterile distilled water using sterile forceps for transferring.

Isolation of Rhizobia

The primary step of the isolation process was to crush the sterile nodules with a blunt tipped forceps in a large drop of sterile water in a Petri dish. One loop full of nodule suspension was streaked on Yeast Mannitol Agar (YMA) plates. After 24 h of growth at 30˚C, the isolates were sub cultured on YMA agar to obtain pure culture. The purity of the Rhizobium isolates was detected by adding Congo red in YMA media (0.25g/100ml of EtOH; 10ml stock /liter of YMA) in YMA media (Somasegaran *et al*.1982). Most Rhizobium absorbs the dye only weakly whereas contaminant including Agrobacteria takes it up strongly. All the rhizobial isolates were subjected to their morphological, cultural and biochemical characterization (Vincent 1970; Creager *et al* 1990; Cappuccino and Sherman, 1992)

Stress Tolerance Studies

Response of all rhizobial isolates to various environmental stresses were monitored. To the basal medium of YMA 2%, 2.5%, 3%, 4%, 5%, 6% and 8% NaCl were added, and each plate were streaked with a loopful of each of different rhizobial isolates freshly grown in yeast-extract-mannitol broth (YEM). After incubating the plates for 48-96 hrs at 30^{o}C, bacterial growth was recorded as positive (visible growth) or negative (no growth). The concentration 0.1 % NaCl was used as control, which was the concentration of NaCl in the basal YMA medium.

Rhizobial isolates were inoculated into YMA medium incubated at different temperatures (25˚C, 37˚C, 45˚C and 55˚C respectively) to test their maximum growth temperatures. After 48-96 hrs of incubation, bacterial growth was recorded by

visual observation compared to control treatments incubated at 30 °C.

Rhizobial isolates were tested for their ability to grow at different pH values of YMA adjusted to pH 4.5, 5.5, 6.5 (with 1M HCL) and pH 8.5, 9.5, 10.5 (with 0.1N NaOH). After inoculation and incubation at 30˚C for 48-96 hrs, bacterial growth was recorded by visual observation compared to control treatments incubated at pH 7.

RESULTS AND DISCUSSION

Rhizobia are soil bacteria that colonize the rhizosphere of legumes and other plants. They are rather diverse group of bacteria than might be supposed, but are united by their ability to form nodules on legumes and occasionally non-legume plants, e.g., *Parasponia (*Ulmaceae).

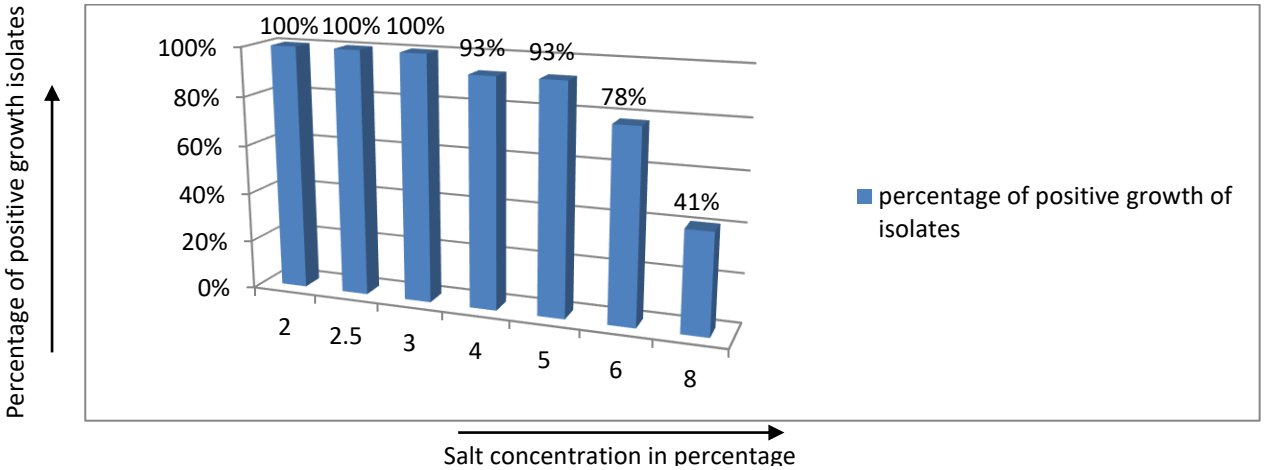

Figure 1 Graphical presentation of different salt concentration's effect on test isolates

These bacteria are among the most intensively studied groups of microorganisms (Sessitsch *et al*. 2002), mainly due to their N_2-fixing ability and their potential to replace N-fertilizers, with emphasis on their key role in achieving sustainability of N-poor soils (Zahran 2006, Zahran 2009). However, many of these rhizobia remain unidentified (Novikova *et al*. 1994). A total of 46 rhizobial isolates were isolated from *Sesbania bispinosa*. The isolates were characterized and were found to be fast growers, some growing in less than 20 hrs, and showed variation in their quality and quantity of LPS/EPS production (Akter *et al*., 2016). Growth pattern and cultural characteristics of root-nodule rhizobial isolates in this study, whether being from crop legumes or from the wild herb legume (*T. resupinatum*), were in agreement with the general characteristics of fast-growing rhizobia summarized in Bergey's Manual of Systematic Bacteriology (Jordan 1984) and also in other reports (Akhter, H 1989).

Salt Tolerance:

Tolerance to NaCl stress is a very complex phenotype that involves not only the bacterial ability to tolerate the stress but also the swiftness to respond and adapt to the environmental change. In the current study, decreased growth of rhizobial

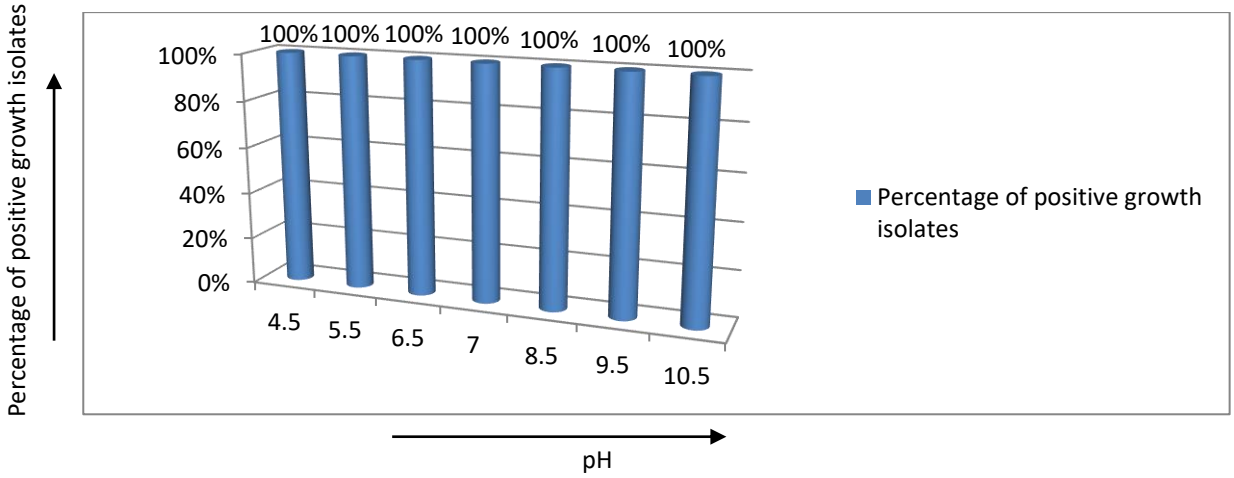

Figure 2: Graphical presentation of different pH effect on test isolates

isolates with increasing salt concentration was observed. Because increasing in salt concentration may have a detrimental effect on rhizobial populations as a result of direct toxicity as well as through osmotic stress (Nagales *et al*., 2002; Thrall *et. al*., 2008). All the 46 isolates were able to tolerate and survived up to 3% salt concentration. However it was observed that with increasing salt concentration the growth of different isolates were found to be decreasing. 8% NaCl was inhibitory for survival of majority rhizobial isolates. The response and adaptation of rhizobia to salt stress is a complex phenomenon implicating many physiological and biochemical processes that notably affect rhizobial colonization of roots and early infection events (Nabizadeh *et al*., 2011).

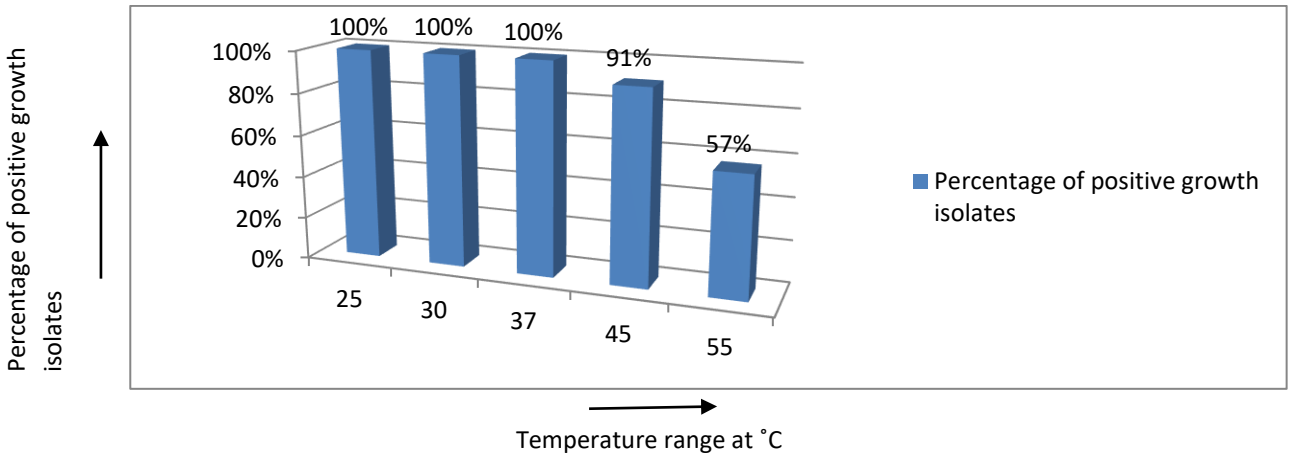

Figure 3: Graphical presentation of different temperature's effect on test isolates

All rhizobia appear to be surviving and varying in their growth ability under acidic and alkali condition. In the current investigation, it was observed that all isolates grew at all pH tested. Surprisingly, it was observed that, acidic and alkaline pH could not suppress the growth of these isolates and all of them were growing at pH 10.5 and also pH 4.5.

Temperature Tolerance

In general, majority of the isolates exhibited excellent growth at the temperature ranging from 25-37˚C. Some previous workers also confirmed that this finding by reporting that optimum temperature for growth of root nodulating bacteria ranged from 25-30˚C (Harwani, 2006; Ali *et al*. 2009). However, 42 isolates out of 46 were able to tolerate 45˚C. The 55˚C temperature was found to inhibit growth of about 43% isolates. However, other studies (Kulkarni *et al*. 2000) showed that rhizobia strains from *Sesbania aculaeta*, survived at 50 °C and 65 °C on YMA at pH 7 for up to 2 and 4 hours. Rhizobial strains obtained in the present study showed a relatively high number of isolates showed growth at 45 °C and 53% showed growth at 55 °C. This finding agreed with the results of previous studies on *R. leguminosarum* strains isolated from Nile Valley of Egypt, which showed tolerance to temperatures between 35-40 °C (Moawad and Beck 1991) and *C. arietinum* rhizobial isolates, which grew at 45 °C (Maatallah *et al*. 2002). The general responses to temperature stress in rhizobia have been reviewed (Alexandre and Oliveira 2012). Temperature can influence not only the survival of free rhizobia but also the exchange of molecular signals between the symbiotic partners (Sadowsky, 2005)

CONCLUSION

In recent decades it has been observed that the biological N fixation is a valuable tool for agriculture in improving plant growth yield and soil quality. The usage of chemical fertilizers may be reduced by exploiting N fixing microorganisms that replaces the usage of chemical fertilizer in turn affects the plant and soil health that causes severe harmful effects to humans. To overcome this problem, the study of BNF organisms needs much more importance to create pollution free agricultural ecosystem. The applications of modern molecular techniques are highly useful to create more potential nitrogen fixing strains with higher potential.

In conclusion, the isolated rhizobial strains are of good traits, e.g., tolerant to high salt levels, resistant to acidic and alkaline pH as well as resistant to high temperature. These rhizobia isolates may have great adaptation mechanism as they could be observed to tolerate stress conditions. This could further be utilized for their

symbiotic effectiveness determination under field conditions.

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