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Morphological and physiological characterization of nitrogen fixing rhizobia isolated from country bean (*Lablaba perpureus*) of Narail, Bangladesh

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ABSTRACT: Nitrogen is one of the important constituents of the plants. It is fixed in legume plants from the atmosphere by the association of beneficial gram negative soil bacteria named rhizobia. This study aimed to distinguish the morpho-physiological characteristics of naturally growing rhizobia having higher environmental and external stress tolerance. Twenty-two isolates were isolated from country bean root nodules and characterized based on different tests. The isolates varied from one another with level of parameters of the treatments. Most of them exhibited cream color colonies and some isolates evolved white and pink colonies. The average colony size of the isolates was ranged between 0.83 to 2.83 mm after one day and 1.33 to 5.00 mm after two days. Among them, 17 isolates were alkali producers, three isolates were acid producer and only two isolates produced neutral mucous. All the isolates showed complete resistance at pH 5.0 to 10.0, 2% NaCl, 10 to 42^{o}C temperature, and 0.1 mM of heavy metallic salt $MnCl_2$, $FeCl_3$ and $PbCl_3$. Nevertheless, most of the isolates also tolerated pH 4.0; 3 to 7% NaCl; 47^{o}C temperature; 0.1 mM $CuSO_4$. On the other hand, a few numbers of the isolates resisted 100 µg/ml and 200 µg/ml ampicillin, kanamycin and tetracycline antibiotics. Therefore, exploration of country bean rhizobia for their environmental stress tolerance capacity might be a core study for the production of cost-effective bio-fertilizer to boost up country bean yield.

Keywords: Country bean, Nitrogen fixation, nodule, rhizobia, symbiosis.

INTRODUCTION

The biological fixation of atmospheric nitrogen is carried out by a symbiotic relationship between the plant and bacteria. It is one of the main sources of naturally nitrogen pool enhancement in cultivation lands. Rhizobia, a group of useful gram negative soil bacteria, induce nodulation on leguminous plants and perform nitrogen fixation through symbiosis process (Zhao-Hai et al., 2007). The bacteria contain some nitrogenase enzymes which reduces atmospheric free nitrogen to ammonia or other nitrogenous complexes. Rhizobia consume required carbon source from the host plant and supply the nitrogenous compounds to the plants. Such type of successful symbiotic association helps rhizobia to survive and promote the adequate number in the soil ecosystem (KÜÇÜK, 2011).

The higher the survival amount of rhizobium in leguminous nodule, the greater the yielding rates of legume product. *Lablaba perpureus* is a leguminous species native to Asia and Africa, used in Bangladesh as commercially cultivating winter vegetable, known as 'Sheem' (Pengelly and Maass, 2001; Saha and Haque, 2005). As *L. perpureus* contains high amount of protein, it can be harvested to reduce the protein scarcity existing in the developing and underdeveloped countries (Adebowale and Lawal, 2003). The high productivity of *L. perpureus* can be done by increasing their natural biological nitrogen fixation (BNF) capability. To enhance this biological nitrogen fixation (BNF) potential of this crop, the selection

and evaluation of new *rhizobial* strains must be carried out. Although chemical nitrogen fertilizer can be used as an alternative, but the higher production cost and the problems associated with its application may reduce biological nitrogen fixation (Lum and Hirsch, 2004). Similarly, rhizobia symbiosis based on biological nitrogen fixation poses more efficient and desirable utility than chemical fertilizers (Elboutahiri et al., 2010). Unlike chemical fertilizers bio-fertilizers' overdose does not hamper the productivity and ecosystem. Besides, symbiotic plants and soil rich absorbent nitrogen would be useful for planting other plants as well (Herridge et al., 2001).

It is very important to study the indigenous population of rhizobia specific for *L. perpureus* to enhance the yield. The aim of this study was to evaluate the phenotypic and morphological characteristics of rhizobia isolated from the roots of *L. perpureus* and to investigate their tolerance to salinity, acidity, some heavy metals and antibiotics.

MATERIALS AND METHODS

Sample collection

The country bean root nodules were collected from different bean fields stand beside the river 'Nabaganga' of 'Narail' (a South western district in Bangladesh). The nodules were washed with 70% ethanol for one minute followed by Sodium hypochlorite (NaClO) for 5 minutes. Then, the nodules were washed using dH_2O for several times. The surface sterilized nodules were crashed individually with autoclaved glass rod sand multi well mortar and made as suspension.

Rhizobia isolation

One loop of crashed nodule suspension was streaked across Petri dishes containing 20 ml of Congo Red Yeast Extract Mannitol Agar (CREYEMA). Bacterial single colony was identified followed by the subculture process. After sufficient growth of rhizobia, further a loop of culture was streaked repeatedly until pure single colonies were counted (Vincent, 1970).

Morphological and phenotypic characterization

The colony morphology of isolated isolates were observed after 24 hours and 48 hours of incubation at 28°C. Millimeter graph paper was used to measure the colony size and colony colours were detected with naked eyes. The fluidity and sticky nature of the isolates were also determined.

Acid-alkali production of rhizobia

To find out the acidic or alkaline nature of rhizobia, 1µl overnight culture of each isolation 10% Bromothymol Blue (BTB) (v/v) containing YEMA plates was pipetted and incubated at 28°C for 24 to 48 hours.

pH tolerance test of rhizobia

The pH tolerance capacity of the isolates were tested by incubating rhizobial culture (1µl) on pH 4.0, 5.0, 9.0 and 10.0 maintained YEMA plates and grew at 28°C for 24 to 48 hours.

Salt tolerance test of rhizobia

Different concentrations of salt (sodium chloride: 1.0 to 8.0%) were prepared in YEMA (w/v). Then, pure 1µl overnight culture of each isolate was inserted on the mentioned salt containing plate sand incubated at 28°C for 24 to 48 hours.

Temperature tolerance test of rhizobia

Rhizobial isolates bellow room temperature (4°C, 10°C and 20°C) and above room temperature (37°C, 42°C, 47°C and 52°C) were tested. The isolates were grown on YEMA plates by incubating at different temperature for 24 to 48 hours.

Heavy metal test

For the evaluation of heavy metal tolerance of the isolates, 1 mM of Ferric chloride ($FeCl_3$), Copper (II) sulfate ($CuSO_4$), Manganese chloride ($MnCl_2$) and Lead (III) chloride ($PbCl_3$) were added in YEMA plates.

Antibiotics test

Three well known antibiotics named ampicillin, kanamycin and tetracycline were used at100 µg/ml and 200 µg/ml in concentrations. The antibiotics were adjusted in YEMA after auto clave sterilization at tolerable temperature (~60°C) under laminar air flow hood. Overnight culture of each isolate was inoculated on different treatment plates at 28°C for 24 to 48 hours.

RESULT

A total of 22 rhizobial isolates (CBR-01 to CBR-22) were isolated from country bean root nodules (Table 1) and characterized based on their physical and biochemical properties.

Morphological characteristics

All the rhizobial isolates were not morphologically similar;

Table1. Colony morphology of the rhizobial isolates.

Isolates	Colony size (mm)	Colony color	Viscosity
CBR-01	1.17	White	Sticky
CBR-02	1.00	Cream	Non-viscous
CBR-03	1.83	Cream	Non-viscous
CBR-04	1.17	Cream	Non-viscous
CBR-05	2.83	White	Non-viscous
CBR-06	2.67	Cream	Non-viscous
CBR-07	1.17	Pink	Sticky
CBR-08	1.83	Cream	Sticky
CBR-09	1.17	White	Non-viscous
CBR-10	1.33	White	Sticky
CBR-11	1.83	Cream	Sticky
CBR-12	1.83	Cream	Sticky
CBR-13	1.67	Cream	Non-viscous
CBR-14	1.67	Cream	Sticky
CBR-15	0.83	Cream	Non-viscous
CBR-16	1.33	Cream	Sticky
CBR-17	2.50	White	Non-viscous
CBR-18	2.00	Cream	Non-viscous
CBR-19	1.83	Cream	Non-viscous
CBR-20	1.17	Pink	Sticky
CBR-21	0.83	Cream	Sticky
CBR-22	1.00	Cream	Non-viscous

there were some changes among their colony size, colony color and viscosity of mucous. Such as most of the isolates (15 out of 22) showed cream colony, where the colony color of five isolates was white and CBR-07 and CBR-20 were pink in color (Figure 1). The isolates developed colony size ranging from 0.83 to 2.83 mm after 24 hours and from 1.33 to 5.00 mm after 48 hours of streaking at 28^{o}C (Figure 2). The least colony was yielded by CBR-15 and CBR-21 (after one day) while the highest colony was produced by CBR-05 (after one day) and CBR-06 (after two days). The growth rate of all the isolates were not linearly double after double time. Twelve (12) isolates were detected with fluidly mucous and other 10 isolates with sticky mucous after sufficient growth.

Acid and alkali production of the isolates

The isolates yielded acidic, neutral and alkaline mucous after BTB test. Most of the isolates (15 out of 22) produced acidic mucous, they exposed yellow color at 10% BTB containing YEMA media. The isolates CBR-01, CBR-11, CBR-21 and CRB-22 produced neutral mucous which showed green color; and CBR-02, CRB-04 and CBR-09 produced alkaline mucous which showed blue color on the media (Figure 3).

pH test

The rhizobial isolates tolerated and grew normally at both lower and higher pH ranged between pH 5.0 to pH 10.0 with normal growth. Nevertheless, the isolates CBR-01, CBR-02, CBR-09, CBR-11 and CBR-22 were totally sensitive along with CBR-04 showed poor growth at pH 4.0.

Salt tolerance

All the isolates resisted to 1% salt (NaCl) but CBR-01, CBR-02 and CBR-04 showed poor growth at 2 to 3%. However, at least 18 isolates kept their normal growth at 4 to 6%. Nevertheless, 50% isolates stopped their growth and the other isolates survived with poor growth (except CBR-15) at 7% salt (Table 2); and none of them tolerated at 8% salt.

Temperature tolerance

The rhizobial isolates did not grow at 4^{o}C but they were found to grow at 10^{o}C though most of them (16 out of 22) had very poor growth (mentioned as '±' in table) (Table 3). Majority of the isolates (19 out of 22) evolved their normal growth (mentioned as '+') at 20^{o}C. All of the isolates retained their normal growth up to 37^{o}C and 90.90% isolates prolonged this normal growth till 42^{o}C. However, at 47^{o}C, 36.37% isolates did not grow at all, 40.91% isolates sustained with poor growth but 22.72% isolates kept their normal growth. Furthermore, no single isolate grow at 52^{o}C and above.

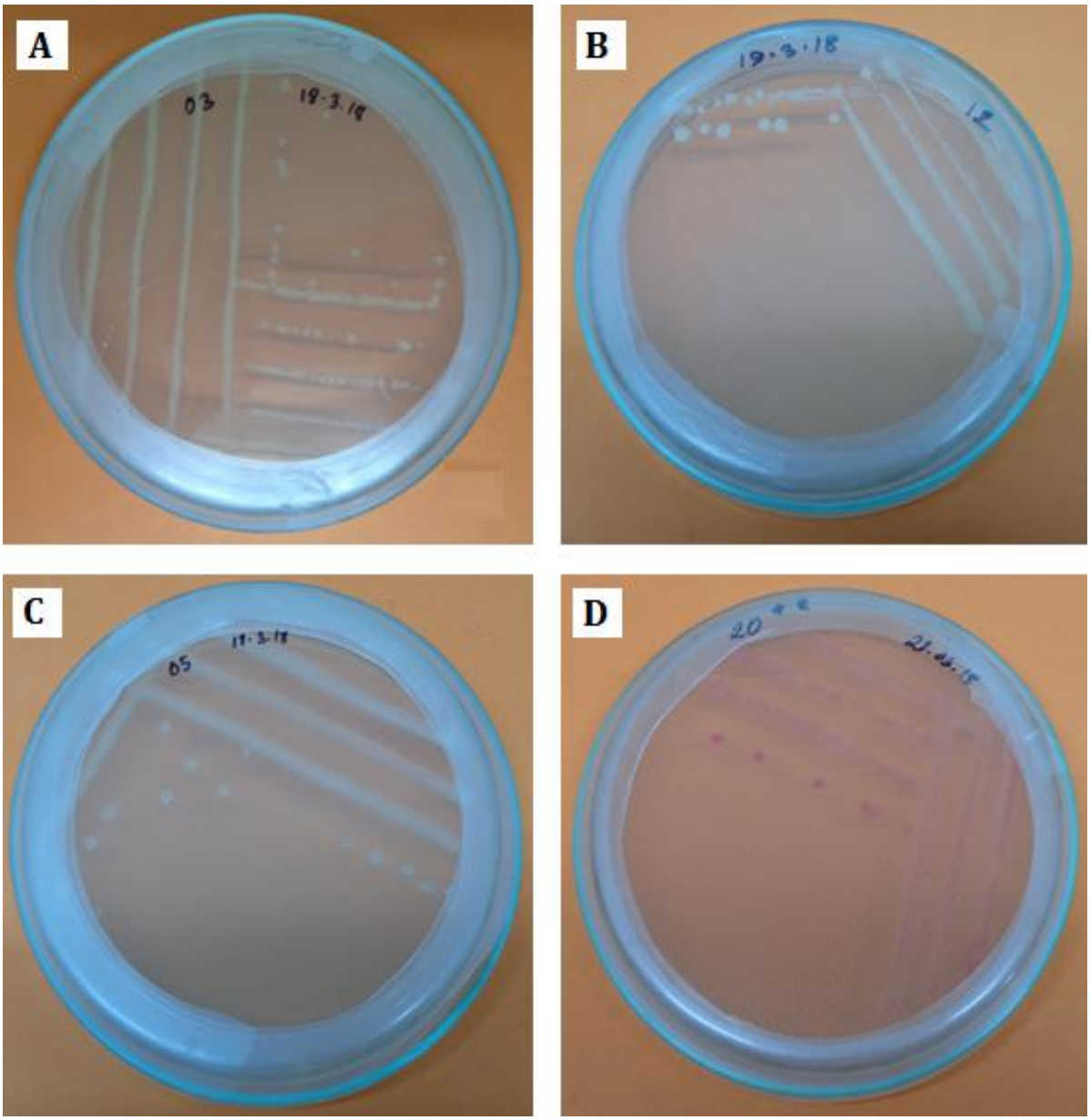

Figure 1. Color and colony morphology of rhizobia. Plate A and B represent cream color isolate (CBR-03 and CBR-12) after one and two days respectively; Plate C represents white color isolate (CBR-05) after one day; and Plate D represents pink color isolate (CBR-20) after two days.

Heavy metal

All the isolates had normal resistance at 1.0 mM of some heavy metallic salt such as Manganese chloride ($MnCl_2$), Iron chloride ($FeCl_3$), Lead chloride ($PbCl_3$) though CBR-02 showed poor growth at that concentration. However, four isolates (CBR-09, CBR-12, CBR-13 and CBR-14) were totally sensitive at 1.0 mM Copper sulfate ($CuSO_4$), nine isolates (CBR-02, CBR-03, CBR-04, CBR-05, CBR-06, CBR-11, CBR-17, CBR-21, CBR-22) showed resistance with poor growth and other nine isolates were completely resistant at $CuSO_4$ with normal growth.

Antibiotics resistance of the isolates

The isolates showed both resistance and sensitivity at 100 µg/ml and 200 µg/ml ampicillin, kanamycin and tetracycline. Here, 40.91%, 31.81% and 27.27% isolates were resistant at 100 µg/ml ampicillin, kanamycin and tetracycline respectively. Nevertheless, the number of resistant isolates became almost half when they were

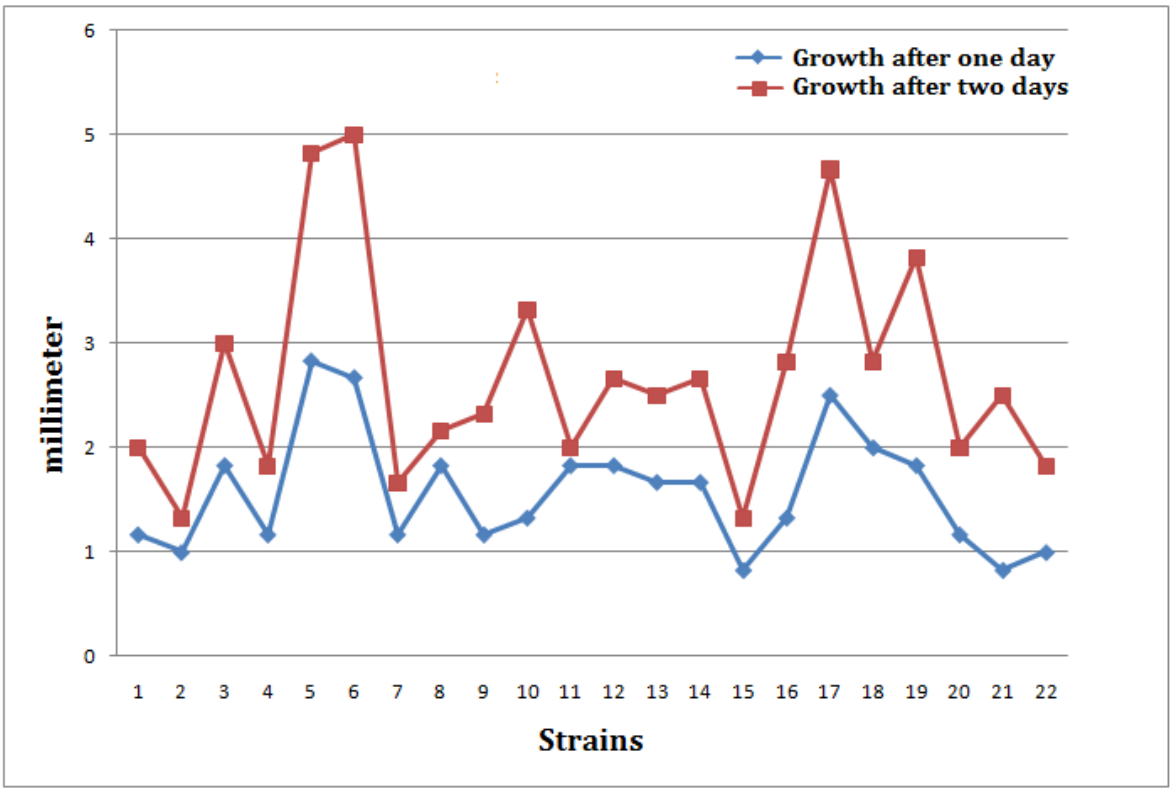

Figure 2. The graph of the growth rate of rhizobial isolates after one day and two days.

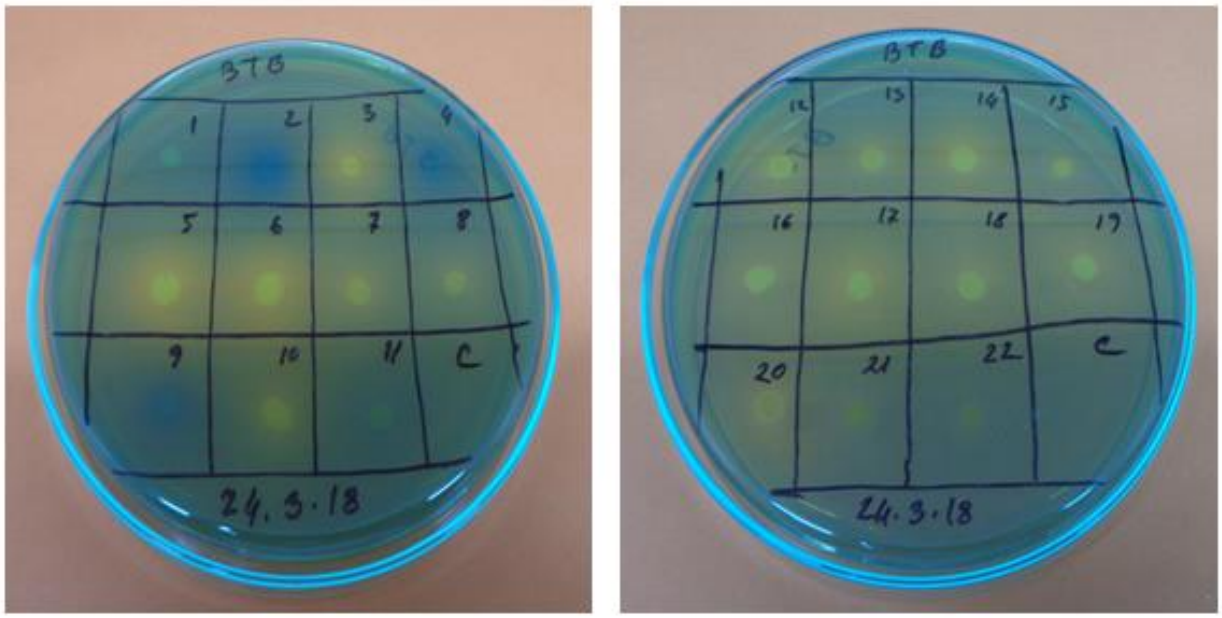

Figure 3. Plates exhibited the rhizobial cultures at 10% BTB containing YEMA after one day.

treated at 200 µg/ml of each antibiotic. There were some isolates survived with poor growth (mentioned as ±, Table 4).

DISCUSSION

In this study, according to colony size, it was observed that

Table 2. Salt (NaCl) tolerance profile of the isolates.

Isolate	1%	2%	3%	4%	5%	6%	7%	8%
CBR-01	+	+	±	-	-	-	-	-
CBR-02	+	±	-	-	-	-	-	-
CBR-03	+	+	+	+	+	+	±	-
CBR-04	+	±	±	-	-	-	-	-
CBR-05	+	+	+	+	+	+	±	-
CBR-06	+	+	+	+	+	+	-	-
CBR-07	+	+	+	+	+	+	-	-
CBR-08	+	+	+	+	+	+	-	-
CBR-09	+	+	+	±	-	-	-	-
CBR-10	+	+	+	+	+	+	±	-
CBR-11	+	+	+	+	+	±	-	-
CBR-12	+	+	+	+	+	+	±	-
CBR-13	+	+	+	+	+	+	±	-
CBR-14	+	+	+	+	+	+	±	-
CBR-15	+	+	+	+	+	+	+	-
CBR-16	+	+	+	+	+	+	±	-
CBR-17	+	+	+	+	+	+	±	-
CBR-18	+	+	+	+	+	+	±	-
CBR-19	+	+	+	+	+	+	±	-
CBR-20	+	+	+	+	+	+	-	-
CBR-21	+	+	+	+	+	+	-	-
CBR-22	+	+	+	+	+	+	-	-

N.B. + indicated positive growth, - indicated negative growth and ± indicated poor growth.

Table3. Temperature tolerance profile of the isolates.

Isolate	4ºC	10ºC	20ºC	37ºC	42ºC	47ºC	52ºC
CBR-01	-	±	+	+	±	-	-
CBR-02	-	±	±	+	±	-	-
CBR-03	-	±	+	+	+	±	-
CBR-04	-	±	+	+	+	±	-
CBR-05	-	±	+	+	+	+	-
CBR-06	-	+	+	+	+	+	-
CBR-07	-	+	+	+	+	-	-
CBR-08	-	+	+	+	+	±	-
CBR-09	-	±	+	+	+	-	-
CBR-10	-	+	+	+	+	±	-
CBR-11	-	±	+	+	+	+	-
CBR-12	-	±	+	+	+	±	-
CBR-13	-	±	+	+	+	±	-
CBR-14	-	±	±	+	+	+	-
CBR-15	-	±	±	+	+	-	-
CBR-16	-	±	+	+	+	-	-
CBR-17	-	±	+	+	+	±	-
CBR-18	-	+	+	+	+	±	-
CBR-19	-	+	+	+	+	±	-
CBR-20	-	±	+	+	+	-	-
CBR-21	-	±	+	+	+	+	-
CBR-22	-	±	+	+	+	-	-

N.B. + indicated positive growth, - indicated negative growth and ± indicated poor growth.

Table4. Antibiotic tolerance profile of the isolates.

Isolate	Ampicillin (100µg/ml)	Ampicillin (200µg/ml)	Kanamycin (100µg/ml)	Kanamycin (200µg/ml)	Tetracycline(100µg/ml)	Tetracycline (200µg/ml)
CBR-01	+	±	±	±	-	-
CBR-02	+	+	+	-	-	-
CBR-03	-	-	-	-	-	-
CBR-04	+	+	-	-	-	-
CBR-05	+	-	-	-	-	-
CBR-06	+	-	-	-	-	-
CBR-07	-	-	-	-	+	+
CBR-08	-	-	+	+	-	-
CBR-09	-	-	-	-	-	-
CBR-10	-	-	-	-	±	-
CBR-11	-	-	-	-	-	-
CBR-12	-	-	-	-	-	-
CBR-13	-	-	-	-	-	-
CBR-14	-	-	-	-	-	-
CBR-15	+	+	+	+	-	-
CBR-16	-	-	-	-	±	-
CBR-17	+	±	+	+	-	-
CBR-18	+	-	+	-	-	-
CBR-19	-	-	-	-	+	-
CBR-20	+	-	-	-	+	+
CBR-21	-	-	-	-	±	-
CBR-22	-	-	±	-	-	-

N.B. + indicated positive growth, - indicated negative growth and ± indicated poor growth.

the growth rate of all isolates were not the same, some grew rapidly and some slowly. It is the natural endophytes of rhizobia as described by Degefu et al. (2018a). Mucous production and generation time vary the colony size of rhizobia and the mean generation time for rapid growing rhizobia is between 2 and 4 hours; and for slow growing rhizobia is 6 hours (Singh et al., 2013). Extracellular polysaccharides might increase the viscosity of mucous (Degefu et al., 2018b). Rhizobia produce both acidic and alkaline mucous and exhibit yellow and blue color respectively at BTB containing YEMA media (Vincent, 1970; Wolde-meskel et al., 2004; Demissie et al., 2018). It was also observed that four isolates showed green color at BTB which indicated that the isolates produced neutral (pH 7.0) mucous at the media (Brasca et al., 2018; Rahman et al., 2018). Stephen and Bohlool (1985) and Chen (2000) also observed that the alkali producing strains showed blue color but the fast growing acid producing strains showed yellow color.

Rhizobia can tolerate a wide range of pH between 4.0 and 10.0 (Correa and Barneix, 1997; Sharma et al., 2010). Most of the isolates of this studies were acid producers but they tolerated at alkaline pH 9.0 to 10.0, but more noticeable matter was that alkali producers and neutral mucous producers did not tolerate lower pH 4.0, though they tolerated pH 5.0. Perhaps acidic mucous reacted with alkaline pH and vice versa, thus they made the media neutral. Similarly, Degefu et al. (2018a) tested that the rhizobial strains could survive at pH values ranging between 5.0 and 11.0.

Rhizobia may be both resistant and sensitive to salt as Abo-Aba et al. (2015) described that some rhizobial strains tolerated salt (NaCl) ranging from 0.5 to 4%. The rhizobia were isolated from different country bean fields stand beside the river Naboganga which flows fresh water but during summer session it bears some saline water of Bay of Bengal. That's why most of the isolates were competent to tolerate higher level of salt concentrations (5 to 7%). Wei et al. (2004) reported several genes remaining in rhizobia were responsible to adapt salinity. However, the fast growing rhizobia were found to tolerant more salt than slow growing rhizobia (Hua et al., 1982; Zahran, 1999).

Rhizobia can cope up with different indigenous environmental conditions, temperature for instance. According to previous studies, it could be concluded that rhizobia have a capacity to tolerate a wide range of temperature, though their growth varies with the change of temperature (Hungria and Vargas, 2000). For instance, Abo-Aba et al. (2015) isolated three rhizobial strains in Saudi Arabia which grew within a wide range of temperature from 30 to 60^{o}C. Michiels et al. (1994) detected an increased level of 14 heat shock proteins in rhizobial strains, grew within 40 to 45^{o}C, were considered responsible for high temperature tolerance. The bacteria

isolated showed visible growth between 10 and 47°C and this result agreed with the work of Zahran (1999) and Berrada et al. (2012).

The isolates showed resistance at 0.1 mM $MnCl_2$, $FeCl_3$ and $PbCl_3$ metallic salts and some of the isolates were sensitive at 0.1 mM $CuSO_4$. In general, soil contains numerous types of salts, mineral, metals, ions and other components, as a result sometimes microbes increase their resistance with the intrinsic heavy metals (Degefu et al., 2018b). Some rhizobia have the capacity to grow at highly polluted soil (Zhang et al., 1991; Alikhani and Yakhchali, 2010).

Though some isolates showed resistance but most of the isolates were sensitive to 100 and 200 µg/ml concentrations of ampicillin, kanamycin and tetracycline. More noticeable was that isolates CBR-01, CBR-02, CBR-15 and CBR-17 tolerated at 100 µg/ml ampicillin and kanamycin but not at 100 µg/ml tetracycline; where only CBR-20 tolerated both at ampicillin and tetracycline at 100 µg/ml. Besides, no isolate tolerated kanamycin and tetracycline together which indicated that every isolate did not contained all of these three antibiotic resistant genes at a time. Previous reports described that generation time interfered with antibiotic tolerance like fast growing strains were more sensitive to different antibiotics than slow growing strains (Jordan, 1984; Maâtallah et al., 2002).

Conclusion

The ultimate goal of the current study was to explore the intrinsic characteristics of country bean rhizobia in a certain country bean cultivation area in Bangladesh. Here, twenty-two isolates were characterized from numerous nodule samples. Most of the isolates showed significant resistance against different implemented treatments except the antibiotics, though all of them did not show similar tolerance against each treatment. Generally, each specimen dose not tolerate every environmental and exterior stress but the most competent isolates could be recommended for relevant cultivation fields as bio-fertilizer. For example, the isolates were mesospheric and also tolerated a wide range pH could be implied both at subtropical acidic and alkaline lands; but only the higher salt (6 to 7% NaCl) tolerant isolates could be competent for costal area. Similarly, the antibiotics and metallic salts stresses tolerant isolates might be selected for heavy metal rich lands where they could survive and function at optimum level.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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