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Full Length Research Paper

Isolation and selection of *Bradyrhizobium* from the root nodules of indigo plants (*Indigofera tinctoria* L.)

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This research was conducted from 2004 to 2005 to isolate and select *Bradyrhizobium* from the root nodules of indigo plants (*Indigofera tinctoria* L.). For isolation of *Bradyrhizobium*, root nodules were collected from indigo plants. Fourteen *Bradyrhizobium* isolates were identified depending on colony, morphological and biochemical characteristics. Out of fourteen isolates, six (HSTU-IR_2, HSTU-IR_3, HSTU-IR_4, HSTU-IR_9, HSTU-IR_{10} and HSTU-IR_{14}) were found promising with respect to nodulation, shoots and roots weights and N fixation by the indigo plants grown inside the Leonard Bottle Jar Assembly (LBJA).

Key words: *Bradyrhizobium*, isolation, selection, root nodules, indigo plants.

INTRODUCTION

Increasing cropping intensity to meet the demands for food and other necessities for a swelling population has resulted in a gradual deterioration of the soil resource of Bangladesh over the years. In the recent years, intensive crop cultivation using high yielding varieties with imbalanced fertilization has led to mining out the inherent plant nutrients from the soils and thus fertility status of the soils has severely declined in Bangladesh (Karim et al., 1994; Ali et al., 1997), which causes declining crop yields (Cassman et al., 1997). On the other hand, organic matter content of most of the Bangladesh soils is very low (<1.5%) (BARC, 1997) moreover, the addition of organic materials through farm yard manure (FYM), compost and organic residues has been reduced considerably because a major portion of these organic residues (cow dung and crop residue) is used up as fuel by the rural people. These practice resulted in a serious deterioration of soil health showing cumulative negative nutrient balance, which caused declining soil fertility and thereby productivity. Restoring, maintaining and increasing soil fertility are major agricultural priorities in many parts of the developing world where soils are inherently poor in plant nutrients, and the demand for grain food and raw materials is increasing rapidly.

Sustainable production of crops cannot be maintained by using chemical fertilizers alone. Nutrients need to be added from other sources such as organic manure and biofertilizer for providing greater stability in production and improving soil fertility. Bangladesh soils are conspicuously low in nitrogen (N) due to rapid decomposition of organic matter under congenial high temperature and humid conditions, losses of N due to volatilization and denitrification, considerable leaching and high removal by intensive cropping with modern varieties. The continuous application of chemical nitrogenous fertilizers has become detrimental for soil fertility and accelerated environmental pollution. Therefore, the use of organic manure and biofertilizer has been increasing and gaining global attention.

Inclusion of a cereal legume in the cropping pattern, inter or relay cropping of cereal legumes may come into picture to share the dire need of adding organic residues into soils. Indigo may contribute significantly to address this desperate situation. Farmers of the different parts of the world are cultivating indigo for different purposes

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such as improving organic matter content in soil (Barrios et al., 2005, Augustin et al., 1999), production of dye used in textile and preparation of medicine (Ahmed, 2002; Sreepriya et al., 2001; Singh et al., 2001; Kavimani et al., 2000; Dahot, 1999) as well as for green manuring (Garrity et al.,1994; Bantilan et al., 1989). Farmers of Bangladesh under the districts of Nilphamary and Rangpur are cultivating indigo plants as green manure and to control nematode from the tobacco fields (Ahmed, 2002). Indigo has already earned farmers acceptance although there had been no research activities on this important issue for finding out suitable cultivation technology. More surprising is that global literature on indigo is minimum and practically there is none on the isolation of *Bradyrhizobium* from root nodules, their efficacy test, preparation of inoculants for use as biofertilizers-which is so vital for such a potential legume like indigo. Under the above circumstances, this pioneering research programme was planned for Isolation of *Bradyrhizobium* from root nodules of indigo plants, their characterization and confirmation.

MATERIALS AND METHODS

Study area and collection of root nodules of indigo plants

To isolate *Bradyrhizobium,* root nodules of Indigo (*Indigofera tinctoria* L) were collected from two locations namely Rangpur (25°45′ N latitude and 89°15′ E longitude) and Nilphamari districts of Bangladesh. Indigo plants were uprooted with intact soil carefully with the help of a spade so that no nodules were left. The samples were carried to the Microbiology Laboratory of the Department of Soil Science, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh. After good soaking in water in a can, the roots with nodules were washed carefully in a slow stream of tap water and finally cleaned with a soft camel hairbrush to remove the soil particles adhering to the root surface. Healthy, intact, firm and pink nodules were selected. Nodules were separated from the roots by cutting the root about 0.5 cm on other side of the nodule attachment.

Isolation of *Bradyrhizobium*

Surface sterilization of the nodules were done by first immersing those in 95% ethyl alcohol for up to 5 s followed by keeping for 4 to 5 min in 0.1% acidified mercuric chloride. After that, the nodules were rinsed in 6 changes of sterile water, making transfer with alcohol flamed forceps. The washed nodules were then taken in a sterile Petri dish and crushed individually in a small aliquot of sterile water with the help of a sterile glass rod. The fluid from crushed nodules was streaked out on YMA plates with the help of a sterile inoculation needle (Somasegaran and Hoben, 1985). The plates were incubated up to 10 days in an incubator at 28°C. Large gummy colonies of bacteria emerged within 7 to 9 days after incubation. Sub culturing was done for getting the pure culture. For this, a loopful of inoculum was suspended in dispersion buffer saline (Vincent, 1970). The suspension was streaked over fresh YMA plates and incubated in an incubator at 28°C for 5 to 10 days. Colonies were observed and recorded the frequency of different forms of colonies. Their detailed morphological features (shape, size, opacity, elevation, surface, margin, consistency chromo-genesis and odour) were observed. Typical rhizobial colonies were fished out which were in majority, and repeated the technique till colonies of consistent morphology were observed. Growth from a discrete rhizobial type colonies showing consistent colony morphology were transferred to YMA slants in screw-capped test tubes, and were incubated till sufficient growth appeared. Then they were stored in a refrigerator for further tests.

Characterization of *Bradyrhizobium* isolates

The form, margin, surface, optical characteristics, consistency and pigmentation of the colonies were studied on YMA media according to Harry and Paul (1972). The shape of bacteria was examined by direct staining with crystal violet solution. Gram reaction was carried out by modified Hucker and Conn's method (1923). Motility was examined by hanging drop method. Growth on glucose peptone agar (GPA), starch hydrolysis, utilization of citrates, gelatin hydrolysis, Congo red dye absorption and acid/alkaline production tests were done according to Vincent (1970).

Confirmation of *Bradyrhizobium* isolates through nodulation test

Nodulation test by Leonard Bottle Jar Assembly (LBJA) was conducted in the Soil Microbiology Laboratory of the Department of Soil Science, HSTU, Bangladesh in the ambient room temperature. Leonard Bottle Jar Assembly (LBJA) is an instrument having two parts. By cutting the bottom of a wide bottle of 750 ml capacity and plugging the narrow mouth with a long wick made the upper part. The lower part was made by a jar of one liter capacity, having dimension suitable for setting the bottom cut bottle inverted in such a way that the neck of the bottle snugly fits in. The upper parts of the LBJA were filled with 500 g sterilized sieved sand up to 1 cm below the top and saturated with Jenson's seedling solution until the solution began to drip through the wick at the bottom. The lower part of the assembly was filled with the same solution. The sand was covered with a Petri dish and the assemblies were wrapped partially with brown paper. The entire set-up was sterilized in an autoclave and cooled down before use. There were 15 treatments along with an uninoculated control.

Treatment combinations and *Bradyrhizobium* isolates preparation

The treatment combinations were T_1: Uninoculated control, T_2: HSTU-IR_2, T_3: HSTU-IR_3, T_4: HSTU-IR_4, T_5: HSTU-IR_5, T_6: HSTU-IR_6, T_7: HSTU-IR_7, T_8: HSTU-IR_8, T_9: HSTU-IR_9, T_{10}: HSTU-IR_{10}, T_{11}: HSTU-IR_{11}, T_{12}: HSTU-IR_{12}, T_{13}: HSTU-IR_{13}, T_{14}: HSTU-IR_{14}, T_{15}: HSTU-IR_{15}. The experiment was laid out with complete randomized design (CBD) with 3 replications. Indigo (*Indigofera tinctoria* L) was used as test crop. Surface sterilized seed were sown to a depth of 2 to 3 cm in each jar with the help of an aseptic glass rod and a forceps. Uninoculated seeds were sown first and then the inoculated seeds. Fourteen *Bradyrhizobium* isolates were prepared following Vincent (1970) method. Yeast Mannitol Broth was prepared in each of 500 ml conical flasks containing (g/L basis) 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.4 g yeast extract and 1 L of distilled water. The conical flasks with the liquid medium were sterilized in an autoclave at 121°C and 15 PSI for 20 min. The medium was cooled to 40°C. A loopful of the respective culture was transferred aseptically from slant to the liquid medium in the flask with the help of a sterilized inoculation needle. The flasks were placed on a rotary shaker. After 6 to 7 days, when the medium in the flask showed milky white growth, the broth cultures were tested for purity and growth @ more than 1×10^9 cells/ml broth. Thus, they were ready for inoculation.

Each pot except the control was inoculated separately by one ml of broth culture of each isolate with the help of 1 ml pipette at 6

days after sowing of seeds. After germination of the seeds, the Petri dishes removed and were covered with dry sterile gravel to protect air borne contamination. After assessing the vigour, one healthy plant was kept in each jar by removing the other two plants. After 50 days of sowing the seeds, the upper part of LBJA with indigo seedlings was washed with gentle flow of water. The roots of the seedlings were placed in a tray filled with water and the roots were examined for the presence of nodules. After harvesting, the nodules from each plant were collected separately and counted. Then the plant samples were cut at the junction of shoots and roots. The shoots and roots were again cut into small pieces and sun dried for 2 days. The cut pieces of the shoots, roots and nodules were oven dried for 2 days at 70°C and the dry weights recorded by using electric balance. The oven dried shoots, roots and nodules samples were ground and stored in desiccators before they were used for total N determination.

Chemical analysis

Nitrogen content in plant samples (nodules, shoots and roots) was determined by the standard micro-kjeldahl method (AOAC, 1980). Plant samples (0.1 g) were digested with 3 ml concentrated H_2SO_4, 1.1 g catalyst mixture (K_2SO_4: $CuSO_4$. 5 H_2O: Se powder in the ratio of 100: 10: 1) and 2 ml H_2O_2. Nitrogen in the digest was determined by distilling the digest with 10 N NaOH followed by titration of the distillate trapped in H_3BO_3 indicator.

Statistical analysis

Data obtained from the pot experiment were analyzed statistically by the method of analysis of variance of Fisher (1958). The treatment means of different characters were compared following Duncan's new multiple range test (DMRT) as out lined by Gomez and Gomez (1984).

RESULTS

Isolation of *Bradyrhizobium*

Fourteen *Bradyrhizobium* isolates were obtained from the root nodules of indigo plants (*Indigofera tinctoria* L.) on YMA medium in the Soil Microbiology Laboratory, Department of Soil Science, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur. The obtained isolates were named as: i) HSTU-IR_2, ii) HSTU-IR_3, iii) HSTU-IR_4, iv) HSTU-IR_5, v) HSTU-IR_6, vi) HSTU-IR_7, vii) HSTU-IR_8, viii) HSTU-IR_9, ix) HSTU-IR_{10}, x) HSTU-IR_{11}, xi) HSTU-IR_{12}, xii) HSTU-IR_{13}, xiii) HSTU-IR_{14} and xiv) HSTU-IR_{15}.

Characterization of *Bradyrhizobium* isolates

Colony characteristics

The colonies of *Bradyrhizobium* isolates of indigo plants appeared on Yeast Mannitol Agar (YMA) media within 7 to 9 days after inoculation. The colony characteristics of all these *Bradyrhizobium* isolates were very similar. All isolates produced milky white, circular and smooth surface colonies on YMA media with distinct margin around.

Morphological characteristics

All the *Bradyrhizobium* isolates of indigo plants under the present study were rod shaped, motile and Gram negative.

Biochemical characteristics

The biochemical characteristics of different *Bradyrhizobium* isolates have been tested in the laboratory with respect to congo red dye absorption, starch hydrolysis, Bromothymol blue (BTB), glucose pentone agar (GPA), growth in Hofer's alkaline broth, utilization of citrate and hydrolysis of gelatin. None of the *Bradyrhizobium* isolates could hydrolyze starch and gelatin, and failed to utilize citrate. It was observed that all the *Bradyrhizobium* isolates except HSTU-IR_{11} absorbed congo red dye slightly. The isolate HSTU-IR_{11} grew poorly on glucose peptone agar at 48 h of inoculation whereas others did not grow. Among the isolates, none was observed to grow in Hofer's alkaline broth. All these tests mentioned above confirmed that the obtained isolates were of *Bradyrhizobium* of indigo plants (Table 1).

Confirmation of *Bradyrhizobium* isolates through nodulation test

Number of nodules

Bradyrhizobium isolates significantly influenced nodules formation on the roots of indigo plants in LBJA (Table 2). Inoculation of *Bradyrhizobium* isolates helped formation of nodules indicating their effectiveness. On the other hand, the control plants receiving no *Bradyrhizobium* failed to produce nodules. The isolate HSTU-IR_3 produced the highest number of nodules (16.0 plant^{-1}), which was statistically identical to that of the isolate HSTU-IR_4 but superior to all others. However, the isolate HSTU-IR_4 was statistically superior to all other isolates except HSTU-IR_2 and HSTU-IR_{10}. The isolate HSTU-IR9 was also similar to the isolates HSTU-IR_{10} and HSTU-IR_{14}. The isolates HSTU-IR_2, HSTU-IR_3, HSTU-IR_4, HSTU-IR_9, HSTU-IR_{10} and HSTU-IR_{14} produced higher number of nodules than the other isolates.

Nodule weight

The effect of inoculation with different Bradyrhizobium isolates on nodule weight of indigo plants was highly

Table 1. Biochemical characteristics of different indigo *Bradyrhizobial* isolates.

Isolate/strain	Congo red test (dye absorption)		Starch hydrolysis	Citrate utilization	Gelatin hydrolysis	BTB test	Growth on GPA	Hofer`s alkalin broth
	Young culture	Old culture						
HSTU-IR_2	Not absorbed	Weakly absorbed	_	_	_	+++	No growth	No growth
HSTU-IR_3	Not absorbed	Weakly absorbed	_	_	_	+++	No growth	No growth
HSTU-IR_4	Not absorbed	Weakly absorbed	_	_	_	+++	No growth	No growth
HSTU-IR_5	Not absorbed	Weakly absorbed	_	_	_	++	No growth	No growth
HSTU-IR_6	Not absorbed	Weakly absorbed	_	_	_	++	No growth	No growth
HSTU-IR_7	Not absorbed	Weakly absorbed	_	_	_	++	No growth	No growth
HSTU-IR_8	Not absorbed	Weakly absorbed	_	_	_	+	No growth	No growth
HSTU-IR_9	Not absorbed	Weakly absorbed	_	_	_	+++	No growth	No growth
HSTU-IR_{10}	Not absorbed	Weakly absorbed	_	_	_	+++	No growth	No growth
HSTU-IR_{11}	Not absorbed	Strongly absorbed	_	_	_	++	Poorly growth	No growth
HSTU-IR_{12}	Not absorbed	Weakly absorbed	_	_	_	++	No growth	No growth
HSTU-IR_{13}	Not absorbed	Weakly absorbed	_	_	_	++	No growth	No growth
HSTU-IR_{14}	Not absorbed	Weakly absorbed	_	_	_	+++	No growth	No growth
HSTU-IR_{15}	Not absorbed	Weakly absorbed	_	_	_	++	No growth	No growth

- = Negative (presence of bluish purple color), + = slight alkali producer, ++ = medium alkali producer, +++ = high alkali producer.

significant (Table 2). All the inoculated isolates showed higher nodule weights compared to that of the control. Isolate HSTU-IR_3 produced the highest nodule weight (22.40 mg $plant^{-1}$), which was statistically higher than those of all other isolates. Higher nodule weight was recorded for the isolate HSTU-IR_4 that was statistically similar to the weight produced by the isolate HSTU-IR_2. The nodule weight recorded against the isolate HSTU-IR_4 was followed by the nodule weight produced by the isolates HSTU-IR_2, HSTU-IR_{10}, HSTU-IR_9 and HSTU-IR_{14}. All these were found statistically higher than those of other treatments.

Shoot weight

The effect of Bradyrhizobium on the shoot weights was significant (Table 2). The results revealed that all the isolates produced significantly higher shoot weights over the control. The highest shoot weight (651.6 mg $plant^{-1}$) was recorded for the isolate HSTU-IR_3 and it was statistically alike to the shoot weights recorded for the isolates HSTU-IR_2 and HSTU-IR_4. The isolates HSTU-IR_6, HSTU-IR_9, HSTU-IR_{10}, HSTU-IR_{12}, HSTU-IR_{13} and HSTU-IR_{14} were found statistically similar to each other and produced higher amounts of shoot weights over the control. The lowest (184.0 mg $plant^{-1}$) shoot weight was found in uninoculated control.

Root weight

Bradyrhizobium isolates significantly influenced the root weight of the indigo plants in Leonard Bottle Jar Assembly (Table 2). The maximum (171.3 mg $plant^{-1}$) root weight was found for HSTU-IR_3 inoculation and it was statistically similar to that of the isolates HSTU-IR_4 (169.5 mg $plant^{-1}$) and HSTU-IR_2 (157.4 mg $plant^{-1}$).The isolate HSTU-IR_2 was statistically comparable to the isolates HSTU-IR_9 (146.4 mg $plant^{-1}$), HSTU-IR10 (145.2 mg $plant^{-1}$) and HSTU-IR_{14} (143.2 mg $plant^{-1}$) in terms of root weight of indigo plants. The control plants recorded the lowest (44.0 mg $plant^{-1}$) root weight.

Total biomass

Bradyrhizobium isolates significantly influenced the production of biomass of indigo plants in

Table 2. Effect of *Bradyrhizobium* isolates on nodule number, nodule weight, shoot weight, root weight and total biomass of indigo plants in Leonard bottle jar assembly (weights are on dry matter basis).

Treatment	Nodule number (plant^{-1})	Nodule weight (mg plant^{-1})	Shoot weight (mg plant^{-1})	Root weight (mg plant^{-1})	Total biomass (nodule+shoot+root) (mg plant^{-1})
Control	0.0^{g}	0.00^{i}	184.0^{e}	44.0^{g}	228.0^{h}
HSTU-IR$_2$	14.5^{bc}	21.10^{b}	603.5^{a}	157.4^{ab}	782.0^{b}
HSTU-IR $_3$	16.0^{a}	22.40^{a}	651.6^{a}	171.3^{a}	845.3^{a}
HSTU-IR $_4$	15.3^{ab}	21.14^{b}	606.5^{a}	169.5^{a}	797.1^{ab}
HSTU-IR $_5$	10.0^{e}	12.17^{g}	453.0^{c}	118.0^{d}	583.2^{e}
HSTU-IR $_6$	12.3^{d}	15.10^{f}	526.6^{b}	116.2^{d}	657.9^{d}
HSTU-IR $_7$	10.3^{e}	12.00^{g}	380.5^{d}	104.2^{de}	496.7^{e}
HSTU-IR $_8$	9.0^{ef}	10.80^{h}	383.5^{d}	100.5^{ef}	494.8^{e}
HSTU-IR $_9$	13.3^{cd}	18.20^{d}	542.2^{b}	146.4^{bc}	706.8^{c}
HSTU-IR $_{10}$	14.3^{bc}	19.57^{c}	533.8^{b}	145.2^{bc}	698.6^{c}
HSTU-IR $_{11}$	6.7^{ef}	11.25^{gh}	398.2^{d}	95.10^{ef}	504.6^{f}
HSTU-IR $_{12}$	12.0^{d}	16.60^{e}	510.2^{bc}	136.8^{c}	663.6^{d}
HSTU-IR $_{13}$	12.6^{d}	16.80^{e}	510.1^{b}	137.9^{c}	664.8^{d}
HSTU-IR $_{14}$	13.3^{cd}	18.20^{d}	513.8^{b}	143.2^{bc}	675.2^{c}
HSTU-IR $_{15}$	8.3^{f}	10.42^{h}	345.1^{d}	85.00^{f}	440.5^{g}
CV (%)	7.83	4.21	5.87	7.27	5.02

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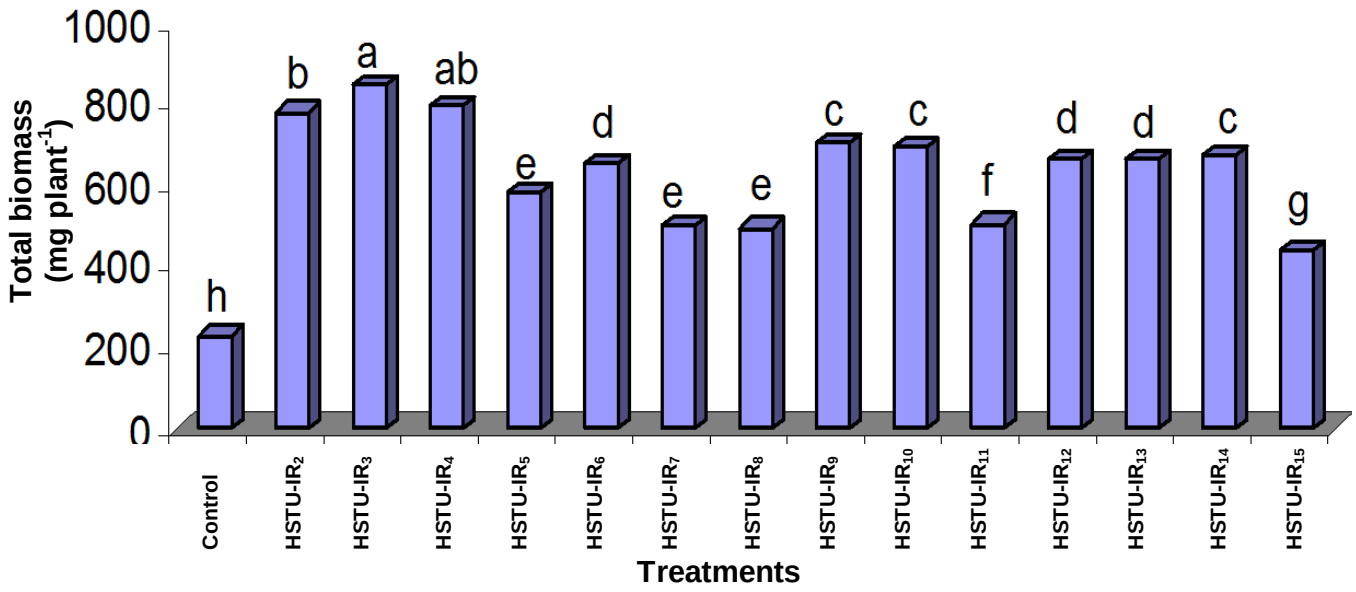

Figure 1. Effect of *Bradyrhizobium* isolates on the total biomass (nodule + shoot + root) of indigo plants in Leonard bottle jar assembly (weights are on dry matter basis).

Leonard Bottle Jar Assembly (Table 2 and Figure 1). All the isolates influenced higher total biomass production over the control. The isolate HSTU-IR$_3$ produced significantly highest biomass (845.3 mg plant^{-1}) and the lowest (228.0 mg plant^{-1}) was in the control. The isolate HSTU-IR$_3$ was statistically similar to the isolate HSTU-IR$_4$ and the isolate HSTU-IR$_2$ was statistically identical to the isolate HSTU-IR$_4$. The isolate HSTU-IR$_9$ was statistically similar to the isolates HSTU-IR$_{10}$ and HSTU-IR$_{14}$.

Nitrogen fixation by the indigo plants

The *Bradyrhizobium* isolates initiated nodule formation

Table 3. Effect of *Bradyrhizobium* isolates on N content in nodule, shoot and root of indigo plants at Leonard bottle jar assembly.

Treatment	Total N in nodule (mg plant^{-1})	Total N in shoot (mg plant^{-1})	Total N in root (mg plant^{-1})	Total N in plants (nodule+shoot+root) (mg plant^{-})	Total N fixation (mg plant^{-1})
Control	-	2.23^{h}	0.30^{k}	2.53^{h}	-
HSTU-IR$_2$	0.578^{ab}	10.57^{b}	1.55^{c}	12.70^{b}	10.17^{b}
HSTU-IR$_3$	0.627^{a}	11.78^{a}	1.74^{a}	14.15^{a}	11.62^{a}
HSTU-IR$_4$	0. 584^{ab}	10.91^{b}	1.70^{b}	13.19^{ab}	10.66^{ab}
HSTU-IR$_5$	0.316^{f}	7.47^{e}	1.09^{g}	8.88d^{e}	6.35^{de}
HSTU-IR$_6$	0.397^{e}	8.42^{d}	1.15^{f}	9.97^{cd}	7.44^{d}
HSTU-IR$_7$	0. 312^{f}	5.99^{f}	0.85^{i}	7.15^{fg}	4.62^{f}
HSTU-IR$_8$	0.270^{f}	5.95^{f}	0.94^{h}	7.16^{fg}	4.63^{f}
HSTU-IR$_9$	0.495^{cd}	9.21^{c}	1.40^{d}	11.11^{c}	8.58^{c}
HSTU-IR$_{10}$	0.532^{bc}	9.08^{cd}	1.41^{d}	11.02^{c}	8.49^{c}
HSTU-IR$_{11}$	0.281^{f}	6.17^{f}	0.82^{i}	7.27f^{g}	4.74^{f}
HSTU-IR$_{12}$	0.415^{e}	7.83^{e}	1.29^{e}	10.54^{c}	8.01^{d}
HSTU-IR$_{13}$	0.435^{e}	8.67^{d}	1.30^{e}	10.41^{c}	7.88^{ef}
HSTU-IR$_{14}$	0.491^{cd}	9.00^{cd}	1.43^{d}	10.92^{c}	8.39^{c}
HSTU-IR$_{15}$	0. 258^{f}	4.93^{g}	0.68^{j}	5.870^{g}	3.34^{g}
CV (%)	8.22	3.9	3.79	8.75	8.57

In the column, figures having similar letter(s) do not differ significantly at 5% level of probability.

on the roots of indigo plants and N_2-fixation occurred in the nodules. The control plants had no nodule and therefore received nitrogen from the seed.

Total N in nodule

There was marked variation among the different *Bradyrhizobium* isolates on total N content in nodules (Table 3). The highest amount of N (0.627 mg plant^{-1}) was found for the isolate HSTU-IR$_3$, which was statistically identical to those of the isolates HSTU-IR$_4$ and HSTU-IR$_2$. The isolate HSTU-IR$_{10}$ showed statistically similar amount of N content compared to those found for the isolates HSTU-IR$_9$ and HSTU-IR$_{14}$. The lowest N content in nodules (0.258 mg plant^{-1}) was in the isolate HSTU-IR$_{15}$ and was statistically comparable to isolates HSTU-IR$_5$, HSTU-IR$_7$, HSTU-IR$_8$ and HSTU-IR$_{11}$.

Total N in shoot

The inoculation of indigo seeds with different *Bradyrhizobium* isolates significantly influenced the total N content in the shoots (Table 3). All the isolates helped to increase the N content in shoots over the uninoculated control. The highest total N content of 11.78 mg plant^{-1} was recorded for the isolate HSTU-IR$_3$, which was statistically superior to all other isolates. The isolate HSTU-IR$_2$ (10.57 mg plant^{-1}) was statistically comparable to the isolate HSTU-IR$_4$ (10.91 mg plant^{-1}). The isolate HSTU-IR$_9$ (9.21 mg plant^{-1}) was also similar to the isolates HSTU-IR$_{10}$ (9.08 mg plant^{-1}) and HSTU-IR$_{14}$ (9.00 mg plant^{-1}). The uninoculated control treatment showed the lowest N content in the shoot (2.23 mg plant^{-1}).

Total N in root

The significant effect of *Bradyrhizobium* inoculation has also been observed in case of N content in the root (Table 2). All the isolates showed higher N content in the roots over no inoculation that is, the control. The N content in the root ranged form 0.30 mg plant^{-1} recorded for the uninoculated control to 1.74 mg plant^{-1} for the isolate HSTU-IR$_3$. The isolate HSTU-IR$_2$ was statistically inferior to both HSTU-IR$_3$ and HSTU-IR$_4$ isolates but superior to the rest of the isolates. The isolates HSTU-IR$_{14}$, HSTU-IR$_{10}$ and HSTU-IR$_9$ recorded statistically identical results.

Total N in plants (nodules + shoots + roots)

The inoculation of the *Bradyrhizobium* isolates significantly increased total N content in the indigo plants (Table 3). All the *Bradyrhizobium* isolates helped significantly higher N content over the control. The total N content in the indigo plants ranged from 2.53 mg plant^{-1} recorded for the uninoculated control plants to 14.15 mg plant^{-1} recorded against the HSTU-IR$_3$ inoculation. The highest N content (14.15 mg plant^{-1}) was noted for the isolate HSTU-IR$_3$, which was statistically identical to that of HSTU-IR$_4$ isolate (13.19 mg plant^{-1}). Total N content in

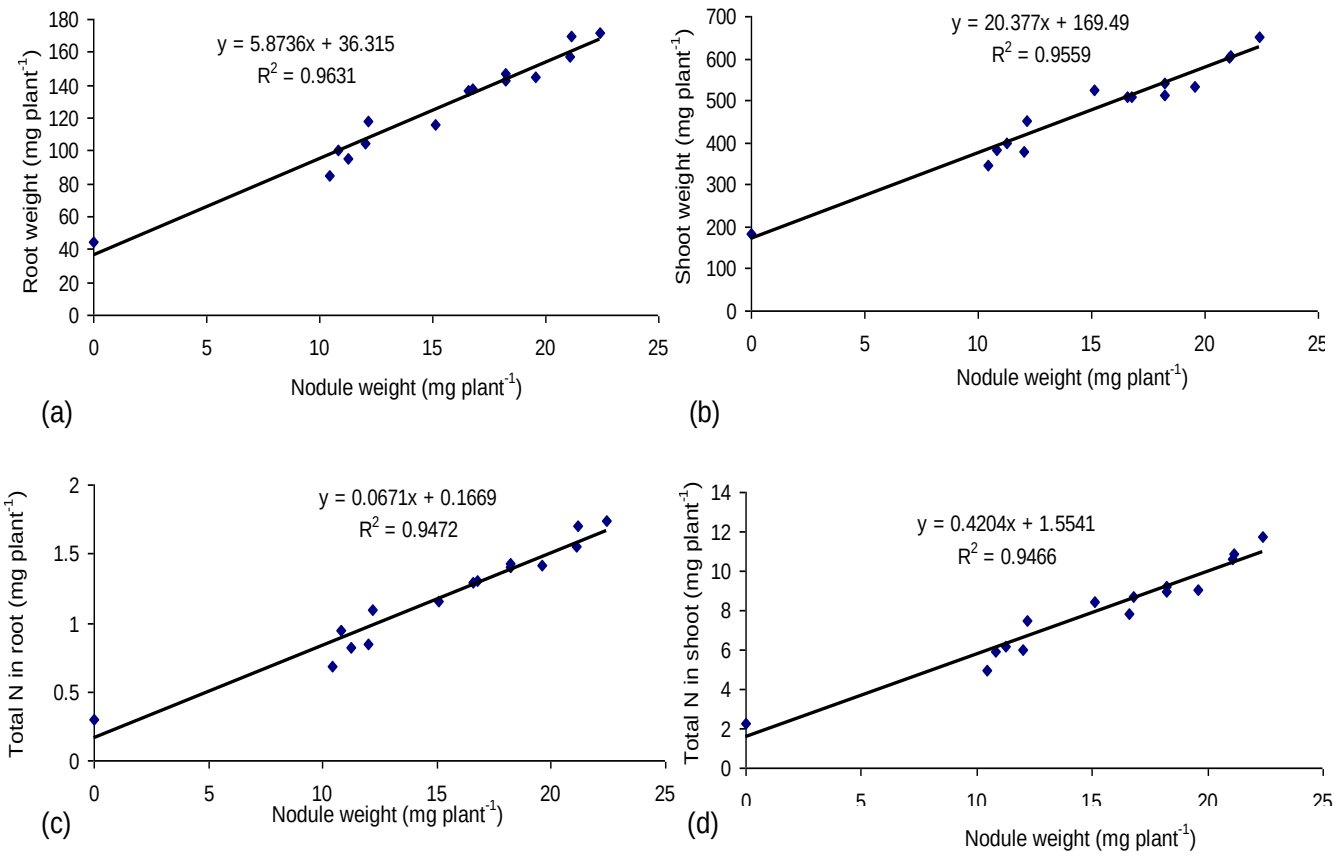

Figure 2. Relationships between (a) nodule weight and root weight (b) nodule weight and weight (c) nodule shoot weight and total N in root (d) nodule weight and total N in shoot.

the plant due to inoculation of isolate HSTU-IR_4 was similar with that of the isolate HSTU-IR_2. The isolates HSTU-IR_9, HSTU-IR_{10}, HSTU-IR_{14}, HSTU-IR_{12}, HSTU-IR_{13} and HSTU-IR_6 showed identical N content in the plants. The isolates HSTU-IR_6 and HSTU-IR_5 were also similar in terms of N content in the indigo plants.

Di-nitrogen (N_2) fixation

Total N fixation has been calculated by deducting N in the biomass of indigo plants grown without *Bradyrhizobium* inoculation from the total N recorded due to inoculation with *Bradyrhizobium* isolates. The isolate HSTU-IR_3 was statistically similar with the isolate HSTU-IR_4. The isolate HSTU-IR_2 was also identical to the isolate HSTU-IR_4 with respect to apparent nitrogen fixation by the indigo-*Bradyrhizobium* symbiosis. The isolates HSTU-IR_9, HSTU-IR_{10} and HSTU-IR_{14} showed similar amounts of nitrogen fixation in association with indigo plants. The lowest amount of N-fixation (3.34 mg $plant^{-1}$) was found due to inoculation with HSTU-IR_{15}. It was observed that uninoculated control plants failed to produce nodules. Inoculation with *Bradyrhizobium* isolates helped formation of nodules and thereby N fixation under the controlled Leonard Bottle Jars Assembly. The highest N_2-fixation (11.62 mg $plant^{-1}$) was recorded for the inoculation of the isolate HSTU-IR_3 and the lowest was in the isolate HSTU-IR_{15}.

Relationship between nodule weight and plant parts as well as nodule weight and total N content in plant parts

Shoot and root weight increased linearly with the increase in nodule weight (Figure 2). This indicates that the influence of nodule on shoot and root weight was more pronounced. Significant coefficient (R^2 = 0.956 and 0.963 for N and K) indicated that about 96% of the shoot and root weight variation could be explained by the linear function of the independent variable (nodule weight). Like shoot and root weight, total N content (mg $plant^{-1}$) of shoot and root was also influenced by the nodule weight. Regression equation indicates that one unit increase of nodule weight, shoot weight, root weight, total N content

of shoot and root would be increased 20.38, 5.87, 0.95 and 0.95 times higher, respectively.

DISCUSSION

I. tinctoria L. is a shrub one to two meters high belonging to the family of Fabaceae. It may be an annual, biennial, or perennial, depending on the climate in which it is grown. It has light green pinnate leaves and sheafs of pink or violet flowers. The plant was one of the original sources of indigo dye (Sreepriya et al., 2001; Singh et al., 2001). It has been naturalized to tropical and temperate Asia, as well as parts of Africa. The plant is a legume, so it is rotated into fields to improve the soil in the same way that other legume crops such as alfalfa and beans are (Barrios and Cobo, 2004). Root nodules of *I. tinctoria* L. were collected from the farmer's fields of two Upazillas of Rangapur district (Sadar and Taragonj) and one Upazilla of Nilphamary district (Sadar). A number of isolates were obtained of which fourteen was selected based on colony, morphological and biochemical characteristics. The second step was to confirm that these fourteen *Bradyrhizobium* isolates were of indigo origin. For confirmation, nodulation test was carried out in sterilized Leonard Bottle Jar Assembly (LBJA). The surface sterilized indigo seeds were inoculated with the broth culture of 14 isolates separately. Under such sterilized system all the isolates produced nodules on the roots whereas, the control plants sprouted out of uninoculated seeds could not produce any nodule.

This nodulation test confirmed that all the isolates were *Bradyrhizobium* of indigo origin. It is an established truth that nodulation is the final test for confirmation of *Rhizobium* or *Bradyrhizobium.* However, no research report on isolation of *Bradyrhizobium* from indigo was so far available even through desperate literature searching. Nevertheless, the repeated tests of nodulation under sterilized system of LBJR unequivocally confirmed that the fourteen isolates were *Bradyrhizobium* of indigo because no inoculation all the time failed to produce any nodule. The effective and promising isolates among 14 were selected on the basis of their nodulation capacity, effects on dry matter production, correlation studies and possible ability for atmospheric dinitrogen (N_2) fixation. The isolate HSTU-IR_3 produced the highest amount of shoot biomass (651.6 mg plant^{-1}), root biomass (171.3 mg plant^{-1}) and total biomass including nodule (845.3 mg plant^{-1}), compared to the corresponding values of 184.0, 44.0 and 228.0 mg plant^{-1}, respectively, in the case of no inoculation. These observations are in agreement with previous reports on inoculation of woody legumes with selected rhizobial strains, which showed increased survival percentage in seedlings and greater biomass production in all inoculated trees (Galiana et al., 1994; Herrera et al., 1993).

Banwari and Sunil (1996) also reported that dry matter yield of all the inoculated plants demonstrated a significant increase over that of the uninoculated plants. The total N in nodules shoots and roots were significantly much higher over those of control plants. The isolate HSTU-IR_2 was found statistically identical to the isolate HSTU-IR_3. The isolate HSTU-IR_4 ranked three in performance. The highest total dinitrogen (N_2) fixation of 11.62 mg plant^{-1} (nodule + shoot + root) was estimated for the inoculation of HSTU-IR_3, which was closely followed by the isolate HSTU-IR_4 (10.66 mg plant^{-1}) and HSTU-IR_2 (10.17 mg plant^{-1}). Since the sand was N-free and no nodules were formed in the control plants of indigo, the N estimated in the total biomass of inoculated indigo plants had obviously been due to biological N_2 fixation in the nodules by the *Bradyrhizobium* isolates. Nevertheless, ^{15}N isotope would have been of high precision. From Figure 1, it was observed that nodules weights was significantly related with the shoots and the roots weights, and total N content in the shoots and the roots which indicate that nodulation influences the growth of indigo plants. On the basis of these observations, six isolates viz. HSTU-IR_3, HSTU-IR_4, HSTU-IR_2, HSTU-IR_{10}, HSTU-IR_9 and HSTU-IR_{14} (in order of performance) were selected for further experimentation.

Conclusion

Nodulation on the roots of indigo plants, due to inoculation of the isolates confirmed that all the isolates were *Bradyrhizobium*. Inoculation of the isolates also helped di-nitrogen (N_2) fixation. On the basis of the overall performance of the isolates in terms of nodulation, N_2-fixation and plant growth (total biomass), the following six isolates have been selected for further study in a bid to select promising ones for inoculants production: HSTU-IR_2, HSTU-IR_3, HSTU-IR_4, HSTU-IR_9, HSTU-IR_{10} and HSTU-IR_{14}.

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REFERENCES

Ahmed M (2002). Indigo farming makes a comeback in Bangladesh. An internate version. Available at: http://archives.dawn.com/2002/06/07/int18.htm (Accessed 25 May 2012).

Ali MM, Shaheed SM, Kubota D (1997). Soil degradation during the period 1967-1995 in Bangladesh. II Selected chemical characters. Soil Sci. Plant Nutr., 43: 879-890.

AOAC (Association of Official Analytical Chemists) (1980). Official Analysis, 13th Ed., Washington, D.C.

Augustin EO, Ortal CI, Pascua SR Jr, Sta CPC, Padre AT, Ventura WB, Obien SR, Ladha JK (1999). Role of Indigo in improving the productivity of rainfed lowland rice-based cropping systems. Special

issue. The Rainfed lowland Rice Research Consortium. Exp. Agric., 35(2): 201-210.
Banwari L, Sunil K (1996). Long term field study shows increased biomass production in tree legumes inoculated with *Rhizobium*. Plant Soil, 184: 111-116.
Barrios E, Cobo G (2004). Plant growth, biomass production and nutrient accumulation by slash/mulch agroforestry systems in tropical hillsides of Colombia. Agrofor. Syst., 60 (3): 255-265.
Bantilan RB, Bantilan CC, Garrity DP (1989). *Indigofera tinctoria* L as a green manure crop in rainfed rice-based cropping systems. Paper presented at the IRRI Saturday Seminar, 1 July, 1989. Int. Rice Res. Ins. Los Banos, Philippines.
BARC (Bangladesh Agricultural Research Council) (1997). Fertilizer Recommendation Guide-1997. Bangladesh Agril. Res. Council. New Airport, Dhaka, 1215: 25.
Cassman KG, De Datta SK, Alcantra J, Sason M, Descalsota J, Dizon M (1997). Yield declined and the nitrogen economy economy of long-term experiment on continuing, irrigated rice system in the tropics. In Lal, R. and Stewart, B. A. (eds). Soil Management: Experimental Basis for Sustainability and Environmental Quality. Lewis Publisher, London, UK, pp. 181-222.
Dahot MU (1999). Antibacterial and antifungal activity of small protein of *Indigofera oblongifolia* leaves. J. Ethnopharmacol., 64(3): 277-282.
Fisher RA (1958). Statistical Methods for Research workers. 13th Ed. Oliver and Boyd, London.
Galiana A, Prin Y, Mallet B, Gnahoua G M, Poitel M, Diem HG (1994). Inoculation *of Acacia mangium* with alginate bead containing selected *Bradyrhizobium* strains under field conditions: Long term effect on plant growth and persistence of the introduced strains in soil. Appl. Environ. Microbiol., 60: 3974-3980.
Herrera MA, Salamanca CP, Burer JM (1993). Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified mediterranean ecosystem. Appl. Environ. Microbiol., 59: 129-133.
Garrity DP, Bantilan RT, Bantilan CC, Tin P, Mann R (1994). *Indigofera tinctoria* : Farmer-proven green manure for rainfed ricelands. Selected papers from the Internal Rice Research Conference. Int. Rice Res. Inst. P. O. Box 933, Manila 1099, Philippines.
Gomez KA, Gomez AK (1984). Statistical Procedures for Agricultural Research. John Wiley and Sons Publishing, New York, USA, pp. 207-215.
Harry WS, Paul JV (1972). Microbes in Action. In: Freeman WH, (ed.). A Laboratory Manual in Microbiology. Amazon Publishing, San Francisco, USA.
Hucker GJ, Conn HJ (1923). Methods of Gram staining. Agri. expt. Stat. Tech. Bull., p. 129.
Karim Z, Miha MMU, Razia S (1994). Fertilizer in the national economy and sustainable environmental development. Asia Pac. J. Environ. Div., 1:48-67.
Kavimani S, Jaykar B, Clercg E de, Pannecouque C, Witvrouw M, Clercg E (2000). Studies of anti-HIV activity of *Indigofera tinctoria*. Hamdard Medicus, 43(1): 5-7.
Sreepriya M, Devaki T, Balakrishna K, Appranantham T (2001). Effect of *Indigofera tin*ctoria Linn. on liver antioxidant defense systems during D-galactosamine/endotoxin induced acute hepatitis in rodents. Indian J. Exp. Biol., 39(2): 181-184.
Singh RP, Gupta MK, Verendra S, Singh V (2001). Studies on root behavior, biomass production and morphological characters of *Indigofera gerardiana* wall in Western Himalayas. Indian For., 116 (7): 584-588.
Somasegaran P, Hoben HJ (1985). Methods in Legume-*Rhizobium* technology. NifTAL Project and Mircen. Univ. Hawaii.
Vincent JM (1970). A manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific Pub. Ltd., Oxford, England.