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Evaluation of N_2 Fixation by Stem and Root Nodules in *Sesbania rostrata* During Rainy and Dry Months

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ABSTRACT

Sesbania rostrata is the most popular green manure legumes in Tamilnadu, India. *S. rostrata* produces both stem and root nodules. Until this time, no experiments had been performed to assess the superiority of one kind of nodule over the other. The levels of nitrogenous compounds, like nitrite, nitrate, total nitrogen, free amino acids, total soluble proteins, total allantoin, total allantoic acid, and total ureides, were determined in both the stem and root nodules during rainy and dry seasons. During the rainy season, the stem nodules possessed heavy deposition of these N-containing compounds than the root nodules. On the other hand, during the dry season, the root nodules accumulated substantial levels of these nitrogenous compounds. In a similar way, the activities of nitrogenase and nitrate reductase were higher in stem nodules during the rainy season, and the trend was reversed during the dry season, the root nodules having more nitrogenase and nitrate reductase. In consonance with the higher activities of enzymes and gorging of nitrogenous compounds, calorific values were more in the stem nodules during the rainy season. The activities of oxygen scavenging enzymes were higher in stem nodules during the dry season, possibly indicating their role in alleviation of H_2O_2 and O_2^- toxicity to the nodules. As regards rhizobial population, the root nodules always contained more *Rhizobia* than that of the stem nodules. The efficiency of the stem and root nodules to fix more nitrogen and synthesize nitrogenous compounds

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is seasonal. In the dry season, the entire role of N_2-fixation seemed to be borne by root nodule *Rhizobia* only, since stem nodules during this period are hardly formed and sparsely distributed.

Index Entries: N_2 fixation; nitrogenous compounds; seasonal effect; *Sesbania rostrata;* stem and root nodules.

INTRODUCTION

Nitrogen fixation plays a key role in the growth, development, and yield of plants. The soil is impoverished of nitrogen nutrition year after year. Application of chemical nitrogenous fertilizers, no doubt, replenishes the nitrogen content of the soil, but unfortunately leads to polluting water bodies, flora, and fauna. Therefore, recourses have been made in recent times through application of Biological Nitrogen Fixers (BNF) and green manures (*1,2*). Application of *Azolla* as biofertilizers (*3*) and *Sesbania rostrata* (*2*) as a green manure is being practiced in rice fields to augment rice production to keep down the cost of application of chemical fertilizers, but also to improve nitrogen utilization efficiency. *S. rostrata,* a stem nodulating green manure legume crop, has attracted much attention in recent times, since attempts are being made to understand the mode of infection, nodulation, nitrogen fixation, and nitrogen contribution by stem nodules (*4*). Green manuring with *S. rostrata* for rice crop considerably improved organic carbon, N, P, and K status of the soil, and Fe, Mn, and Zn by rice crop (*5*). *S. rostrata* can produce nodules on both root and stem, and is able to grow under flooded as well as dry conditions.

The present study evaluates the efficiency of N_2-fixing capacity of stem and root nodules in rainy and dry seasons in *S. rostrata* by way of chemical constituents of nodules with more emphasis on leghemoglobin, total nitrogen, and ureides, the latter being considered to be the predominant storage and transportable form of nitrogen in symbiotic nitrogen-fixing plants (*6*). Oxygen toxicity to nitrogenase (N_2-ase) in both the nodules was also evaluated through oxygen-scavenging enzymes.

MATERIALS AND METHODS

The seeds of *S. rostrata,* Brem and Oberm, obtained from the Tamilnadu Agricultural University, Coimbatore, India, were used in the present study. Healthy and disease-free seeds were selected and sown in the botanical garden of the Bharathidasan University, Tiruchirapalli. Plants were watered daily. March–July comprises the dry season, and October–February comprises the rainy season for this part of the country. At the site of experimentation, during the rainy and dry seasons, there was an average rainfall of 20.18 ± 0.86 mm and 2.75 ± 0.17 mm, respectively, and temperatures of 24.27 ± 1.07°C and 34.55 ± 1.25°C, respectively. Biomass of the plants was determined at different time intervals (50, 75, 100, and 120-d). Plants of *S. rostrata* at different stages of growth were collected and assorted into different organs, like stem, root, stem and root nodules, and kept in an hot air oven at 60 ± 1°C for 48 h until a constant dry weight was obtained, at the end of which the samples were removed and their respective dry weights were determined gravimetrically using electrical balance (Sartorious, Germany). Estimation of chloroplast pigments, basic biochemistry and assay of enzymes like nitrate reductase (NR), superoxide dismutase (SOD), ascorbate peroxidase (AP), catalase, glutathione reductase (GR), and N_2-ase were done in the nodules of 75-d old plants by following the standard methods (*7,8*). The content of leghemoglobin (LHb) was determined by the method of Wilson and Reisenauer (*9*). The intermediary N-compounds, like total ureides, allantoin, and allantoic acid, were determined by following the method of Vogels and Vander Drift (*10*). Assessment of nodule and soil rhizobial population was carried out in yeast extract mannitol agar (YMA) medium by the method suggested by Vincent (*11*). Sevenfold serial dilutions of 4 replications/dilution were used for most probable number (MPN) counts. The total energy content of the nodules was determined by using bomb calorimeter (Advance Research Instruments Company, India). All the data given in the tables are mean values with SE and the range of values given in parenthesis. All the data were statistically analyzed using Student's *t*-test (*12*). The available soil nutrients were N (10.86 mg/g), P (0.63 mg/g), and K (0.88 mg/g) with total organic carbon of 1.99% and total organic matter of 3.12%. The soil had a bulk density of 0.62 g/cc and water-holding capacity of 21.87% with percentage pore space of 36.67. The seedlings were raised in the open field under a natural photoperiod (26 ± 1 W/m^2) with day and night temperatures of 28–32 and 22–25°C, respectively.

RESULTS AND DISCUSSION

Indian soils are known to be deficient in nitrogen. Nitrogen, as is well known, is one of the two major factors governing plant productivity, the other being water, which is also dwindling leading to arid condition. The present work mainly concerns improving nitrogen status of the soil. To boost plant productivity, application of chemical fertilizers is generally resorted to, but continuous application of chemical fertilizers leads to change in the structure of the soil with increase in alkalinity/salinity/sodicity. Therefore, in recent times, more than ever before, biological nitrogen fixation is being presented as an appealing and viable alternative to mitigate the use of synthetic fertilizers (*13*). Particularly in the rice field, advocation of *S. rostrata* is strongly made due to the plant acting as a more promising green manure (*4,5,14*) than other *Sesbania* species.

In the present study, the potency and efficacy of stem and root nodules to fix atmospheric nitrogen were evaluated because such an in-depth study has not been carried out so far. The plants of *S. rostrata* were grown directly in the field. The plant has special characteristic feature of fixing a considerable amount of atmospheric nitrogen through both stem and root nodules; besides nitrogen, the plant is known to supply such elements as Fe, Mn, Cu, Zn, and P (*5*). *S. rostrata* appears to be a fast grower depending on the nature of the environment in which it grows. As indicated in Table 1, during the rainy season (monsoonic periods), the plant produces two times higher biomass on a dry weight basis than during the dry season (nonmonsoonic). In fact, the biomass of different organs (stem, root, and nodules) of the plant was also found to increase significantly during the rainy months.

It is interesting to observe that stem nodules are predominantly formed only under water-logging conditions with simultaneous formation of very few nodules in the roots. The above observation may indicate that the *Rhizobia* of *S. rostrata* may not be totally anaerobic. Therefore, under flooded situations, the *Rhizobia* may be transported upward along the water current and, hence, the formation of a very large number of stem nodules during monsoonic conditions.

Since, *S. rostrata* produced both stem and root nodules, the respective nodules were collected, and the *Rhizobia* isolated and cultured on YMA medium. The population of *Rhizobia* was determined following MPN method. The rhizobial population of the stem and root nodules was always higher during the rainy season (root nodule [RN]—72.54 $\times$ 10^7; stem nodule [SN]—50.17 $\times$ 10^7) than the dry season (RN—66.07 $\times$ 10^7; SN—47.54 $\times$ 10^7). The root nodules always contained more rhizobial population than the stem nodules.

The efficacy of stem and root nodules by way of gorging of biomolecules was studied, and the results are tabulated (Table 2). The chloroplast pigments of the stem nodules were found to more during the rainy season than the dry season. During rainy months, the stem nodules were totally green containing the photosynthetically active antennae pigments, like chlorophyll *a* (0.14 mg/g fw), chlorophyll *b* (0.08 mg/g fw), and total carotenoids (0.05 mg/g fw), whereas during dry season, the levels of chlorophyll *a* (0.07 mg/g fw), chlorophyll *b* (0.04 mg/g fw), and total carotenoids (0.04 mg/g fw) were reduced significantly. In order to evaluate the superiority of the stem and root nodules over each other during rainy and dry seasons, several biomolecules were quantitatively determined. Since at least in members of Fabaceae, LHb creates a microaerobic environment in which N_2-ase functions with complete protection from O_2^- toxicity (*15*), the levels of LHb were measured in both stem and root nodules as an indirect measure of symbiotic nitrogen-fixation efficiency. During the rainy season, total LHb content was significantly higher in the stem nodule compared to the root nodule, whereas during the dry season,

Table 1
Biomass Production of *Sesbania rostrata* Grown in Two Different Seasons*

		Average biomass, g/plant							
		Stem		Root		Root nodules		Stem nodules	
Day	Season	Fresh wt	Dry wt	Fresh wt	Dry wt	Fresh wt	Dry wt	Fresh wt	Dry wt
50	Dry	6.72 ± 0.42	1.90 ± 0.12	0.52 ± 0.07	0.25 ± 0.04	1.32 ± 0.07	0.13 ± 0.01	0.05 ± 0.01	0.01 ± 0.00
	Rainy	8.27 ± 0.56**	2.15 ± 0.24	1.33 ± 0.12***	0.42 ± 0.06**	1.45 ± 0.06	0.15 ± 0.02	0.07 ± 0.01	0.02 ± 0.01**
75	Dry	21.28 ± 1.21	5.32 ± 0.46	6.52 ± 0.96	1.64 ± 0.08	3.00 ± 0.12	0.50 ± 0.04	0.95 ± 0.08	0.24 ± 0.06
	Rainy	47.68 ± 2.16***	28.61 ± 1.21***	10.30 ± 0.98**	3.66 ± 0.21***	3.98 ± 0.24**	0.77 ± 0.09**	2.58 ± 0.10***	0.92 ± 0.08***
100	Dry	34.68 ± 1.89	8.67 ± 0.48	7.77 ± 0.87	2.44 ± 0.26	2.70 ± 0.16	0.25 ± 0.04	1.20 ± 0.09	0.30 ± 0.02
	Rainy	70.42 ± 4.21***	32.00 ± 1.81***	36.33 ± 2.16***	15.30 ± 1.21***	2.98 ± 0.18	0.35 ± 0.05	4.49 ± 0.25***	2.04 ± 0.03***
120	Dry	60.23 ± 3.96	20.74 ± 1.69	9.25 ± 0.87	2.42 ± 0.24	0.60 ± 0.09	0.27 ± 0.04	1.72 ± 0.11	0.24 ± 0.01
	Rainy	99.98 ± 3.93***	35.99 ± 2.17***	52.67 ± 1.98***	17.80 ± 1.17***	1.83 ± 0.09**	0.33 ± 0.04	3.39 ± 0.10***	1.67 ± 0.05***

* The data are mean values of five different experiments ± SE.
** Significant at 5% level.
*** Significant at both 5 and 1% levels.

Table 2
Basic Biochemical Analyses of Root and Stem Nodules of 75-d-old *S. rostrata**

	Season			
	Rainy		Dry	
Biomolecules	Stem nodule	Root nodule	Stem nodule	Root nodule
Total leghemoglobin (mg/g fw)	48.25 (46.26–50.23)	27.42*** (25.14–29.68)	21.75 (19.67–23.83)	36.32*** (34.87–37.71)
Total flavonoids (mg/g fw)	46.17 (44.16–48.18)	20.24*** (19.17–21.31)	24.72 (22.18–27.26)	36.17** (33.17–39.17)
Total water-soluble SH compounds (mg/g dw)	0.50 (0.47–0.53)	0.28*** (0.26–0.30)	0.11 (0.09–0.13)	0.32*** (0.28–0.36)
Total proline (mg/g dw)	1.28 (1.07–1.49)	1.20 (1.04–1.36)	4.09 (3.79–4.39)	1.15*** (1.02–1.28)
Free amino acids (mg/g dw)	35.61 (33.17–38.05)	17.61*** (15.27–19.95)	20.16 (19.86–20.46)	29.71** (26.18–33.24)
Total water-soluble proteins (mg/g dw)	158 (142–174)	135 (129–141)	102 (99–104)	179** (158–199)
Total nitrate (mg/g dw)	6.41 (5.95–6.87)	2.61*** (2.01–3.21)	5.24 (4.95–5.53)	8.83** (7.67–9.98)
Total allantoin (mg/g dw)	16.32 (15.87–16.77)	18.16** (17.67–18.69)	11.18 (10.78–11.58)	18.01*** (17.65–18.37)
Total allantoic acid (mg/g dw)	39.87 (37.17–42.57)	21.02*** (20.07–21.97)	16.89 (15.95–17.83)	42.18*** (39.17–45.19)
Total ureides (mg/g dw)	56.17 (54.28–58.06)	39.18*** (38.17–40.19)	28.07 (26.17–29.97)	60.19*** (57.48–62.94)
Total nitrogen (mg/g dw)	224 (197–251)	112** (108–160)	112 (101–124)	168** (149–188)
Total energy (calories/g dw)	5225 (4985–5465)	2728*** (2517–2939)	2331 (2178–2484)	4996*** (4727–5265)

* The data are mean values of three different experiments with two replicates. The range of values is given in parenthesis.
** Significant at 5% level.
*** Significant at both 5 and 1% levels.

total LHb content was significantly higher in the root nodule than the stem nodule. Legocki et al. (*16*) also observed a substantially higher level of total LHb in stem nodules than in root nodules of *S. rostrata*. Gebhardt et al. (*17*) found higher tolerance of stem nodule *Rhizobia* to O_2^- from the culture studies. O_2^- tolerance to stem nodulating *Rhizobia* could also be confirmed in the present study (Table 3). The level of water soluble SH compounds was more in the stem nodules during the rainy season than the dry season, but root nodules did not show such a drastic difference. Regarding the levels of total ascorbic acid, glycine betaine, and nitrite, no significant difference could be observed. In dry months, free proline was more in the stem nodule than the root nodule, whereas total flavonoids

Table 3
Enzymatic Activities in the Nodules from 75-d-old *S. rostrata**

Specific activities of enzymes	Season			
	Rainy		Dry	
	Stem nodule	Root nodule	Stem nodule	Root nodule
Nitrogenase (nmol C_2H_4 produced/g fw/h)	6687 (6127–7247)	3389*** (3018–3760)	1435 (1398–1472)	2628*** (2517–2739)
Nitrate reductase (mmol NO_2^- released/mg protein/h)	1.62 (1.49–1.75)	0.68*** (0.57–0.79)	0.46 (0.41–0.52)	1.07*** (1.01–1.13)
Superoxide dismutase (U/mg protein/min)	23.24 (21.08–25.41)	35.17** (32.16–38.18)	48.72 (42.15–55.29)	15.13*** (14.97–15.29)
Ascorbate peroxidase (nmol of ascorbate consumed/mg protein/min) mg protein/min)	24.69 (22.18–27.24)	38.71** (34.26–43.16)	42.43 (40.18–44.68)	28.96*** (26.97–30.95)
Catalase (OD U/mg protein/min)	28.04 (26.71–29.29)	45.01*** (43.17–46.85)	48.47 (46.17–50.78)	32.18*** (30.17–34.19)
Glutathione reductase (OD U/mg protein/min)	16.48 (15.97–16.99)	19.65** (18.94–20.36)	26.69 (25.87–27.51)	15.51*** (14.95–16.07)

* The data are mean values of two different experiments with three replicates. The range of values is given in parenthesis.
** Significant at 5% level.
*** Significant at both 5 and 1% levels.

were more in the root nodule than the stem nodule. The interesting observation is that in the root nodule most of these biomolecules were found to be concentrated during the dry season than the rainy season. On the other hand, stem nodules substantially accumulated these compounds only during the rainy season. This is quite evident from the levels of total leghemoglobin, free amino acids, water-soluble proteins, allantoin and allantoic acid, nitrogen and nitrate contents, as well as the energy level of the nodules (Table 2).

From these observations, it is evidently clear that stem nodules function more efficiently than the root nodules during the rainy season. The stem nodules in particular accumulate a significant quantity of LHb possibly to prevent more O_2^- from reaching the active site of N_2-ase (*18*). In the dry season, the stem nodules are hardly formed.

A few enzymes, more innately connected to nitrogen metabolism, such as N_2-ase and NR, and the O_2-scavenging enzymes, such as AP, catalase, SOD, and GR, were assayed. N_2-ase activity was twice higher (6687 nmol) in the stem nodule than the root nodule (3389 nmol) during the rainy season. In contrast, the stem nodule showed the least activity (1435 nmol) during the dry season compared to the root nodules (2628 nmol). NR activity was highest in stem nodule (1.62 μmol) during the rainy season, but lowest (0.46 μmol) during the dry months. Similarly, the activity of enzymes like SOD was highest in the stem nodules (48.72 U/mg protein/min) during the dry months. AP and catalase activities were highest in stem nodules during the dry months, whereas higher activity was observed in the root nodules only during the rainy months. Similarly, GR activity was highest in the root nodules during the rainy period. The higher activities of O_2^--scavenging enzymes in the stem nodules during the dry months than the rainy season possibly indicate their detoxifying mechanism against H_2O_2, and O_2^- radicals portraying meaningful seasonal fluctuation in the functioning of the nodules of *S. rostrata*.

The present observations of the highest activity of N_2-ase, total LHb, total N_2, and high calorific values clearly indicate that the stem nodules are endowed with higher potential of nitrogen fixation. It is strongly advocated from the present study that *S. rostrata* is the right type of leguminous green manure crop for wet-land cultivation. Such a promising green manure as *S. rostrata* would curtail the application of synthetic fertilizers, thereby saving money for farmers and, at the same time, protecting the structure of the soil as well as its dynamic status with nitrogen enrichment. It is tempting to speculate from the experimental evidence that the *Rhizobia* of *S. rostrata* are not of a special type, and erecting a separate generic or even species status seems to be ambiguous and unwarranted. Serological studies might throw further light on this serious point to ponder over.

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