Delpinoa, 25-26: 39-52. 1983-84

Cultivation in temperate climate of *Sesbania rostrata* Bremek. & Oberm. (Fabaceae), a tropical legume with nitrogen-fixing stem nodules

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Riassunto

Sesbania rostrata è una leguminosa originaria dell'Africa nord occidentale caratterizzata dalla presenza di noduli infettati da *Rhizobium* non solo sulle radici ma anche sui fusti. Il doppio sistema di nodulazione permette a *S. rostrata* di fissare azoto in grandi quantità (200 Kg N ha^{-1}).

Gli autori hanno coltivato *S. rostrata* a Napoli (Italia) sia in vaso che in piena terra. Tutte le piante hanno raggiunto l'altezza di 4 m dopo 90 giorni dalla semina. L'attività nitrogenasica è presente nell'intervallo di temperatura di 5°—45°C ed è risultata massima a 27°C; a questa temperatura *S. rostrata* ha prodotto 120 nmol C_2H_4 h^{-1} mg^{-1} per peso secco di nodulo. *Vicia faba*, esaminata comparativamente alle stesse temperature, ha presentato il massimo di attività nitrogenasica a 10°C, producendo 4 nmol C_2H_4 h^{-1} mg^{-1} per peso secco di nodulo.

Per il suo alto valore di crescita, per l'elevata capacità di fissare azoto e per il carattere termofilo, *S. rostrata* rappresenta un biofertilizzante potenzialmente molto efficace se utilizzato in aree con clima mediterraneo o subtropicale.

Key words: *Sesbania rostrata* — Cultivation — N_2-fixation — Biofertilizer — Acetylene reduction assay

Introduction

Sesbania rostrata Bremek. & Oberm. (Fabaceae) is an annual legume fully described in Flore Illustrée du Sénégal (Berhaut, 1976). It is indigenous to north-western Africa where it grows best in waterlogged soils with its most rapid period of growth in June-September (Dreyfus *et al.*, 1983b).

S. rostrata has nitrogen-fixing root nodules, as do many legumes. Moreover, there are three to four vertical rows of protuberances on its stem. These stem protuberances early in development are 0.2-0.4 mm wide and resemble adventitious roots primordia. After one to two days the central area appears bumpy and structures resembling adventitious roots are visible. These structures may remain dormant, but if they come into contact with water they may develop into normal adventitious roots. The differentiation of the dormant structures may fallow a very different path if a specific *Rhizobium* is available. In this case, the adventitious root primordia form stem nodules morphologically very similar to underground root nodules (Dreyfus *et al.*, 1983b; Duhoux and Dreyfus, 1982). Two distinct *Rhizobium* strains are able to nodulate *S. rostrata*. An unspecific *Rhizobium* strain infects roots whereas a second strain (ORS571) infects both underground roots and adventitious roots on stems (Dreyfus *et al.*, 1983b). In addition, the former is able to infect roots only when the soil is not flooded, the latter when the soil is flooded (Dreyfus *et al.*, 1983b).

Such structural features are known only in a few legumes. Besides *S. rostrata, Aeschynomene* spp. and *Neptunia oleracea* have *Rhizobium* infected stem nodules (Duhoux and Dreyfus, 1982; Eaglesham and Szalay, 1983).

The « double-nodulation » system permits *S. rostrata* to fix nitrogen in large quantities, estimated as equal to 600 μmol C_2H_4 h^{-1} per plant. This corresponds to about five times the maximum nitrogen fixing ability of soybean (120 μmol C_2H_4 h^{-1} per plant). After a growth period of 50 days, *S. rostrata* can yield, in optimal conditions, up to 200 Kg N ha^{-1} (Dreyfus *et al.*, 1983b; Rinaudo *et al.*, 1983). Another unusual feature of *S. rostrata* is its ability to nodulate and fix nitrogen even in the presence of high amounts of mineral or organic nitrogen in the soil (up to 200 Kg N ha^{-1}). This feature makes *S. rostrata* unique among higher plants (Dreyfus *et al.*, 1983b; Dreyfus and Dommergues, 1980).

Because of these particular characteristics, *S. rostrata* could be an important source of organic nitrogen in agriculture. Experiments aimed at verifying the increase of seeds and rice straw yield in rice cultivation, using *S. rostrata* as a nitrogen source, were carried out in Senegal. It was possible to obtain a 50% increase in grain yield, in dry weight and in nitrogen content in rice straw in plots where *S. rostrata* was used as green manure, as compared to the yield obtained in plots where ammonium sulfate fertilizer was used (Dreyfus *et al.*, 1983b). The most common utilization of *S. rostrata* as green manure consists in cutting manure plants (1.5 m high) into pieces 10-20 cm long and burying them lengthwise in soil to a depth of 10-15 cm (Dreyfus *et al.*, 1983b). These and other results (Dreyfus *et al.*, 1983b; Rinaudo *et al.*, 1983) obtained by using *S. rostrata* in agricultural practices in tropical areas gave evidence of its very high potential as a green manure. Further research would certainly be worthwile.

For this purpose, we undertook experiments to determine if *S. rostrata* could also have applications in temperate climate agriculture. The first objective was to determine if *S. rostrata* could be adapted to climatic conditions differing from those in which it normally grows. The second objective was to conduct physiological studies to determine if its ability to nodulate and fix nitrogen was impaired under growing conditions like those present in summer in temperate areas. The adaptation tests were carried out in experimental plots at the Botanical Garden of Naples (latitude 40° 50' N), Italy. Physiological tests related to modality and extent of nodule infection and the measurement of the nitrogenase activity as a function of some ecological parameters were also undertaken in the laboratory. Finally, some data were obtained on the morphology of stem nodules using light and electron microscopy.

Materials and methods

Cultivation of S. rostrata

Viable seeds from Senegal were provided by Dr. H. F. Diara (Warda-Adrao, Richard-Toll, Senegal) and by Mr. W. Baudoin (F.A.O., Rome, Italy).

Before sowing, the seeds were treated for 30 min in concentrated H_2SO_4 and then washed with tap water. The seeds were sown in clay pots, 40 cm wide and 10 cm high, that had been filled with a mixture of 50% sand and 50% peat. The pots were placed in containers with 5 cm of water, and the containers with the pots were in turn placed in sand on a heating mat at 25°C in greenhouse.

The first sowing was at the end of April, 1983. After one month, when the plants were about 50 cm high, they were transplanted outside in the open ground in rows 50 cm apart with plants 30 cm apart in each row. This experimental plot was continuously flooded with water. Some plants were transferred from original clay pots to bigger pots (40 cm in diameter × 40 cm high) which were buried in the experimental plot. At the end of August, these pots were again transferred to the greenhouse in order to avoid low temperatures and allow further growth. A second sowing experiment was made at the beginning of March, 1984, with seeds from Senegal, as in 1983, and also with seeds produced by the plants from the first sowing. The procedures described above were again followed with seeds and seedlings of the two sources maintained in separate plots. A third experiment was begun at the beginning of June, 1985, with seeds from Senegal and seeds harvested from the 1984 experiments. All other procedures were the same as described for the second experiment.

The cultivation of *S. rostrata* in Naples was carried out without any kind of fertilizer and insecticide.

Inoculation of roots and stems with Rhizobium

The inoculation with *Rhizobium* was carried out on plants obtained from 1984 and 1985 sowings. For the successful induction of nodulation of both roots and stems, *Rhizobium* strain ORS571 was used (Dreyfus *et al.*, 1983b). The lyophilized *Rhizobium* (received from Dr. C. Elmerich, Pasteur Institute, Paris, France), was transferred to a YLS liquid culture medium (Na-lactate 10 g, $(NH_4)_2SO_4$ 1 g, K_2HPO_4 1.67 g, KH_2PO_4 0.87 g, $MgSO_4.7H_2O$ 0.409 g, NaCl 0.1 g, $FeCl_3$ 0.004 g, yeast extract 1g, distilled water 1l) (Dreyfus *et al.*, 1983b), at 25°C. Very dense cultures (approximately 10^8 cells per ml) were used. To induce

root nodulation, seeds of *S. rostrata* were steeped in the *Rhizobium* cultures before sowing. After the seeds were sown, the remaining liquid cultures were poured into the pots containing the sown seeds to infect also the soil. To induce stem nodulation, seedlings 80-100 cm tall were sprayed at the beginning of July with the liquid culture medium according to the procedure described by DREYFUS *et al.* (1983b).

Recovering of Rhizobium from roots and stems

Nodules were excised from the plants and washed for 30 min in running water, immersed for a few moments in 95% ethanol, and sterilized in a solution of $HgCl_2$ (1 g l^{-1}) and 0.1% HCl for 4 min. Then the nodules were washed six times in sterile distilled water, cut into pieces, and the pieces placed on YLS solid medium (VINCENT, 1970). After three to four days, normal colonies developed.

Light and electron microscopy

This investigation was carried out on stem nodules from plants grown in 1984. For light microscopy, stem nodules of *S. rostrata* were excised, fixed in FAA (1:1:18 v/v formalin-acetic acid-70% ethanol), dehydrated in an ethanol-tertiary butil alcohol series, embedded in Paraffin (53°C), sectioned on a rotary microtome at 16 μm, and stained either with Safranin O and Fast Green or with Milovidnov reagent (JOHANSEN, 1940). For electron microscopy, stem nodules were excised and fixed in 2.7% glutaraldehyde in 0.2 M phosphate buffer for 2 hrs at 0°C, dehydrated in an ethanol-propylene oxide series, and embedded in Epon. The sections were stained with lead citrate and uranyl acetate.

Nitrogenase activity

Measurements of nitrogenase activity were made in September-October, 1984, on both root and stem nodules of *S. rostrata*, and on nodules of *Vicia faba* grown in the ground at the

Botanical Garden of Naples. The nodules were excised and placed in 10 ml tubes containing wet filter paper. The tubes were then hermetically sealed and prepared for gas chromatography according to the method of acetylene reduction (HARDY *et al.*, 1968). 0.5 ml of the atmosphere was extracted from each tube and analysed by gas chromatography. In the case of the stem nodules, only nodules from the lower 20-30 cm of the stem were analysed because it has been demonstrated that there are significant differences in the amount of acetylene reduced in nodules of the lower part of the stem as compared to the upper part (DREYFUS and DOMMERGUES, 1981).

Measurements of nitrogenase activity were also made on *Rhizobium* recovered from stem nodules and grown on the YLS liquid medium. This medium was modified in order to eliminate any nitrogenous constituent that could inhibite nitrogenase activity. Thus, the yeast extract was eliminated and biotine (0.01 g l^{-1}), nicotinic acid (0.01 g l^{-1}) and pantothenic acid (0.01 g l^{-1}) were added (DREYFUS *et al.*, 1983a). The preparation of material for gas chromatography was the same as outlined for nodules with the following modification: the air in the tube was removed and an atmosphere consisting of 3% O_2 and 97% N_2 was introduced (DREYFUS *et al.*, 1983a).

Gas chromatography was carried out in a 1.5 m × 4 mm glass column packed with silica gel 100-120 mesh. Isothermal 75°C, carrier gas was N_2 at 30 ml min^{-1}. Injector and FID detector temperature was 100°C. Sample peak heights of ethylene were compared with prepared and analysed standards. Calibration curves were drawn and response factors determined. Peak areas were determined by triangulation.

RESULTS AND DISCUSSION

Cultivation and growth of S. rostrata

From the April 1983 sowing we obtained 50 plants. The germination rate was 90%. One month after sowing the plants reached a height of 50 cm. By August, the plants reached a height of approximately 4 m (Plate Ia). In September, those individuals of *S. rostrata* which had been planted in the ground suffered a

setback because of a temporary cold wave. The individuals left in their original clay pots and returned providently to the greenhouse at the end of August, continued their vegetative cycle. They flowered at the end of November (Plate Ib) and were bearing fruit at the beginning of Dicember (Plate Ic). By the following February, seeds were mature.

In June 1983, we used a lateral branch of a plant growing outside to prepare cuttings. Some cuttings were placed in water, others were placed directly in flooded soil. All cuttings developed abundant adventitious roots (Plate Id). The cuttings from the flooded soil grew very strong with numerous lateral branches. In contrast, the cuttings rooted in tap water tended to die soon after being transferred to soil.

The growth of plants obtained from the 1984 and 1985 sowings generally showed the same pattern as those sown in 1983 except that in 1984 and 1985 the maximum period of growth occurred at the end of July. Flowering and fruiting occurred again only on individuals transferred to the greenhouse. Seedlings from all sources exhibited in the second and third sowing the same growth vigour and patterns both in the open and in the greenhouse.

A part of the seeds collected at the end of the 1983 season was sown directly into the ground and watered daily. The germination rate of this group of seeds was 50%. Only 10% of seedlings followed the normal growth cycle, and maximum height of plants was 60-70 cm.

Although none of *S. rostrata* seeds was treated with insecticides, no insects were ever observed, in contrast to some observations made in Senegal (Dreyfus *et al.*, 1983b).

Morphology of plants grown in the Botanical Garden of Naples

General morphology of *S. rostrata* plants grown in the Botanical Garden of Naples did not differ substantially from that reported by Berhaut (1976). However, individuals from the Botanical Garden of Naples were taller and stronger in all respects, reaching an average height of 4-5 m as opposed to the 2 m reported by Berhaut. The size and vigour of plants grown in Naples were more similar to those reported for plants cultivated and examined by Dreyfus *et al.*, (1983b) in Senegal.

Nodule formation on roots and stems

Rhizobium not infected plants from the 1983 sowing did not form any kind of nodules.

Rhizobium infected plants from 1984 and 1985 sowings formed nodules. Both roots and stems began to form nodules in September, regardless of time in which two organs were infected. Nodule number and size increased continuously until mid November. *Rhizobium* was present in all examined nodules. Stem nodulation was not very abundant and the placement of nodules along vertical rows on stems was irregular in contrast to very high nodule number and the regular stem nodule placement observed in Senegal (DREYFUS *et al.*, 1983b). For 20 cm of root (Plate IIa) or stem (Plate IIb) we counted, on average, 15 and 40 nodules, respectively.

Light and electron microscopy of stem nodules

The Molovidnov method was the best for demonstrating the presence of bacteria in the nodules. A transverse section of a nodule (Plate IIc, d) shows clearly the region of *Rhizobium* colonization. The electron micrograph in Plate IIe shows cells of the central tissue of a nodule infected by *Rhizobium*. The bacteroids are enclosed in membrane envelopes distributed through the cytoplasm. Each envelope contains one or two bacteroids with prominent granules of β-OH-butyrate.

Nitrogenase activity

Measurements of nitrogenase activity, as a function of temperature and time, were carried out on both stem and root nodules.

Nitrogenase activity values obtained for root and stem nodules at tested temperatures are showed in Fig. 1a. At extreme temperatures, 5° and 45°C, nitrogenase activity was near zero but still detectable; at 10°C the activity was very low. At intermediate temperature values, a Gaussian curve was obtained, with optimum nitrogenase activity for both nodule types occurring at

27°C. Root nodules resulted slightly less thermophilous than stem nodules. The similarity of the two curves probably depends on the fact that the same bacterial strain was used to inoculate both plant organs.

Fig. 1b shows the nitrogenase activity values achieved for nodules excised from *Vicia faba* and examined at the same temperatures as *S. rostrata*. The resulting curve is quite different from the one observed for *S. rostrata* (Fig. 1a). Optimum temperature for nitrogenase activity is 10°C and ethylene amounts produced at various temperatures are much lower than those produced by *S. rostrata*.

Fig. 1c shows ethylene accumulation curves for root and stem nodules examined as incubation time in closed tubes. For both nodule types a plateau is reached after about 30 hours.

The curve resulting from measurements of decreasing nitrogenase activity after *S. rostrata* stem nodules were excised and kept in the open tubes, from a minimum of zero to a maximum of 24 hours, is showed in Fig. 1d. It can be seen that activity decreases rapidly in the first 2-4 hours, then the activity exhibits a uniform decrease in the following hours with all activity ceasing after 24 hours.

In Fig. 1e, the upper curve shows the nitrogenase activity for stem nodules excised every 4 hours up to a total of 48 hours. The plants used to provide the nodules were kept outside. The temperature and humidity values at the time of nodules excision are reported at the top of Fig. 1e. The curve clearly indicates that nitrogenase activity is greater in daylight than at night. Maximum activity occurs at 4.00 p.m.; minimum activity occurs at 4.00 a.m. Therefore, it can be considered that *S. rostrata* exhibits a typical day-night cycle of nitrogenase activity. At the bottom of Fig. 1e similar data are given on excised nodules of *V. faba* in order to compare the activity of these two nitrogen fixing taxa. *V. faba* exhibits a very low relative nitrogenase activity per dry weight, as compared to *S. rostrata*. The optimum temperature for nitrogen fixation in *S. rostrata* in this experiment is 20°C as opposed to the optimum of 27°C shown in Fig. 1a. Probably, this discrepancy is because the data presented in Fig. 1a are from excised nodules whose nitrogenase activity was affected only by temperature whereas those presented in Fig. 1e were obtained from nodules affected by manifold factors (temperature,

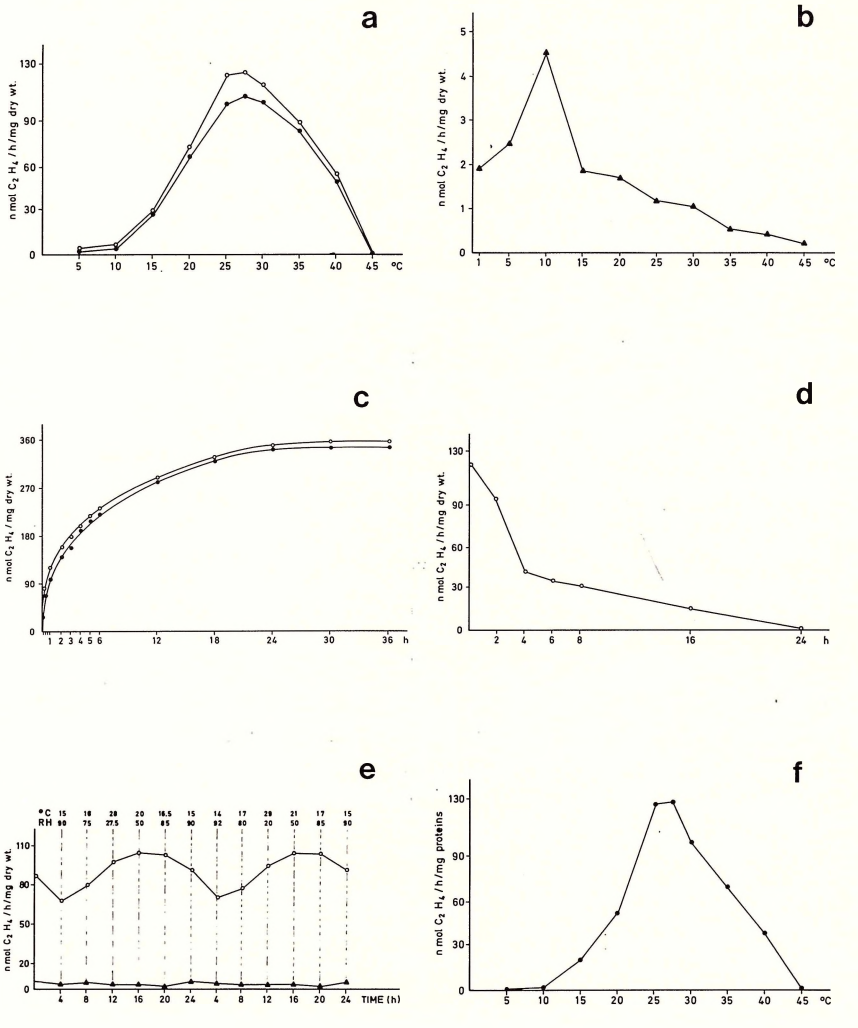

Fig. 1. a) Nitrogenase activity at various temperatures by stem (O) and root (●) nodules of *S. rostrata*. b) Nitrogenase activity at various temperatures by root nodules of *Vicia faba*. Each point represents the activity of four excised nodules placed in each tube and incubated for 1 h.
c) Nitrogenase activity by stem (O) and root (●) nodules of *S. rostrata* as a function of incubation time. Four tubes with four excised nodules each were sealed and incubated at 27°C; each tube was used for three or four measurements. Time values do not include the incubation time (1 h).
d) Nitrogenase activity by excised stem nodules of *S. rostrata* as a function of time in the open tubes at 20°C, with natural light and 60% relative humidity. e) Nitrogenase activity by stem nodules of *S. rostrata* (O) and root nodules of *Vicia faba* (▲) excised and examined every 4 hours for a total time of 48 hours. Note conditions of temperature (°C) and relative humidity (RH) at top. Each point represents the activity of three nodules in each tube. Time values do not include the incubation time (1 h).
f) Nitrogenase activity by *Rhizobium* recovered from stem nodules of *S. rostrata* as a function of temperature. Each point represents the average of three measurements. Incubation time 1 h.

humidity, day-night cycle, plant metabolic state) that changed at the time of each excision.

The curve of nitrogenase activity by *Rhizobium* isolated from *S. rostrata* stem nodules and examined at various temperatures is showed in Fig. 1f. The curve differs slightly from the one obtained for the excised nodules (Fig. 1a). Activity is absent at extreme temperatures of 5° and 45°C and only slightly observable at 10°C. As in the case of the nodules, maximum activity occurs at 27°C, but the isolated bacteria show a temperature plateau (25°-27°C) in which the nitrogenase activity is almost constant.

Conclusions

From this study conducted over three summers in a typical Mediterranean climate, it appears that *S. rostrata* has a significant potential as a green manure when an adequate moisture is available. Seed germination is very high, reaching 90% in controlled conditions. The *S. rostrata* growth is rapid, with plants that may reach a height of 4 m in 60 days. If plants are properly inoculated, the nitrogen fixation rate is very high. Further research is needed to test direct seeding techniques in the field to find the proper seeding time (particularly in relation to germination temperature), optimal plant spacing, the water (irrigation) requirement for optimal growth, etc.; that is, all the agronomic techniques involved in normal cultivation in the open field.

There appears to be a significant disadvantage. Like most equatorial species, *S. rostrata* flowers and fruits under short days and when critic temperatures are reached. Therefore, seed setting can only be achieved in our climate if plants are kept in greenhouses. To overcome this problem there are several options: direct import of seed from tropical areas; natural occurrence and isolation of day-neutral individuals; induction of a mutation involving day-length response through mutagenic treatment of seeds with physical or chemical agent. Thus, in terms of the first objective stated in the introduction, although a satisfactory vegetative growth can be obtained when *S. rostrata* is cultivated in a temperate area, nevertheless there appear to be significant

problems to overcome in the adaptation of this tropical species outside its natural range relatively to the flowering and fruiting.

As regads the second objective, *S. rostrata* showed the capability to form nodules and fix nitrogen in Naples, even though the nodules were not as abundant as those obtainable in Senegal. The present study has demonstrated that the nitrogen fixing ability of *S. rostrata* far exceeds that of *V. faba*, an indigenous legume widely used as a green manure to increase nitrogen level in the soil. Where water is available in relatively high amounts, poor soils can be enriched with organic matter containing a high amount of nitrogen for the following winter crops, particularly cereals. Mineral fertilization can be limited to superphosphate, before cultivation of *S. rostrata*.

Besides the high nitrogen producing ability of *S. rostrata*, there would appear to be other advantages for using it as a green manure in subtropical and Mediterranean climates. It has the ability (1) to produce nitrogen in greater quantities over a greater temperature range than other legumes (Dreyfus *et al.*, 1983b; Rinaudo *et al.*, 1983; Nutman, 1976), (2) to produce a greater quantity of phytomass in the same time period than other legumes, (3) to add a significant quantity of fixed nitrogen from the stem nodules, and (4) to contribute organic material when the aerial portion of the plant is turned over in the soil. In addition, the rapid growth rate of *Sesbania* plants indicates that significant quantities of fixed nitrogen can be added to the soil in a short time and that a field need not lie fallow for as long a time as with other legumes.

Although the present study demonstrates valuable insights into adaptation of *S. rostrata* cultivated outside its original range, due to problems cited above the present data should still be considered preliminary. Further research is needed to measure biomass produced under optimal conditions in open fields and thus to evaluate the economic feasibility for utilization of *S. rostrata* in warm temperate areas.

Acknowledgements

We wish to thank the following people for their help in conducting and completing this research: Dr. H. F. Diara (Warda-

ADRAO, Richard-Toll, Senegal) and Mr. W. Baudoin (F.A.O., Rome, Italy) for supplying seeds, Dr. C. Elmerich (Pasteur Institute, Paris, France) for supplying the *Rhizobium*, and Mrs. J. W. Stevenson (New York Botanical Garden, New York, USA) for supplying much needed editorial assistance.

SUMMARY

Sesbania rostrata produces in north-western Africa *Rhizobium* containing nodules on both roots and stems. These nodules are the sites of nitrogen fixation at a rate of up to 200 Kg N ha^{-1} even in the presence of nitrogen in the soil.

We cultivated *S. rostrata* both in pots and in the ground in Italy. Plants reached a height of 4 m after 90 days from seed sowing. Nitrogenase activity occurred from 5° to 45°C with maximum (120 nmol C_2H_4 h^{-1} mg^{-1} dry weight of nodules) at 27°C. This was in contrast to that we observed in *Vicia faba* which had a maximum nitrogenase activity (4 nmol C_2H_4 h^{-1} mg^{-1} dry weight of nodules) at 10°C. Thus, for the fast growth rate and high nitrogen-fixing capability as well as for the thermophilous behaviour, *S. rostrata* can offer considerable potential as green manure in areas with a Mediterranean or subtropical climate.

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PLATE I

a) Habit of *Sesbania rostrata* as grown in the ground.
b-d) Flowering shoot (b), two fruits (c) and cutting (d) of *S. rostrata*. Note vertical file of adventitious roots. Scale bar 3 cm.


a
b
c
d

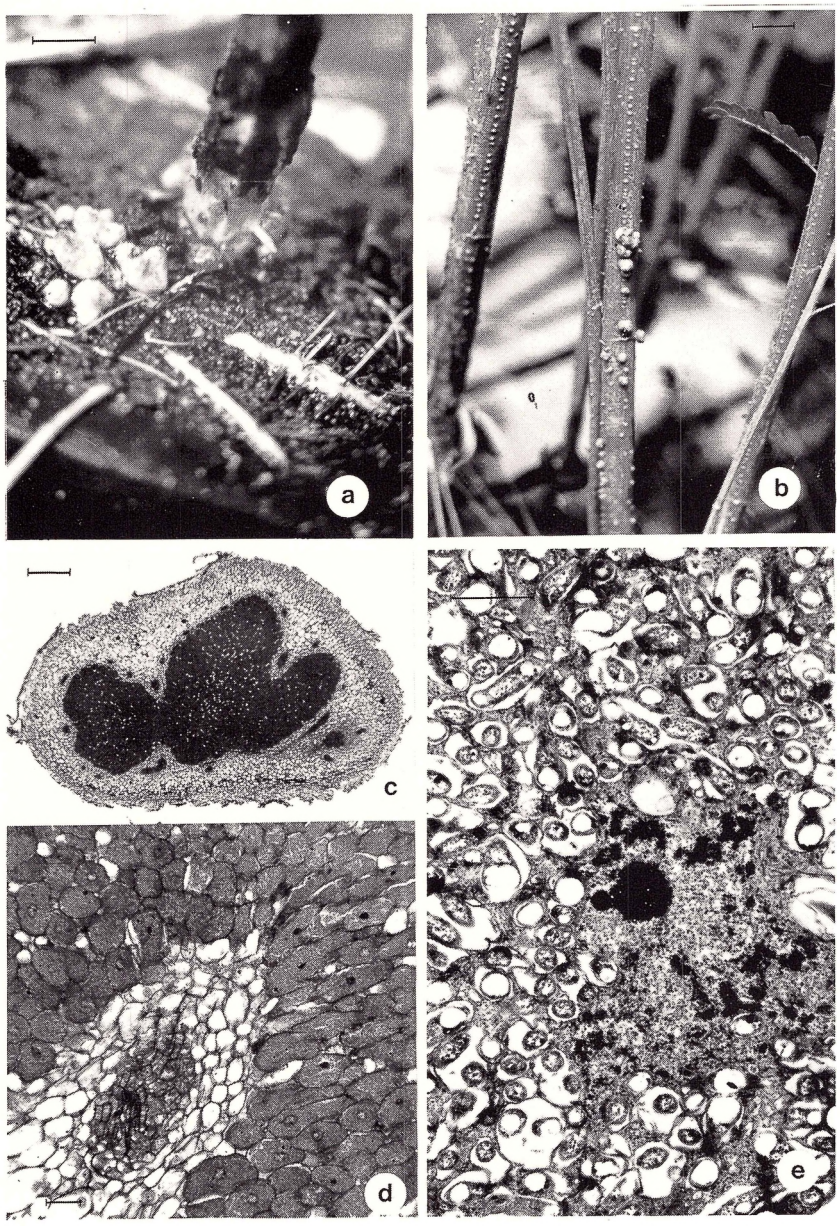
PLATE II

a-b) Root nodules (a) and stem nodules (b) of *S. rostrata*. Scale bar 1 cm.

c) Transection of a stem nodule of *S. rostrata*. The dark area in the center is the area infected by *Rhizobium* which is surrounded by a series of vascular bundles. Scale bar 0.5 mm.

d) Greater magnification of a portion of c with *Rhizobium* infected cells and one of the adjacent vascular bundles. Scale bar 0.3 μm.

e) Electron micrograph of a *Rhizobium* infected cell. Nucleus with nucleolus in center. Scale bar 0.3 μm.


a
b
c
d
e