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Isolation of Both Fast and Slow Growing Rhizobia Effectively Nodulating a Medicinal Legume, *Mucuna pruriens*

N.K. ARORA, V. KUMAR, and D.K. MAHESHWARI*
Department of Botany and Microbiology, Gurukul Kangri University, Hardwar 249 404, U. P., India, Tel. +91-133-416767, Fax. +91-133-416366
E-mail. akataria@hwr.bhel.co.in

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Abstract

Strains of root nodulating bacteria were isolated from the root nodules of a medicinal legume plant, *Mucuna pruriens* (Kaunch), growing wildly in the foothills of Himalayas. The strains were morphologically, physiologically and biochemically characterised according to the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). According to their generation time the strains were both fast and slow growers. Both fast and slow growing strains showed marked differences in morphology, physiology and biochemical characteristics. Morphological, cultural, biochemical characters, G+C mole percent and *in vivo* infectivity on their original host and other plants confirmed that the fast growing isolates were *Rhizobium meliloti* while the slow growers belong to the *Bradyrhizobium* sp. (*Mucuna*). The fast growing strains were highly salt tolerant being able to tolerate 850 mM NaCl concentration *in vitro*. Slow growing strains showed growth at wide temperature range, being tolerant up to 45°C.

Keywords: *Mucuna pruriens, Rhizobium meliloti, Bradyrhizobium,* salt-tolerance

*The author to whom correspondence should be sent.

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1. Introduction

Legumes and their symbiotic bacteria make the maximum contribution to global nitrogen fixation. The *Rhizobium*-legume symbiosis, because of its agricultural importance, has ensured continuing research support world wide and is presently one of the best understood plant-microbe interactions (Somasegaran and Hoben, 1994). Despite this, only about 57% of the genera and 20% of the species have been examined for nodulation and most of those which have not been examined are from tropical areas (de Faria et al., 1989). As pointed out by Young (1996), the taxonomy of rhizobia is in a state of flux. *Mucuna pruriens* (Kaunch) is an annual herbaceous legume growing wildly in the Himalayan foothills. It is a plant with immense medicinal value in the Ayurvedic form of medicine (Sharma, 1996). *Mucuna pruriens* is also prominent among the herbaceous legumes, being promoted in the moist savanna region of the western Africa for use as green manure for soil fertility improvement and weed control (Versteeg and Koudokpon, 1990). Kaunch soil improving effects have also been reported in India and south Asia (Buckles, 1994).

In this study strains of root nodulating bacteria have been isolated from nodules of wildly growing Kaunch plants in the foothills of Himalayas. These strains were morphologically, biochemically and physiologically characterised to ascertain their taxonomic position. The newer isolates of the root nodulating bacteria may be beneficial for conserving this tropical medicinal plant besides improving soil fertility, being better adapted to the local conditions. As known most of the rhizobial isolates are capable of nodulating more than one legume species, and one legume species may also yield more than one species of *Rhizobium* (Young, 1996). This study reports for the first time isolation of both fast and slow growing rhizobia effectively nodulating *Mucuna pruriens*. The study also shows that with the examination of more and more leguminous plants, especially of the tropical and subtropical regions our knowledge on the taxonomy of the family Rhizobiaceae will get advanced.

2. Materials and Methods

Mucuna pruriens plants were collected in July, 1997, from the banks of river Ganga in the district of Hardwar, about 200 km northeast of New Delhi. This area is a deciduous rain forests with a temperature range of 4°C to 45°C. Strains of root nodulating bacteria were isolated from the root nodules of *M. pruriens* according to Vincent (1970). The strains were incubated on yeast extract mannitol (YEM) agar at 28°C and stored on YEM agar slants at 4°C. Twenty

strains were isolated and were characterised according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Cell dimensions, morphology and type of flagellation of the isolates was determined by scanning electron microscopy according to Miller and Pepper (1988). Generation time was calculated in YEM broth at 28°C (Kumar et al., 1999). Catalase and oxidase activities were determined according to the methods of Graham and Parker (1964) and Kovaks (1956), respectively. The ability to hydrolyse urea and gelatin were determined according to the methods of Lindstrom and Lehtomaki (1988) and Sadowsky et al. (1983) respectively. The ability to utilise citrate was determined according to Koser (1923). Growth on Hofer's alkaline broth was checked after inoculation with log phase cultures (10^8 cells) of the isolates (Hofer, 1935). Strains were tested for the ability to grow on glucose peptone agar (Klezkowska et al., 1968). Ability to tolerate 2% NaCl was observed according to Graham and Parker (1964). Precipitate formation in calcium glycerophosphate was determined according to Hofer (1941). DNA base composition was calculated by the thermal denaturation temperature determined with Shimadzu UV-VIS spectrophotometer model, UV-1601, as given by Marmur and Doty (1962).

Antibiotic resistance was detected by using antibiotic discs (HiMedia, Mumbai). YEM agar plates were spread with exponentially growing cultures of the isolated strains and antibiotic discs placed over the surface. The resistance to an antibiotic was detected by the inhibition zone formed around the discs. The following antibiotics were used: ampicillin, (10 µg); bacitracin, (10) µg; carbenicillin, (100 µg); chloramphenicol, (30 µg); erythromycin, (15 µg); furazolidone, (30 µg); gentamicin, (30 µg); kanamycin, (30 µg); neomycin, (30 µg); streptomycin, (10 µg); tetracycline, (15 µg); amoxycillin, (15 µg;) doxycycline, (10 µg) and cotrimoxazole, (10 µg).

To determine the carbon and nitrogen source utilisation, 80 µl of 10% w/v filter sterilised solutions of the carbohydrates and amino acids were added to 5 ml YEM broth in which yeast extract was reduced to 50 mg/l. The medium was then inoculated by the addition of exponentially (10^8 cells/ml) growing cultures of the isolates (Kumar et al., 1999). The inoculated broth were incubated at 30°C and kept at 150 rpm in an incubator shaker. Optical density was taken at 610 nm after 7 d incubation for measuring the growth.

Tolerance of acidic and alkaline pH was determined in YEM broth with pH adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. Buffers used to adjust the pH were citrate phosphate (3.0 to 7.0 pH), tris HCl buffer (8.0 to 9.0 pH) and glycine NaOH buffer (10.0 to 11.0 pH). Log phase culture was inoculated in the YEM broth with adjusted pH and growth observed by taking optical density as above.

Symbiotic study

All the isolates were checked for nodulation on their host, *M. pruriens* and a range of other plants. Nodulation study was carried out in earthen pots of half kilogram capacity. Seeds were collected from Kaunch plants during January, 1997. Earthen pots were filled with sterile soil and surface sterilised seeds of Kaunch and other plants were sown in the pots. The exponentially grown cultures of the isolates (10^8 cells/ml) were inoculated during seedling stage. A similar booster dose of the inoculum was given one week after the germination of seeds. Plants were watered with sterile water and were uprooted carefully after 30 and 60 days to observe for nodulation, seedling biomass, nodule number, nodule weight and *in situ* nitrogenase activity. Symbiotic nitrogenase activity was measured by the acetylene reduction (AR) assay according to Hardy et al. (1968).

Effect of temperature on growth

The effect of temperature on growth of isolates was observed in 50 ml YEM broth taken in flasks. The broth was inoculated with 0.1 ml of exponentially grown cultures. Flasks were placed inside temperature controlled incubator shaker at 10°, 20°, 25°, 30°, 35°, 40° and 45°C at 150 rpm. The optical density was measured every 4 h on Shimadzu UV-VIS spectrophotometer model, UV-1601, at 610 nm. Specific growth rate was determined according to Stanier et al. (1985) and plotted against temperature.

Effect of salinity on growth

The effect of salinity on the isolates was studied in YEM broth containing 0 to 900 mM NaCl. Exponentially growing cultures of the isolates were added to the NaCl amended broth and kept in the incubator shaker at 150 rpm and 30°C. The optical density was recorded every 4 h at 610 nm. Specific growth rate was determined (Stanier et al., 1985) and plotted against salinity.

3. Results

All the isolates were motile, Gram-negative, non-spore forming rods. Out of the 20 isolates, 12 were fast growers, with mean generation time of 2.8 h, and 8 were slow growers with mean generation time of 6.0 h. Fast growing strains formed white colored, rounded, mucoid colonies with 2–4 mm diameter after 48 h incubation at 28°C, on YEM agar media. On the other hand, slow growers formed white, rounded, opaque, convex and granular colonies with diameter ≤ 1

mm after 5–7 days of incubation. Scanning electron microscopy showed 2–5 peritrichous flagella in fast growing strains and single subpolar flagella in slow growers (Fig. 1).

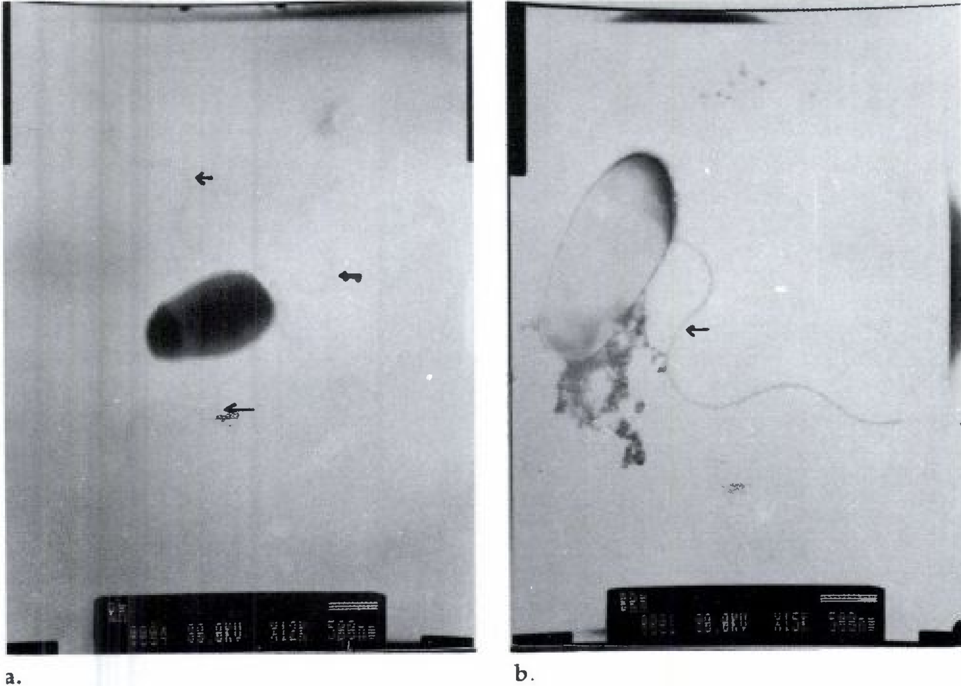


Figure 1. a. Scanning electron micrograph of fast growing strain. b. Scanning electron micrograph of slow growing strain.

All the isolates were catalase and oxidase positive. While the fast growing strains produced acid from glucose, the slow growers produced alkali. None of the strains showed growth on glucose peptone agar. Most (80%) of the strains were positive for urease and were able to grow in Hofer's alkaline broth. All the isolates were negative for gelatinase and lactase. None of the strains could utilise citrate. All the fast growing strains were able to tolerate 2% NaCl. Fast growing strains showed formation of precipitate in calcium glycerophosphate. The average G+C content of DNA was 62.6 mol % for fast growing strains and 64 mol % for slow growers (Table 1). Both fast and slow growing strains showed almost similar tolerance to pH with most (90%) of the strains tolerating pH from 5.0 to 11.0.

Table 1. Biochemical characterisation of the isolates from *M. pruriens*

Characteristic	Fast growing RMP												Slow growing BMP							
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8
Gram reaction	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Flagellar arrangement																				
i) 2-5 peritrichous	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–
ii) Single subpolar	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
Generation time (h)	2.8	2.9	2.8	3.0	2.8	2.8	2.8	2.8	2.7	2.8	2.6	2.7	5.8	6.0	6.0	6.0	6.1	6.1	6.0	5.9
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–
Growth on GPA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Urea hydrolysis	+	+	+	–	+	+	+	+	+	+	–	+	+	+	+	–	–	+	+	+
Growth on HAB	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lactase	–	–	–	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–
Citrate utilisation	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8% KNO_3 tolerance	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	–	+	+	+
2% NaCl tolerance	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	–
Ppt. in cal. glycerophosph.	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–
G+C content (mol %)	62.5	62.4	62.7	62.7	62.6	62.7	62.7	62.8	62.6	62.6	62.4	62.6	64.1	64.0	64.0	64.1	64.0	63.9	64.0	63.8

+ = Positive; – = Negative; GPA = Glucose peptone agar; HAB = Hofer's alkaline broth.

Table 2. Intrinsic antibiotic activity shown by the isolates

Antibiotic	Fast growers	%	Slow growers	%
Ampicillin	+	100	+	100
Bacitracin	+	100	+	100
Carbenicillin	–	0	–	0
Chloramphenicol	+	90	+	100
Erythromycin	+	100	–	25
Furazolidone	+	100	–	0
Gentamicin	–	0	+	100
Kanamicin	–	0	+	100
Neomycin	–	0	+	100
Streptomycin	–	0	+	75
Tetracycline	–	0	+	100
Amoxycillin	–	0	+	100
Doxycycline	–	0	–	0
Cotrimoxazole	–	0	–	0

+ = Resistant; – = Sensitive; %= Percent strains resistant.

Of all the antibiotics taken in the study both the fast and slow growing strains (except RMP_3), were resistant to ampicillin, bacitracin and chloramphenicol. Fast growers were also resistant to erythromycin and furazolidone. Slow growers were found resistant to gentamicin, kanamicin, neomycin, tetracycline, amoxycillin and streptomycin (except BMP_3 and BMP_5). Strains BMP_2 and BMP_6 were also resistant to erythromycin (Table 2).

Fast growing strains were able to utilise a majority of carbon and nitrogen sources tested. Majority of fast growers were able to utilise hexoses (glucose, mannitol, dextrose, rhamnose and sorbitol), pentoses (arabinose and xylose), disaccharides (trehalose, lactose and sucrose) and trioses (glycerol). Slow growing strains were mostly unable to utilise disaccharides and trioses, but were able to utilise pentoses and hexoses (Table 3). Fast growers were also able to utilise majority of amino acids except valine, threonine, glycine and methionine. However, slow growing strains were also able to utilise majority of amino acids except glycine, methionine, cystine and phenylalanine.

Symbiotic study

All the strains showed nodulation in *M. pruriens* and *Medicago sativa*. The fast-growing strains also showed nodulation in *Trigonella foenumgraecum*. Seedling biomass of the *M. pruriens* plants inoculated with slow growing strains

was maximum (13.2 g) after 60 days. Fast growing strains also increased seedling biomass over uninoculated controls. The nodule number in the plants inoculated with slow growing strains was less than in the plants inoculated with fast growing strains. However, nodules formed by slow growers were bigger and more in weight (per plant). The difference between seedling biomass of inoculated and uninoculated (control) plants increased considerably after 60 days (Table 4).

Plant nodulated by fast and slow growing strains showed significant nitrogenase activity. The AR activity, per plant, followed the trend of nodule fresh weight. Thus higher nitrogenase activity was observed in the plants nodulated by slow growing strains (Table 4).

Effect of temperature

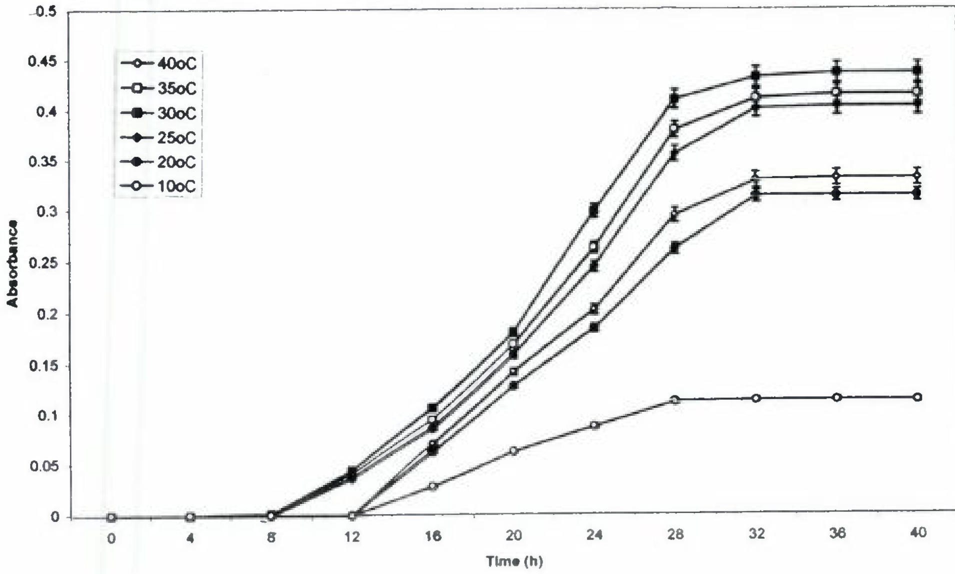
The optimum growth temperature for fast growing strains was 28°C and for slow growers 30°C. All of the fast growing strains were tolerant up to 35°C, while the slow growers, as confirmed by the considerable growth rate, could tolerate up to 45°C (Fig. 2). The growth rate of slow growers increased up to 30°C and then declined (Fig. 2b).

Effect of salinity

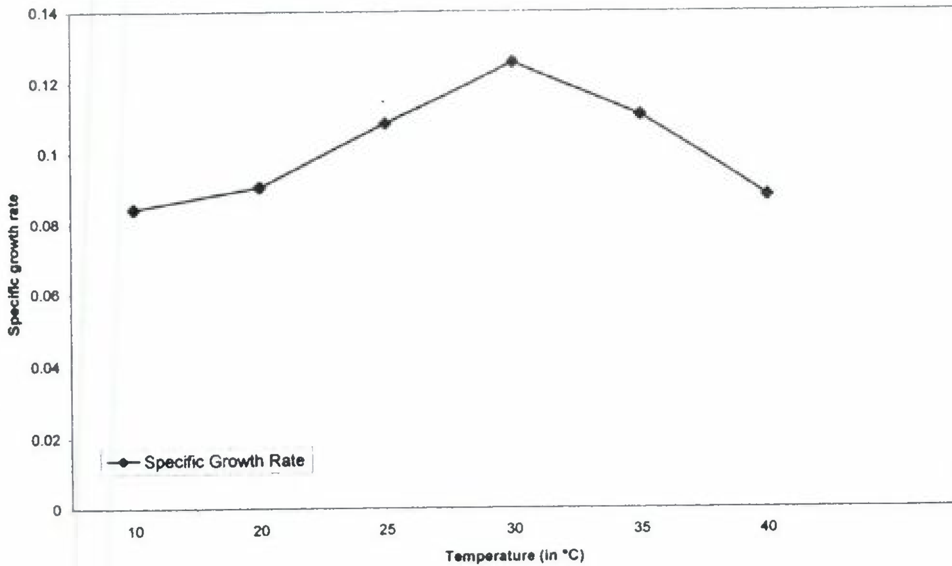
Fast growing strains were highly salt-tolerant. Nine of the fast growing strains were able to tolerate NaCl concentration up to 850 mM, in YEM broth (Fig. 3a). Strains RMP_4, RMP_6 and RMP_9 were tolerant up to 750 mM NaCl. Fast growing strains showed maximum specific growth rate at 300 mM NaCl concentration which declined with further increase in the NaCl concentration (Fig. 3b). Slow growing strains were able to tolerate up to 350 mM NaCl.

4. Discussion

Root nodulating bacteria isolated from the roots of *M. pruriens* were found to be both fast and slow growing. Both fast and slow growers formed effective nodules on the roots of their original host, *M. pruriens*. Both types of strains also showed effective nodulation in *M. sativa*. The morphological, physiological, biochemical characteristics, G+C mole percent and *in vivo* infectivity confirmed that the fast growers belong to the species *Rhizobium meliloti* and the slow growers to the species *Bradyrhizobium* sp. (*Mucuna*) (Holt et al., 1994). Fast growing strains showed precipitate formation in calcium glycerophosphate and were able to tolerate 2% NaCl, which is in accordance with the characteristics of *Rhizobium meliloti* (Jordan, 1984). Fast

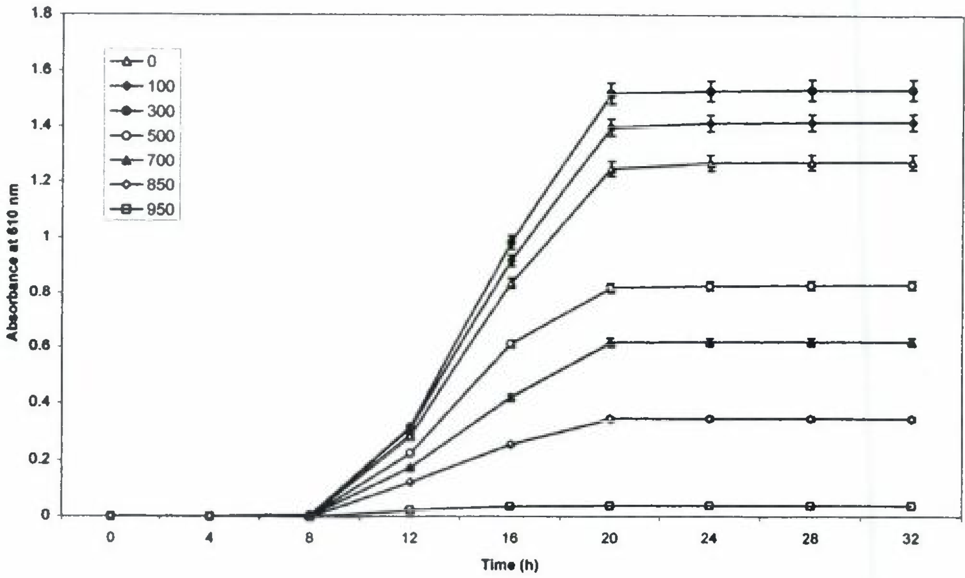


a.

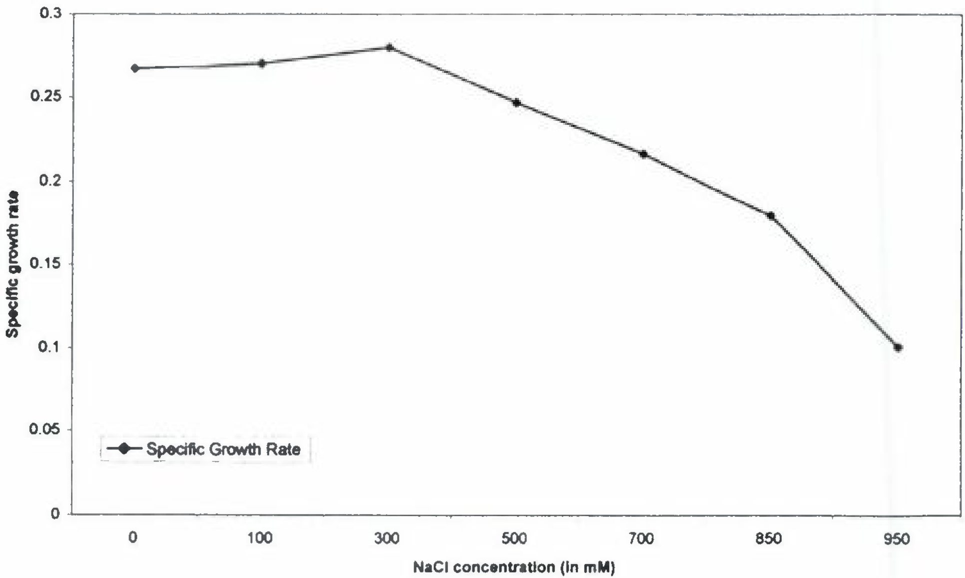


b.

Figure 2. a. Effect of temperature on growth of slow growing strains. b. Effect of temperature on specific growth rate of slow growing strains.



a.



b.

Figure 3. a. Effect of salinity (in mM) on growth of fast growing strains. b. Effect of salinity (in mM) on specific growth rate of fast growing strains.

growing strains have now been isolated from several other plants previously thought to be effectively nodulated only by slow growers as in soybean (Sadowsky et al., 1983; Scholla and Elkan, 1984; Keyser et al., 1982; Broughton et al., 1984), lotus (Cooper, 1982), hyacinth bean (Trinick, 1980) and lupin (Jordan, 1984). However, the slow growing strains were more effective, as confirmed by the seedling biomass, nodule number, nodule fresh weight and *in situ* nitrogenase activity. Fast growing strains although less effective in comparison to slow growers, showed a significant enhancement in the above parameters over uninoculated controls. The increase in difference between seedling biomass of inoculated and uninoculated plants with time clearly indicates the effectiveness of the strains. The relationships between nodule fresh weight and AR activity were positive. Similar results were reported for *Mucuna* by Reddy et al. (1984). Sangiga et al. (1996) reported the association of both slow and fast growing rhizobia with *M. pruriens* but only the nodules formed by slow growing isolates were effective in fixing N_2, as shown by increase in dry matter by their inoculation. However, the present study reports for the first time effective nodulation by both fast and slow growing strains in *M. pruriens*, as confirmed by increase in seedling biomass and nitrogenase activity of inoculated plants over uninoculated controls.

The results show that the slow growing strains (*Bradyrhizobium*) are much more resistant to antibiotics than the strains of *Rhizobium*. This is in accordance with the characters of *Bradyrhizobium* and *Rhizobium* (Elkan, 1992; Padmanabhan et al., 1990). Fast growing strains were able to utilise wide range of carbohydrates including hexoses, pentoses, disaccharides, trioses and organic acids. On the other hand, slow growers were able to utilise only pentoses and hexoses. This is also in accordance with the earlier findings that the fast growing rhizobia are capable of growing on a variety of carbon substrates while slow growing rhizobia are limited in their ability to use diverse carbon sources (Jordan, 1984; Stowers, 1985; Elkan, 1992). These results further confirm the taxonomic position of the bacterial isolates.

The optimum growth temperature for fast and slow growing rhizobia was 28° and 30°C respectively. All the fast growers were able to tolerate 35°C of temperature. Four of the fast growing strains were tolerant up to 40°C temperature. Slow growing strains were tolerant up to 45°C although the specific growth rate was reduced. Karanja and Wood (1988) and Hartel and Alexander (1984) suggested that rhizobia from hot dry areas are more temperature- and dessication-tolerant than strains from cooler regions. Tolerance of temperature up to 45°C can be useful in hot and dry conditions commonly encountered in tropical and subtropical regions (Padmanabhan et al., 1990). None of the isolates were tolerant to temperature below 10°C, which is in accordance with the results of Graham (1992).

Both fast and slow growing strains showed similarities in pH tolerance. Optimum pH for growth for both the strains was 7.0 and almost all the strains were tolerant to pH range from 5.0 to 11.0. The tolerance to similar pH range shows the effect of habitat on the isolates. Both fast and slow growing strains being from similar habitat were tolerant to similar pH range. Aguilar et al. (1998) observed that strains of *Sinorhizobium meliloti* were able to nodulate alfalfa at pH 5.0. Del Papa et al. (1999) also reported the isolation of acid tolerant rhizobia (upto pH 5.0) from the root nodules of alfalfa. However, a gradual loss of effectiveness was observed at acidic pH. The acid tolerant rhizobia can be of great agricultural significance to reclaim and increase the fertility of acidic soils.

Nine of the twelve fast growing strains were tolerant up to 850 mM NaCl concentration *in vitro*. Kumar et al. (1999) reported NaCl tolerance up to 700 mM and Lal and Khanna (1995) also observed salt tolerance by certain rhizobial strains upto 850 mM NaCl. Singleton et al. (1982) and Abd Alla and Abdel Wahab (1995) even reported that rhizobia isolated from arid lands could actually grow in solutions with salinity as high as that of seawater. Kassem et al (1985) observed that strains of *Rhizobium meliloti* are able to grow in presence of 4.5% NaCl. The study therefore reports the isolation of highly salt-tolerant strains. The in vivo study will be carried out to assess the capability of salt-tolerant strains in saline soils.

In the present study the specific growth rate of fast growing strains was enhanced up to 300 mM NaCl concentration, in YEM broth. Pillai and Sen (1973) reported that the growth rate of *Rhizobium* spp. increased by the addition of 1% NaCl in broth. Hua et al. (1982) also observed stimulation of growth of *Rhizobium* sp. strain WR1001, at 100 to 200 mM NaCl in medium. Highly salt tolerant strains (=850 mM NaCl) may be very useful in the reclamation of saline soils. However, further study is needed to determine the effect of salinity *in vivo* conditions on nitrogen fixation ability and nodulation capability of the strains. Slow growing strains were tolerant only up to 350 mM NaCl concentration. Singleton et al. (1982) and Hua et al. (1982) reported that slow growing strains are less tolerant to salt than fast growing strains.

This study according to the properties of rhizobia (Jordan, 1984; Elkan; 1992; Holt et al., 1994) confirms that the fast growing isolates from the root nodules of *M. pruriens* belong to the species *Rhizobium meliloti* and slow growing to *Bradyrhizobium* (*Mucuna*) species. These strains, specially *Bradyrhizobium* can thus be used in increasing the productivity of an important medicinal plant, *M. pruriens*. This study also reports the isolation of competent, salt, acidity and temperature tolerant strains which can be utilised in future, for land reclamation and increasing productivity of hot arid and saline soils.

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