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REGULAR ARTICLE

SCREENING OF RHIZOBIAL ISOLATES OF *LEUCAENA LEUCOCEPHALA* LAM. FOR MIMOSINE DEGRADATION ABILITY

Syed Farook Ali[1*], L.S. Rawat[1], M.K. Meghvansi[1,2] and S.K. Mahna[1]
[1]*Department of Botany, Maharshi Dayanand Saraswati University, Ajmer-305 019, India*
[2]*Present Address: Defence Research Laboratory, Post Bag 2, Tezpur- 784 001, Assam, India*

SUMMARY

Rhizobia can use mimosine as sources of carbon and nitrogen. In this study, an attempt has been made to evaluate the eleven rhizobial isolates of *Leucaena leucocephala* under arid and semi arid regions of Rajasthan for their relative mimosine degradation ability. It was observed that higher concentrations of mimosine adversely affected the growth, in terms of CFUs of rhizobial isolates. However, the trend of growth reduction was uneven and a few rhizobial isolates could tolerate the mimosine considerably. It provides growth advantage to *Rhizobium* strains that can utilize mimosine. Further, large scale field trials at different geographical locations are required for testing the effectiveness of these rhizobial isolates.

Key words: Mimosine degradation, Leucaena leucocephala, Rhizobia

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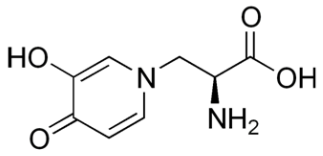
*Corresponding Author, Email: farookali2008@gmail.com

1. Introduction

Mimosine(ß-[N-(3-hydroxy-4-pyridone)]-α -aminopropionic acid) is a free abnormal toxic amino acid found in the leguminous plants of the genera *Mimosa* and *Leucaena* (See Figure 1 for chemical structure). All parts of the *Leucaena* plants including roots and root nodules, contain this abnormal amino acid, which is known to have antimitotic activity (Jones, 1979; Soedarjo and borthakur, 1996) that inhibits growth and protein synthesis in microorganisms. Besides microorganisms, mimosine has been found to exert toxic effects on the most terrestrial animals and plants (Patrick *et al.*, 2002). Structurally, it is an analog of Dihidroxy-phenyl alanine with a 3-hyddroxy-4-pyridine ring instead of a 3,4- dihydroxy-phenyl ring. It is also secreted in the root exudates of *Leucaena* (Soedarjo and borthakur, 1998). There are various demonstrated mechanisms of Mimosine toxicity (Lin et al., 1996; Mikhailovn *et al.*, 2000; Oppenheim *et al.*, 2000). Mimosine can affect DNA metabolism in eukaryotes (Tsai *et al.*, 1971; Gilbert *et al.*, 1995; Wang *et al.*, 2000).

Figure 1: Chemical structure of Mimosine



Leucaena leucocephala Lam. de wit belonging to Mimosoideae, is very common leguminous fodder tree in arid and semi arid regions of Rajasthan. In recent years the potential for economic use of *L. leucocephala* for various commercial purposes has been well recognized. It's wood and twigs can be used for a paper industry. It also used for land reclamation, erosion control, reforestration, shade and hedge in many parts of the world (Duke, 1983). Because of it's high tolerant to drought, resistant to pest and diseases and the ability to grow in a wide variety of soil type, it has become an important tree in agro forestry. The analyzed nutrient composition indicated that *Leucaena* seeds are potential source of protein and energy (Ahmed and Abdelti, 2009). *Leucaena*

is nodulated by several types of rhizobia, among these some can degrade mimosine (Mid^+ strains) and use it as a sources of carbon and nitrogen, while some cannot degrade mimosine (Mid^- strains) (Soedarjo *et al.*, 1994; Marutani *et al.*, 1999). Inhibition of growth of Mid^- strains was also noticed, when grown on trypton extract media incorporated with mimosine as a carbon and nitrogen source (Soedarjo and borthakur, 1996). The ultimate objective of the present investigation was to screen rhizobial isolates obtained from *Leucaena leucocephala* under arid and semi arid region for their mimosine degradation ability. The knowledge of the level of *Leucaena* rhizobial isolates to degrade mimosine can help in tracing the isolates introduced into the soil, in studies of competition for nodule infection sites and in ecological studies.

2. Materials and Methods

Experimental site

Two regions (A-Ajmer and B-Bikaner) of Rajasthan were selected representing the typical semi-arid and arid zone, respectively with sparse vegetation.

Region A: Ajmer

Ajmer district is located in the centre of Rajasthan state between 25° 38' and 26°58' north latitudes and 73° 54' and 75° 22' east longitudes. The average maximum temperature recorded is 46.0 degrees Celsius. The normal annual rainfall is 60.18cm. Ajmer district occupies an area of 8480 km^2, and is located between 25°38': 26°58' north latitude and 73°54': 75°22' east longitude. The annual rainfall is below 500mm, showing a semi-arid climate (Khan, 1999). The northwestern part is covered with sand dunes and rest of the area is generally flat. Hydro- geologically, the major part of the region is occupied by crystalline rocks comprising of calc-schist, amphibolites/calc-gneiss and biotite schist (all Precambrian); sand and alluvium of younger age are other important formations (GSI, 1977; Srivastava, 2001).

Region B: Bikaner

Bikaner district lies in the north-west of Rajasthan in heart of 'Thar' desert between the latitude 27011'03'' to 29003' north and longitude 71054' to 74012' east comprising a total geographical area of 27,244 sq km (CAZRI, 1990). Climate of the district ranges from arid in the east to extremely arid in the west. The mean rainfall of the district is 247mm varying from 300mm in the east to 180mm in the west. The annual potential evapo-transpiration is 1770 mm (Gheesa, 1999). The mean maximum temperature ranges from 24.4 to 43.80°C and mean minimum for 7.3 to 31.0°C. Frequent droughts once in 2.5 years is a common phenomenon. Soils of this district are predominately light textured, weak structured and well drained. All the soils are calcareous, amount of calcium carbonate increases with depth merging at lower depths with lime concretionary zone particularly in the flat aggraded older alluvial plains and the flat interdunal plains (CAZRI, 1974).

Collection of root nodules and isolation of rhizobia

Root nodules of *Leucaena leucocephala* were collected from Ajmer and Bikaner regions and were transported to the laboratory in plastic bags along with seedlings, where bacterial strains were isolated. In the process, nodules were separated from the roots and washed in sterilized distilled water for several times. Following serial dilution agar plate technique as described by Somasegaran and Hoben, (1994) using YEMA (Teast Extract Mannitol Agar) medium containing 0.0025 % Congo red dye (Vincent, 1970), bacterial isolation was carried out. After that these plates were incubated at 28±1°C and observed daily. Bacterial colonies appeared after 2-3 days were picked up and streaked on YEMA plates. Pure cultures were obtained with one or more further sub-culturing steps. All the rhizobial isolates were subjected to their morphological, cultural and biochemical characterization (Vincent, 1970; Creager *et al.*, 1990; Cappuccino and Sherman, 1992). Furthermore, all the isolates were subjected to authentication test before performing any experiment.

Rhizobial isolates were nomenclatured so as to indicate their site of origin, name of legume and isolate numbers. In this pattern first letter A or B indicate the site of origin (A- Ajmer, and B-Bikaner), and numeric figure given in the end show the specific strain assign number.

Screening of rhizobial isolates for utilization of mimosine

Eleven rhizobial isolates of *Leucaena lecocephala* were screened for their ability to utilize mimosine ([ß-*N*-(3-hydroxy-4-pyridone)-α-aminopropionic acid) as sole source of carbon and nitrogen by inoculating them on *Rhizobium* Mimosine (RM) solid medium containing varying concentrations of mimosine (1mM, 2mM, 3mM, 4mM and 5mM). In order to study the comparative effect of mimosine, rhizobial isolates were also plated on standard YEMA medium, which served as control having no mimosine content. For inoculation, rhizobial isolates were serially diluted and a dilution of 10^{-6} was drop inoculated (as per Mishra Droplet method) on RM medium and on standard YEMA media. Triplicate plates were prepared for each inoculation. Results were recorded as number of viable colonies as colony forming units (CFUs) after incubation at 28±1°C for 72h and data have been furnished in Table (1). Logarithm (base 10) was calculated for viable CFUs for narrowing down the variation for graphical representation.

Table 1: Growth of rhizobial isolates on RM media supplemented with different concentrations of mimosine

Mimosine concentration	Rhizobial isolates CFUs × 33* × 10^6 cells.ml^{-1}										
	ALL-1	ALL-2	ALL-3	ALL-4	ALL-5	ALL-6	BLL-1	BLL-2	BLL-5	BLL-6	BLL-7
Control#	52±0.577	86±1.453	76±2.027	52±1.527	45±1.732	80±0.882	43±1.201	56±1.000	39±0.333	68±1.764	82±1.732
1 mM	40±1.201	12±0.882	06±1.201	16±0.667	05±0.882	41±1.202	29±2.081	22±0.882	11±0.577	56±0.333	30±0.882
2 mM	17±1.453	03±0.000	--	09±0.333	01±0.333	14±0.577	20±0.667	08±0.333	02±0.333	40±0.577	13±1.155
3 mM	03±0.882	--	--	--	--	04±0.333	07±0.882	--	--	27±1.333	04±0.577
4 mM	--	--	--	--	--	--	--	--	--	14±1.000	--
5 mM	--	--	--	--	--	--	--	--	--	04±0.667	--

Data are mean of three replicates ±S.E.
#Standard YEMA media (without mimosine)
*one drop (0.03ml) from dilution 10^{-6} was plated on each plate. 33 (1/0.03=33) is conversion factor to convert CFU count in ml

3. Results

It was observed that brownish yellow color of the RM medium turned colorless, which indicated the utilization of mimosine by rhizobial isolates. In the current investigation, higher concentrations of mimosine caused a significant reduction in number of CFUs as compared to those on control media. However, the trend of growth reduction was uneven (Fig. 2 to 4). Maximum CFUs (86 x 10^6) was recorded for ALL-2, followed by ALL-6 (80x10^6 CFUs) and BLL-7 (82 x10^6) at standard YEMA medium (without mimosine). When 1mM final concentration of mimosine was incorporated in the RM media, isolate BLL-6 formed maximum viable cells (56x10^6 CFUs). However, CFUs of rhizobial isolates were much less as obtained on standard YEMA media. All the isolates utilized 1mM mimosine and the utilization by rhizobial isolates was observed to be in the order BLL-6 > ALL-6 > ALL-1 > BLL-7 > BLL-1 > BLL-2 > ALL-4 > ALL-2 > BLL-5 > ALL-3 > ALL-5. Poor to very good growth of rhizobial isolates was observed on RM media having 2mM mimosine. Seven rhizobial isolates (ALL-1, ALL-4, ALL-6, BLL-1, BLL-2, BLL-6 and BLL-7) demonstrated noticeable growth, whereas growth of three rhizobial isolates i.e.

ALL-2 (CFUs 3 x10^6), ALL-5 (CFUs 1 x10^6), and BLL-5 (CFUs 2 x10^6) showed much toxic impact. Moreover, viable cell count for ALL-3 was undetectable on this concentration. In the current study, five rhizobial isolates i.e. ALL-1 (CFUs 3 x10^6), ALL-6 (CFUs 4 x10^6), BLL-1 (CFUs 7 x10^6), BLL-6 (CFUs 27 x10^6) and BLL-7 (CFUs 4 x10^6) exhibited growth on RM medium containing 3mM mimosine, while six rhizobial isolates viz., ALL-2, ALL-3, ALL-4, ALL-5, BLL-2 and ALL-5 did not show any growth. Furthermore, only isolate BLL-6 degraded mimosine in the bioassay (few rhizobial colonies) when 4mM and 5mM mimosine was supplemented in media.

Figure 2: Comparison of rhizobial growth (Log 10 CFUs/ml) on standard YEMA media and 1mM mimosine supplimanted RM media

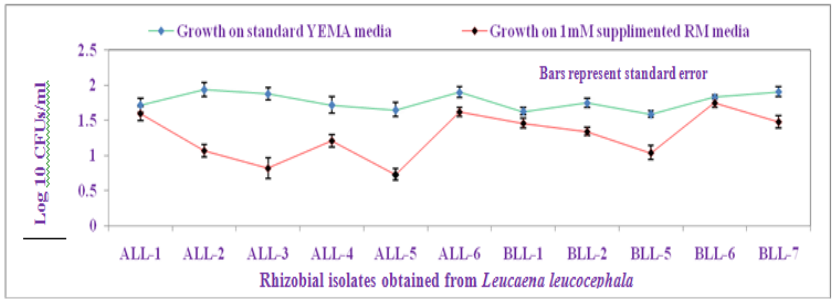


Figure 3: Comparison of rhizobial growth (Log 10 CFUs/ml) on standard YEMA media and 2mM mimosine supplimanted RM media

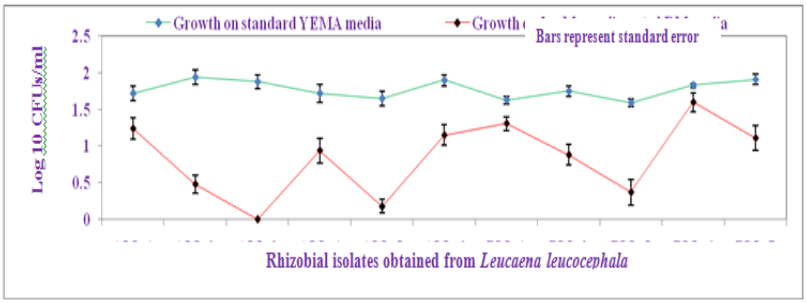
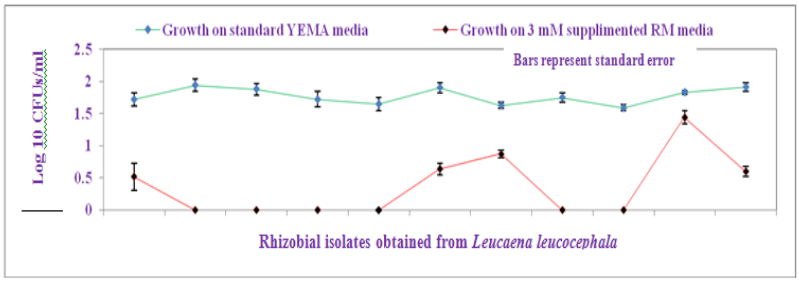


Figure 4: Comparison of rhizobial growth (Log 10 CFUs/ml) on standard YEMA media and 3mM mimosine supplimanted RM media



4. Discussion and conclusions

Mimosine, a toxin found in large quantities in the seeds, foliage and roots of the genera *Leucaena* and *Mimosa* induces a mimosine degrading enzyme activity in some strains of *Rhizobium* that nodulate *Leucaena* (Soedarjo *et al.*, 1994). Some *Leucaena* – nodulating *Rhizobium* strains can utilize mimosine as a selective growth substance (Soedarjo *et al.*, 1994). However, growth of most *Rhizobium* strains is inhibited by mimosine. In the present work, it was

observed that higher concentrations of mimosine adversely affected the growth, in terms of CFUs of eleven rhizobial isolates. This submission aligned correctly with previous findings of several other workers (Soedarjo *et al.,* 1994; Soedarjo and Borthakur, 1996; Marutani *et al.,* 1999). Notwithstanding, in the current study, all the isolates could utilize mimosine when inoculated on RM medium containing 1mM and 2mM . Similar to the present results, Soedarjo and Borthakur, (1996) found that most of the strains isolated from the nodules of *Leucaena leucocephala* in Hawaii completely degraded low concentration of mimosine (mid+) on RM medium. However, the growth performance of tested rhizobial isolates was quite variable. Soedarjo *et al.* (1994) while studying the mimosine degrading enzyme activity of some rhizobial strains obtained from various *Leucaena* species and NifTAL Project, Hawaii, also reported variability in the performance of different rhizobial strains in term of growth on mimosine medium. Soedarjo and Borthakur, (1996) determined the toxic effects of mimosine on the growth of most strains of *Rhizobium* by inoculating the strains in TY broth containing 3mM mimosine and observed that only strains TAL 1145 and MS 1246 showed growth. Marutani *et al.* (1999) also isolated indigenous *Rhizobium* strains from *Leucaena* leucocephala (Lam.) de Wit and reported that when rhizobial strains were grown on RM medium, few strains out of many, showed the ability to catabolise mimosine. Similar to above study also, out of eleven rhizobial isolates, only five isolates could grow on RM medium supplemented with 3mM mimosine and less value of CFUs were recorded. Moreover, in the RM medium containing 4mM and 5mM mimosine, only one isolate (BLL-6) could grow. This is the first report to demonstrate that rhizobial isolates obtained from *Leucaena leucocephala* grown in arid and semi-arid regions of Rajasthan have the variable potential to degrade mimosine. Thus, it also indicates the genetic diversity among rhizobial isolates of *L. leucocephala* being grown in Ajmer and Bikaner regions.

According to Soedarjo and Borthakur (1996), the mimosine degrading ability of rhizobia is not essential for nodulation of *Leucaena* species, but it provides growth advantage to *Rhizobium* strains that can utilize mimosine and it suppresses the growth of the strains that are sensitive to this toxin. Moreover, the result indicates that the variability in rhizobia might be an important factor to be considered in strain selection and preservation of culture for inoculants production. Large scale field trials at different geograophical locations in the semi-arid and arid regions are required to test the effectiveness of these rhizobial isolates.

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