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Mimosine produced by the tree-legume *Leucaena* provides growth advantages to some *Rhizobium* strains that utilize it as a source of carbon and nitrogen

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Abstract

Growth of most *Rhizobium* strains is inhibited by mimosine, a toxin found in large quantities in the seeds, foliage and roots of plants of the genera *Leucaena* and *Mimosa*. Some *Leucaena*-nodulating strains of *Rhizobium* can degrade mimosine (Mid^+) and are less inhibited by mimosine in the growth medium than the mimosine-non-degrading (Mid^-) strains. Ten Mid^+ strains were identified that did not degrade 3-hydroxy-4-pyridone (HP), a toxic intermediate of mimosine degradation. However, mimosine was completely degraded by these strains and HP was not accumulated in the cells when these strains were grown in a medium containing mimosine as the sole source of carbon and nitrogen. The mimosine-degrading ability of rhizobia is not essential for nodulation of *Leucaena* species, but it provides growth advantages to *Rhizobium* strains that can utilize mimosine, and it suppresses the growth of other strains that are sensitive to this toxin.

Introduction

Leucaena spp. are very common leguminous trees in many tropical and subtropical countries. In recent years the potential for economic use of *Leucaena* spp. for various commercial purposes has been recognized. Its wood and twigs can be used for a paper industry. Because of its high tolerance to drought, resistance to pests and diseases and the ability to grow in a wide variety of soil types, it has become an important tree in agroforestry. However, *Leucaena* plants contain a toxin, mimosine, which is known to be harmful to animals (Brewbaker and Hylin, 1965; Hegarty et al., 1964). Structurally, mimosine [*β-N*(3-hydroxy-4-pyridone)-a-aminopropanoic acid] is an analog of dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxyphenyl ring.

Mimosine has general antimitotic activity that blocks the cell cycle at late G1 (Boehme and Lenardo, 1993; Khanna and Lavin, 1993). It was found to arrest cell division in cultured human cells (Hoffman et al., 1991; Telfer and Green, 1993; Watters et al., 1994) and in Chinese hamster cells (Mosca et al., 1992). It also inhibited replication in yeast (Levenson and Hamlin, 1993) and cultured petunia leaf cells (Perennes et al., 1993). Recently, Feldman and Schonthal (1994) have shown that mimosine prevents the serum-stimulated synthesis and activation of histone H1 kinase, a crucial regulator of cell cycle progression. It is also known to chelate metals, bind pyridoxal phosphate and inhibit the enzymes tyrosine decarboxylase, tyrosinase (Thomson et al., 1969) and ribonucleotide reductase (Dai et al., 1994).

We found that young leaves and pods of *Leucaena* contain as high as 8–10% mimosine on a dry weight basis (Soedarjo and Borthakur, 1996). The toxicity of mimosine has been shown particularly in connection with animal husbandry. Because of the toxic effect of mimosine, sheep unaccustomed to *Leucaena* shed their wool approximately 7 to 14 days after feeding on it (Hegarty et al., 1964; Reis et al., 1975). Similarly, mice having 10% *Leucaena* seed or 1% mimosine in their diet showed loss of hair (Crounse et al., 1962). In spite of the high toxic effects of mimosine on animals, *Leucaena* leaves are used as a fodder for sheep and cattle in some countries since ruminants in some parts

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of the world contain a mimosine-degrading bacterium that degrades mimosine to nontoxic products (Allison et al., 1992).

Enzymes in the macerated leaves of *Leucaena* can rapidly degrade mimosine to 3-hydroxy-4-pyridone (HP) (Lowry et al., 1983). Previous studies of the metabolism of mimosine established that in the rumen of some *Leucaena*-fed ruminants mimosine is converted to HP before being completely degraded (Allison et al., 1992). Recently, we showed that mimosine induces a mimosine-degrading enzyme activity in some strains of *Rhizobium* that nodulate *Leucaena* (Soedarjo et al., 1994). In the present work, it is shown that mimosine provides growth advantages to some *Leucaena*-nodulating *Rhizobium* strains that utilize it as a source of carbon and nitrogen, and suppresses the growth of other strains that cannot catabolize it.

Materials and methods

Media and growth conditions

Rhizobium strains were grown in YEM, TY, RM and RP media as described previously (Soedarjo et al., 1994). In order to determine the toxic effects of mimosine, *Rhizobium* strains were grown on TY broth containing 3 m*M* mimosine. The cell densities were measured every 10 to 12 h for 2 days. Toxic effects of mimosine on *Rhizobium* was also determined by spotting 10 μL aliquots from serial dilutions, 10^{-1} to 10^{-6}, of a fresh culture grown in TY broth onto YEM agar containing 1 m*M* mimosine.

Rhizobium strains were screened for their ability to utilize mimosine or HP as the sole source of carbon and nitrogen by streaking them on RM agar plates and incubating them at 28°C for 4 days. The growth rate of *Rhizobium* strains in liquid RM medium with different mimosine concentrations was determined by inoculating 50 mL RM medium in 250 mL screw-cap bottles with 0.5 mL of fresh stationary phase *Rhizobium* culture. Cultures were grown at 28°C with shaking and growth was determined by measuring the cell density as absorbance at 600 nm in a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, NY). Aliquots were also plated from each culture to check for possible contamination. Each treatment was replicated three times. Cultures were maintained until the stationary phase was achieved.

Mimosine and HP determination

Mimosine was purchased from Sigma Chemical Co., St. Louis, Mo. HP was a gift from Dr Thomas K Hemscheidt. The mimosine or HP concentration in a sample was determined by means of HPLC (Beckman model 110A) using a C_{18} column (Ranin Instrument Co., Woburn, MA) and an absorbance detector at 280 nm (Tangendjaja and Wills, 1980). Elution of mimosine or HP was obtained using a solvent system of 0.2% orthophosphoric acid in deionized water at a flow rate of 1 mL/min. In this system mimosine and HP peaks appeared approximately at 2.7 min and 4.8 min, respectively.

Results

Mimosine is toxic to most strains of Rhizobium

Strains belonging to various *Rhizobium* spp. were streaked on YEM agar containing 1 m*M* mimosine. Although almost all strains grew, most of them did not form single colonies and grew much slower than on YEM without mimosine, suggesting that mimosine is toxic to most strains. When 10 to 10^4 freshly-grown cells in 10 μL of these strains were spotted on YEM agar containing 1 m*M* mimosine, growth was not visible in most spots where fewer than 10^3 bacteria were used. When more than 10^3 cells of a sensitive strain were plated on the mimosine-containing medium, aggregates of many cells together could grow overcoming the toxic effects of mimosine. Such inhibitory effects of mimosine were also seen on TY agar. However, some *Leucaena*-nodulating strains of *Rhizobium* did not show inhibitory effects due to mimosine when they were spotted on YEM agar containing mimosine. Later, it was found that these strains degraded the toxin and used it as a source of carbon and nitrogen (Mid^+). TAL1145 is such a *Leucaena*-nodulating Mid^+ strain that was originally isolated from the nodules of *Leucaena diversifolia* in Australia. We isolated a mutant MS1246 that cannot degrade mimosine (Mid^-) by Tn3Hog*us*-insertion in the *midD* gene of TAL1145. Strains TAL1145 and MS1246 were grown in TY broth containing 3 m*M* mimosine. Compared to the growth in TY broth without mimosine, the growth of TAL1145 in TY with mimosine was inhibited by 30% (Figure 1). However, an 85% inhibition was observed in the growth of the Mid^- strain MS1246 when its growth was compared in TY medium with and without mimo-

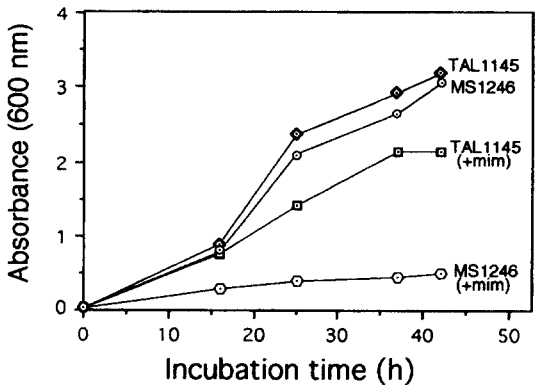

Figure 1. The effects of mimosine on the growth of the strain TAL1145 and MS1246. These strains were grown in TY broth with 3 m*M* mimosine (+ mim) and the growth of these cultures was compared with cultures grown in TY broth without mimosine.

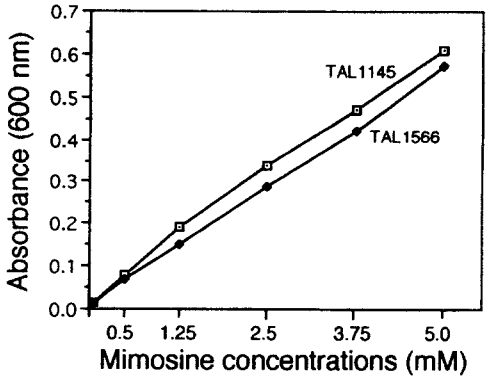

Figure 2. Growth of *Rhizobium* strains TAL1145 and TAL1566 in RM medium containing 0–5 m*M* mimosine. The cells were grown at 28°C for 48 h with shaking. It took 30–36 h for the cultures to attain stationary phase when 5 m*M* mimosine was present in the RM broth.

sine. Analysis of the culture supernatants by HPLC showed that strain MS1246 did not utilize any mimosine while TAL1145 utilized it completely. In another experiment, TY broth containing 3 m*M* mimosine was co-inoculated with an equal amount of cells of MS1246 and TAL1145 and grown for 72 h. The mixed culture was then diluted and plated on YEM agar containing different antibiotics to distinguish the colonies of the two strains. More than 90% of the colonies were of TAL1145, suggesting that mimosine in the medium provided a growth advantage to this strain by suppressing the growth of MS1246.

Some Leucaena-*nodulating* Rhizobium *strains can degrade mimosine*

Strains of different *Rhizobium* spp. were streaked on RM medium that contains mimosine as the sole source of carbon and nitrogen. Strains that can degrade mimosine (Mid^+) grew on the RM agar plates in 3–4 days. These strains were also tested in RM broth. Of the 92 strains originally isolated from the nodules of various *Leucaena* species, only 37 degraded mimosine (Table 1). When mimosine was completely utilized in the RM medium, the cultures turned from light yellow to colourless. This was verified by HPLC analyses of the culture media. *Rhizobium* NGR234 and *R. tropicii* CIAT899 that nodulate *Leucaena* did not utilize mimosine. Similarly, none of the *Rhizobium* strains isolated from the nodules of other mimosine-containing plants such as *Mimosa invisa* and *M. pigra* degraded mimosine. More than 200 strains belonging to different *Rhizobium* spp. were tested for growth in RM medium. None of these strains utilized mimosine.

When *Rhizobium* strains TAL1145 and TAL1566 were grown in RM medium, the final cell densities, determined by absorbancy at 600 nm, depended on the mimosine concentrations in the medium (Figure 2). The disappearance of mimosine from RM medium inoculated with these strains was monitored by HPLC. The time taken to utilize mimosine completely in the RM broth depended on the concentrations. For example, 1.25 m*M* mimosine disappeared in 15–18 h while 2.5 m*M* mimosine was completely utilized in 18–24 h. In the RM broth containing the highest concentrations of mimosine (5 m*M*), the entire amount disappeared completely in less than 36 hours whereas the mimosine concentrations of the RM broth containing the least concentration of mimosine (0.5 m*M*) inoculated with the Mid^- strains remained unchanged even when the incubation was extended to 48 hours (data not shown).

The Mid^+ strains were screened for their ability to degrade HP which is known to be a degradation product of mimosine. Ten strains were identified that cannot utilize HP as a source of carbon and nitrogen (Pid^-) (Table 1). In this way, the *Leucaena*-nodulating rhizobia were classified into three groups on the basis of mimosine and HP degrading abilities: (i) Mid^+Pid^+, (ii) Mid^+Pid^-, and (iii) Mid^-Pid^-.

Table 1. Degradation of mimosine and HP by *Leucaena*-nodulating rhizobia collected from different geographic origin

Strains	Legume host	Origin	Mimosine	HP
TAL82	*Leucaena leucocephala*	Hawaii, USA	+	+
TAL582	*L. leucocephala*	Australia	+	+
TAL589	*L. leucocephala*	Hawaii, USA	+	+
TAL595	*L. leucocephala*	Hawaii, USA	+	+
TAL598	*L. leucocephala*	Hawaii, USA	+	+
TAL599	*L. leucocephala*	Hawaii, USA	+	+
TAL721	*L. leucocephala*	Colombia	+	+
TAL995	*L. leucocephala*	Philippines	+	+
TAL996	*L. leucocephala*	Philippines	+	+
TAL1005	*L. leucocephala*	Philippines	+	-
TAL1143	*L. leucocephala*	Hawaii, USA	+	-
TAL1145	*L. diversifolia*	Australia	+	+
TAL1564	*L. diversifolia*	Hawaii, USA	+	-
TAL1566	*L. diversifolia*	Hawaii, USA	+	+
TAL1571	*L. shannoni*	Hawaii, USA	+	-
TAL1861	*L. retusa*	Puerto Rico	+	+
TAL1862	*L. retusa*	Texas, USA	+	+
TAL1863	*L. retusa*	Texas, USA	+	+
TAL1864	*L. retusa*	Texas, USA	+	+
TAL1943	*L. macrophylla*	Honduras	+	-
TAL1951	*L. macrophylla*	Honduras	+	-
MS1	*L. leucocephala*	Hawaii, USA	+	+
MS2	*L. leucocephala*	Hawaii, USA	+	-
MS3	*L. leucocephala*	Hawaii, USA	+	-
MS4	*L. leucocephala*	Hawaii, USA	+	+
MS5	*L. leucocephala*	Hawaii, USA	+	-
MS8	*L. leucocephala*	Hawaii, USA	+	+
MS11	*L. leucocephala*	Hawaii, USA	+	+
MS12	*L. leucocephala*	Hawaii, USA	+	+
MS14	*L. leucocephala*	Hawaii, USA	+	+
MS15	*L. leucocephala*	Hawaii, USA	+	+
MS16	*L. leucocephala*	Hawaii, USA	+	+
MS17	*L. leucocephala*	Hawaii, USA	+	+
MS18	*L. leucocephala*	Hawaii, USA	+	+
MS19	*L. leucocephala*	Hawaii, USA	+	+
MS22	*L. leucocephala*	Hawaii, USA	+	-
MS23	*L. leucocephala*	Hawaii, USA	+	+

+, degrading; -, nondegrading. Degradation of mimosine and HP was assayed in RM medium RP medium containing 2 m*M* mimosine and 2 m*M* HP, respectively.

Physiological differences between Mid$^+$Pid$^+$ and Mid$^+$Pid$^-$ strains

MS22, a strain that could not utilize HP (Mid$^+$Pid$^-$) was studied in more detail. When grown in RM medium, this strain grew to the same cell density as TAL1145, indicating that MS22 utilized mimosine as efficiently as TAL1145 (Figure 3A). Strain 8002 which does not degrade either mimosine or HP (Mid$^-$Pid$^-$) was used as a negative control. To determine whether HP accumulates in MS22 cells, the cells grown in RM medium were harvested and lysed. HP could not be detected in the lysate of either MS22 or TAL1145, suggesting that mimosine was completely degraded by both strains (data not shown). When these two strains were grown in medium to which HP was added instead

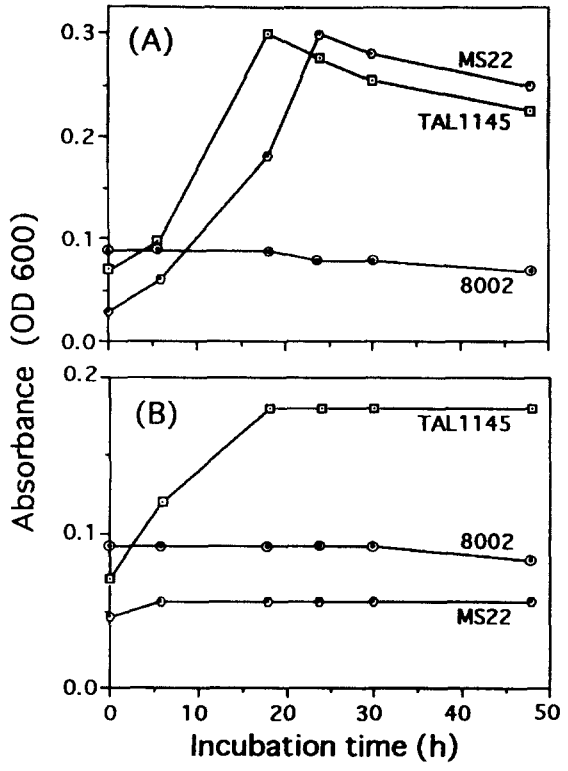

Figure 3. Utilization of mimosine and HP by Mid$^+$Pid$^+$ strain TAL1145 and Mid$^+$Pid$^-$ strain MS22. Mid$^-$Pid$^-$ strain 8002 was used as a control. Strains were grown in RM medium containing 2 m*M* mimosine (**A**) and RP medium containing 2 m*M* HP (**B**) as described in the Materials and methods section.

of mimosine, strain TAL1145 (Mid$^+$Pid$^+$) grew to a cell density of 0.18 within 18 hours whereas the cell density for the Mid$^+$Pid$^-$ strain MS22 did not increase (Figure 3B). These cultures reached the stationary phase at a relatively low cell density because the amount of HP was exhausted.

Discussion

Mimosine is a naturally-occurring toxic compound present in the shoots and roots of *Leucaena*. We also found mimosine in the nodules of *Leucaena*. Mimosine or a mimosine-like compound is present in the root exudate of *Leucaena* (M Soedarjo and D Borthakur, unpublished results). However, only a limited number of *Rhizobium* strains can utilize mimosine as a selective growth substance. The ability to catabolize mimosine is not required for nodulation or nitrogen fixation since many strains that cannot catabolize mimosine, such as CIAT899 and MS13, can form nitrogen-fixing nodules on *Leucaena*. Mimosine utilization may be a specialized mechanism that some rhizobia and other microorganisms living in the rhizosphere of *Leucaena* have developed to survive. It is conceivable that the ability to catabolize mimosine provides a competitive advantage to Mid$^+$ strains of *Rhizobium* in the rhizosphere. We have shown that mimosine provides a growth advantage to the Mid$^+$ strain TAL1145 over the Mid$^-$ strain MS1246 by suppressing the growth of the latter. Ability to degrade mimosine may provide another advantage to the Mid$^+$ strains by providing a source of carbon and nitrogen for growth. However, the Mid$^+$ strains do not utilize mimosine when they are growing in a medium containing tryptone and yeast extract. The mimosine-degrading enzyme activity in *Rhizobium* may be suppressed in the presence of other preferred sources of carbon and nitrogen. Recently, we did competition experiments using the Mid$^+$ strain TAL1145 and its Mid$^-$ mutants and found that the Mid$^-$ mutants are competition-defective (M Soedarjo and D Borthakur, unpublished results). This suggests that the ability to utilize mimosine provides a competitive advantage to strain TAL1145 in the rhizosphere. Mimosine may provide a source of carbon and nitrogen to the Mid$^+$ rhizobia and suppress the growth of other mimosine-sensitive microorganisms in the rhizosphere of *Leucaena*. Thus the *Leucaena* plants may provide a selective advantage to the Mid$^+$ strains by releasing mimosine in the root exudate.

We found that most Mid$^+$ strains also degrade HP which is a degradation product of mimosine. In our growth studies, strains such as MS22 that degrade mimosine but not HP were expected to accumulate HP when grown in RM medium. However, we did not detect HP in cells of MS22 growing on mimosine. On the other hand, strain MS22 grew to the same cell density as the Mid$^+$Pid$^+$ strain TAL1145 in RM medium. Since exogenous HP is not utilized by the strain MS22, we postulate that mimosine but not HP may be transported by this strain. Recently, we isolated several Mid$^-$ strains of TAL1145 that cannot degrade mimosine but degrade HP, suggesting that mimosine degradation by *Rhizobium* is completed in at least two steps (D Borthakur and M Soedarjo, manuscript in preparation). In the first step, mimosine is converted to HP which may be then degraded to pyruvate. In the mutants that we have recently isolated, the first step of mimosine degradation is blocked. We used strain TAL1145 in most experiments because this is an effective and competitive strain for nodulation of *Leucae-*

na and it is genetically well characterized (George et al., 1994; Parveen and Borthakur, 1994; Pooyan et al., 1994). We are currently doing genetic analysis of mimosine and HP degradations in TAL1145.

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