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Biochemical Mechanisms of Mimosine Toxicity to *Sclerotium rolfsii* Sacc.

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Abstract

Mimosine when added at a final concentration of 2·5 mM to potato dextrose broth reduced the mycelial growth of *S. rolfsii* by 40–60% and prevented the formation of sclerotia. This inhibitory action of mimosine was alleviated by 80% by 5 mM pyridoxal-5-phosphate and by 50% by tryptophan and 5 mM tyrosine. Phenylalanine had little effect. Among the metal ions, Fe^{3+} at 10 mM most effectively decreased mimosine's inhibitory effect by 93%, Al^{3+} at 5 mM decreased it by 80% while Fe^{2+} and Cu^{2+} at 5 mM exhibited the least effect (about 25 and 9%, respectively).

Mimosine decreased the specific activities of aspartate aminotransferase and polyphenol oxidase by 37 and 87%, respectively, and that of α-amylase by only 25%. Mimosine inhibited aspartate aminotransferase non-competitively (K_I = 0·056 mM) and polyphenol oxidase competitively (K_I = 0·089 mM).

ATP production in cells grown in the presence of 2·5 mM mimosine was reduced by 70%. Decreased synthesis of DNA was shown by lower incorporation of [^{3}H]thymidine. Mimosine had little or no effect on RNA synthesis.

Cell-free extracts of *S. rolfsii* degraded mimosine slightly into 3-hydroxyl-4(1H)-pyridone (DHP), mimosinic acid and an unidentified compound. A large amount of undegraded mimosine remained. No further metabolism of DHP was observed.

Introduction

Mimosine [β-(*N*-3-hydroxyl-4-pyridone)-α-aminopropionic acid] is a non-protein amino acid which is highly concentrated in the seeds and foliage of two legume genera, *Mimosa* and *Leucaena*. Some of the effects of mimosine and/or its metabolites include inhibition of growth of mung bean seedlings (Smith and Fowden 1966; Ling *et al.* 1969), low weight gains, hair loss and goitre in adult cattle (Jones *et al.* 1976), general ill-health, hair loss and stunted growth in sheep (Hegarty *et al.* 1964*b*), mortality of chicken embryos (Hatchcock and Labadan 1975) and growth inhibition of *E. coli* (Suda 1960).

More recently, Ebuenga *et al.* (1979) reported that 0·03–0·3% (w/v) mimosine infused in the growth medium caused inhibition of mycelial growth of *Colletotrichum lindemuthianum* (Sacc. & Magn.) Bri. & Cav., *Sclerotium rolfsii* Sacc., *Cercospora canescens* (Ell. & Mart.), *Diplodia natalensis* (P. Evans) and an *Alternaria* sp. It also inhibited conidial germination of *Cercospora personata* (Berk. & Court) Ell. & Everh. and uredospore germination of *Phakopsora pachyrhizi* Syd. Further studies supported the biocidal properties of mimosine against these legume pathogens (Mendoza and Ilag 1980, 1982; Ilag *et al.* 1981).

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Toxicity of mimosine in animals has been shown to result from chelation of metals essential for enzyme activity (Chang 1960; Tsai 1961), from interaction of mimosine with certain pyridoxal phosphate enzymes (Crounse *et al.* 1962; Lin *et al.* 1962*a*) and from inhibition of DNA synthesis (Tsai and Ling 1971). In plants, the chelating property of mimosine to biologically bound metals was found to account for mimosine toxicity (Ling *et al.* 1969). Except for some studies on mimosine effect on *E. coli* showing its antagonistic action against tyrosine and tryptophan (Suda 1960), no other studies have been made on the mechanisms of action of mimosine in microorganisms.

This study was conducted to investigate the biochemical mechanisms of mimosine toxicity to *S. rolfsii*, an economically important fungal pathogen of mung bean.

Materials and Methods

Materials

Crystalline L-mimosine was purchased from Sigma Chemical Co., Inc., and dissolved in 0·1 M HCl after which the pH of the solution was adjusted to *c.* 6·5. The solution was kept at 4°C until required. 3-Hydroxyl-4(1H)-pyridone (DHP) was prepared from mimosine following the method of Hegarty *et al.* (1964*a*).

Radioisotope materials such as [^{3}H]thymidine (37×10^6 Bq/ml, sp. act. $1 \cdot 33 \times 10^{10}$ Bq/mole), [^{3}H]uridine ($3 \cdot 7 \times 10^6$ Bq/ml, sp. act. $82 \cdot 9 \times 10^{10}$ Bq/mole) and [^{14}C]phenylalanine ($3 \cdot 7 \times 10^6$ Bq/ml, sp. act. $1 \cdot 98 \times 10^{10}$ Bq/mole) were purchased from New England Nuclear, Massachusetts, U.S.A.

S. rolfsii was obtained from the Pathology Laboratory, Institute of Plant Breeding, University of the Philippines at Los Bãnos, Laguna, The Philippines.

Growth Experiments

S. rolfsii was grown still on potato dextrose broth (PDB) prepared according to a modified procedure of Tuite (1969). The growth of the microorganism in the absence (control) or presence of mimosine at room temperature (25–29°C) was monitored by measuring its dry weight as follows. Two uniformly sized sclerotia were aseptically placed in sterilized Erlenmeyer flasks containing 10 ml PDB with or without mimosine. At certain time intervals, the fungus was harvested and filtered, rinsed with distilled water, placed in an oven at 100°C for 4 h and the dry weight obtained. To test the effect of some substances on the growth of *S. rolfsii* in the presence or absence of mimosine, their percentage inhibition of growth was determined after 90 h. These experiments were replicated three times.

Effect of Mimosine on Enzymes

Aspartate aminotransferase

Aspartate aminotransferase (aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) was extracted according to Jenkins *et al.* (1959) and its activity was determined using the method of Reitman and Frankel (1957). One enzyme unit is defined as the amount of enzyme that will convert 1 μmol of substrate per minute under the specified conditions. Specific activity is defined as unit activity per milligram protein for the three enzymes studied.

For *in vitro* kinetic studies, the enzyme extract prepared from cultures grown in the absence of mimosine was used. To determine K_I and the nature of its inhibitory action, mimosine at a final concentration of 0·016 mM was incubated with the reaction mixture for one hour before activity was measured. K_M and K_I values were calculated from Lineweaver–Burk plots.

Polyphenol oxidase

Polyphenol oxidase (EC 1.14.18.1) was extracted according to the method of Bouchillox *et al*, (1963) and assayed by following the oxidation of tyrosine at 280 nm as suggested in the Worthington Enzyme Manual (1977). One unit of polyphenol oxidase is defined as the amount of enzyme which

oxidizes 1 μmol of tyrosine to *o*-quinone per minute at 25°C and is equal to an increase in absorbancy of 0·001 per minute under specified conditions. For kinetic studies, mimosine at 0·09 mM was added to the reaction mixture after the lag period of the reaction had occurred.

α-Amylase

The procedure of Soyter and Schramm (1962) was used to extract α-amylase (EC 3.2.1.1) from cultures of *S. rolfsii*. α-Amylase was assayed according to the procedure of Bernfeld (1951). One unit of activity is defined as the amount of reducing group (calculated as μmol maltose) liberated from soluble starch per minute at 25°C.

Effect of Mimosine on ATP Formation in vivo

Cultures grown with or without 2·5 mM mimosine were homogenized in 20 ml distilled water in a Waring Blendor set at speed No. 7 for 1 min. The homogenate was boiled for 15 min, cooled and then centrifuged at 12 000 *g* for 1 min. The supernatant was immediately used for ATP determination. ATP was measured following the method of Bücher (1947) as modified by Adams (1963) based on the coupled phosphoglycerate phosphokinase and glyceraldehyde phosphate dehydrogenase assay for ATP.

Effects of Mimosine on DNA, RNA and Protein Synthesis

S. rolfsii was cultured on PDB in the presence or absence or 2·5 mM mimosine for 24 h and then transferred to a fresh medium of PDB containing radioisotope. The cultures harvested at timed intervals were subjected to different treatments to extract labelled DNA, RNA and proteins before counting in a liquid scintillation counter (Beckman LS-100C). These experiments were done in duplicate.

DNA Synthesis

[^{3}H]Thymidine was added to the medium at a concentration of $3{\cdot}7 \times 10^3$ Bq/ml. DNA was extracted according to the methods of Tsai and Ling (1971). The incorporated [^{3}H]thymidine was determined by liquid scintillation spectrometry. The amount of extracted DNA was measured by hydrolysing the extracted DNA precipitate with 1·0 ml TCA (5%) and then subjected it to the Dische diphenylamine reaction (Schneider 1957).

RNA Synthesis

In this study, [^{3}H]uridine was added to the culture medium at $3{\cdot}7 \times 10^3$ Bq/ml. RNA was extracted following the method for DNA extraction except that the KOH solution was treated with 1 M $HClO_4$, after which an aliquot of the supernatant (100 μl) was measured for incorporated radioactivity. For the quantitative determination of RNA, 1·0 ml of the extracted RNA supernatant was subjected to the orcinol reaction (Schneider 1957).

Protein Synthesis

[^{14}C]Phenylalanine was added to the culture medium at $1{\cdot}85 \times 10^3$ Bq/ml. The cell extract was prepared according to Cowie *et al.* (1959) and protein extracted following the method of Greenberg and Rothstein (1957). The resulting precipitate was solubilized with tissue solubilizer (Protosol, New England Nuclear), and after addition of scintillation liquid, measured for radioactivity. Protein was measured by the method of Lowry *et al.* (1951).

Metabolism of Mimosine by Cell-free Extracts of S. rolfsii

About 5 g of freshly harvested *S. rolfsii* grown in the absence of mimosine was homogenized in 5 ml of 0·05 M potassium phosphate buffer, pH 8·0. The homogenate was centrifuged at 18 000 *g* for 20 min. One-half ml aliquots of the supernatant were separately incubated with 0·5 ml of mimosine (4 mg/ml) and 0·5 ml of DHP (4 mg/ml) for 18 h at 37°C. The reaction was terminated by adding 2 ml of 95% v/v) ethanol and the resulting mixture centrifuged. The supernatant was concentrated to about 0·4 ml and subjected to descending paper chromatography using Whatman No. 3 MM

filter paper, at 25°C for 10 h. The solvent system used was butanol: acetic acid: water (4 : 1 : 2 v/v/v). The resulting chromatograph was dried in an oven at 80–90°C and the metabolites visualized by spraying with 1% (w/v) $FeCl_3.5H_2O$ in 0·1 M HCl. Authentic mimosine, DHP and mimosinic acid were run with the samples.

Results and Discussion

Effect of Mimosine on the Growth of S. rolfsii

Even at the low mimosine concentration of 2·5 mM, the mycelial growth of *S. rolfsii* was reduced by 40–60% (Fig. 1) and the mycelia were thin and brownish. Furthermore, sclerotia were not formed even after prolonged incubation of *S. rolfsii* with 2·5 mM mimosine. This is significant in considering the possible use of mimosine as a biocide since the effectiveness of a biocide lies in its ability to partially or completely cut-off the life cycle of an organism.

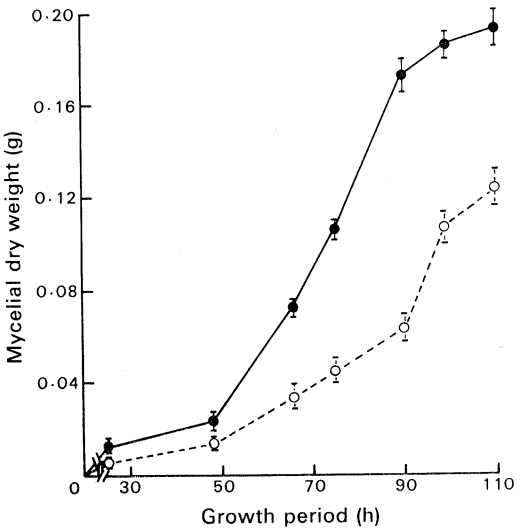


Fig. 1. Effect of 2·5 mM mimosine on the mycelial growth of *S. rolfsii*. ● Control. ○ With mimosine. Means of three replications ±standard deviation (vertical lines).

Counter-effect of some Substances on Growth Inhibitory Action by Mimosine

The inhibitory action of mimosine was lowered by 80% by 5 mM pyridoxal-5-phosphate, by 45% by 5 mM tryptophan and by 56% by 5 mM tyrosine (Table 1). Phenylalanine had little or no antagonistic action against mimosine.

Fe^{3+} (10 mM) and Al^{3+} (5 mM) were very effective in reducing the toxicity of mimosine to *S. rolfsii* (Table 1), Fe^{2+} and Cu^{2+} causing only a slight decrease.

Tyrosine at 2·5–10 mM reduced the fungal growth by 36% (Table 1) although its overall effect was still to decrease mimosine toxicity. Pyridoxal-5-phosphate (2·5 mM) and tryptophan (5·0 mM) also inhibited fungal growth by 17 and 16%, respectively. The other substances had little inhibitory or stimulatory effect on the growth of *S. rolfsii*.

The reduction of mimosine toxicity to *S. rolfsii* by pyridoxal-5-phosphate and metal ions such as Fe^{3+}, Al^{3+} and Fe^{2+} could be due to the strong complex formation between mimosine and the cofactor/metal ion (Lin *et al.* 1965; Tsai and Ling 1973). Lin *et al.* (1964) similarly observed that tyrosine and tryptophan but not phenylalanine

(all structural analogues of mimosine) were effective in counteracting mimosine toxicity in rats. Suda (1960) observed that mimosine exerted an antimetabolic action on *E. coli* which was greatly antagonized by tryptophan and to a lesser degree, tyrosine and phenylalanine. However, Tsai and Ling (1972) and Smith and Fowden (1968) reported that simultaneous addition of L-phenylalanine or L-tyrosine and mimosine to cultures of human epithelial cells and mung bean seedlings, respectively, showed no alleviation of mimosine toxicity.

Table 1. Effect of 2·5 mM mimosine on the mycelial growth of *S. rolfsii* in the presence of varying concentrations of different substances

Each value was obtained from the means of three replications. Average standard deviation for 64 means was ±9·90. Values in parentheses refer to percentage reduction (−) or stimulation (+) of fungal growth in the presence of the substance and without mimosine

Substance	Inhibition (%) at concentrations (mM) of:			
	0	2·5	5	10
Pyridoxal-5-phosphate	43·3	19·0(−17)	8·8(−4)	10·5(−6)
Tyrosine	55·3	31·7(−36)	24·6(−35)	37·5(−32)
Tryptophan	47·6	26·0(−9)	37·5(−16)	37·1(0)
Phenylalanine	48·2	46·3(−4)	43·4(−5)	41·8(+8)
Fe^{3+}	43·3	56·9(+3)	39·3(+4)	3·3(0)
Cu^{2+}	48·5	44·9(−3)	44·0(−1)	52·0(−3)
Fe^{2+}	44·3	36·3(−6)	32·6(−5)	41·1(−2)
Al^{3+}	44·6	22·5(+9)	8·0(−5)	21·3(0)

The antagonistic effect against mimosine inhibition of the growth of *S. rolfsii* by metal ions reported in this study corresponds well with the stability of their chelates (using a spectrophotometric method by Tsai and Ling 1973) which was found to be as follows:

$$Fe^{3+} > Al^{3+} > Cu^{2+} \gg Pb^{2+} > Zn^{2+}, Ni^{2+} > Co^{2+} > Ca^{2+}, Mg^{2+}.$$

Effects of Mimosine on Some Enzymes

In the presence of 2·5 mM mimosine, specific activities of aspartate aminotransferase and polyphenol oxidase in *S. rolfsii* decreased by 35 and 87%, respectively, while specific activity of α-amylase decreased by 25% (Table 2). Mimosine inhibited aspartate aminotransferase non-competitively as shown in Fig. 2, with a K_I of $5{\cdot}6 \times 10^{-5}$M. Since aspartate aminotransferase is a pyridoxal phosphate enzyme, mimosine toxicity to *S. rolfsii* may result from complex formation between mimosine and pyridoxal phosphate moiety of this and other pyridoxal phosphate enzymes. Inhibition by mimosine of pig heart aspartate aminotransferase (Lin *et al.* 1962*b*) but not of plant aminotransferase (Lin *et al.* 1962*a*; Smith and Fowden 1966) had been reported earlier.

On the other hand, polyphenol oxidase, a Cu^{2+}-containing enzyme which acts on a substrate (tyrosine) that is structurally similar to mimosine, was inhibited competitively by mimosine (Fig. 3) with a K_I for mimosine of $0{\cdot}89 \times 10^{-4}$M. This indicates that mimosine most likely inhibits polyphenol oxidase by binding the active site of the enzyme. It is feasible, however, that inhibition is caused by chelation of Cu^{2+} of the enzyme by mimosine.

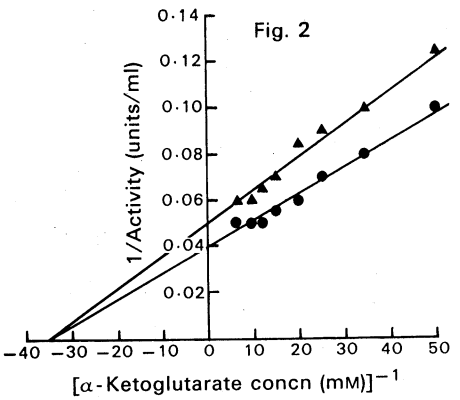
The low decrease (25%) in α-amylase activity of *S. rolfsii* cells grown with mimosine may be due to the unstable complex mimosine forms with Ca^{2+} (Tsai and Ling 1973). Ca^{2+} is required for activation of α-amylase.

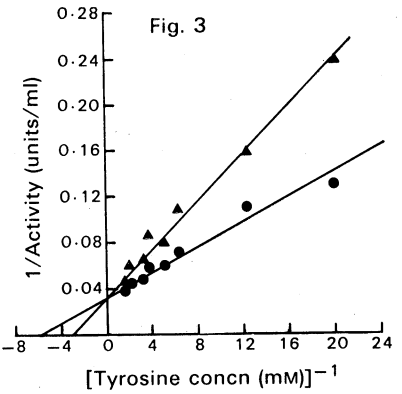
Table 2. Effect of mimosine on the specific activities of aspartate aminotransferase, polyphenol oxidase, α-amylase and on the production of ATP in *S. rolfsii*

Values are means of two replications ±standard deviation

	Control	With 2·5 mM mimosine
Aspartate aminotransferase (units/mg protein)	26·8±3·1	16·9±2·4
Polyphenol oxidase (units/mg protein)	52·1±3·0	7·0±1·0
α-Amylase (units/mg protein)	34·5±4·7	25·5±4·9
ATP production (μmol/g cells)	0·28±0·007	0·08±0·014

These results indicate that mimosine could act as an inhibitor of these enzymes *in vivo*, thus contributing to the decreased growth of *S. rolfsii* in the presence of mimosine.





Figs 2 and 3. Lineweaver–Burk plot showing the effect of mimosine on aspartate aminotransferase (Fig. 2) and polyphenol oxidase (Fig. 3) activity. ● Control (Figs 2 and 3). ▲ With 0·016 mM mimosine (Fig. 2) or 0·09 mM mimosine (Fig. 3).

Effect of Mimosine on ATP Production

Production of ATP by mycelium of *S. rolfsii* grown in the presence of mimosine was reduced (0·08 μmol/g) compared with control cultures (0·28 μmol/g) (Table 2). This is the first report on the effect of mimosine on ATP production. Since ATP production is largely mediated by several metal- and sulfhydryl-containing enzymes, this decreased ATP production could be due to complex formation between mimosine and the metal cofactor.

Effect of Mimosine on DNA, RNA and Protein Synthesis

In the presence of mimosine, DNA synthesis was reduced by up to 56% at maximum growth rate as shown by lower [^{3}H]thymidine uptake (Fig. 4*a*). The drop in [^{3}H]-thymidine observed after 60 h could be attributed to the dilution of the labelled compound as the growth of the microorganism increased exponentially. A similar decrease in DNA synthesis was observed in human epithelial cells grown in the presence of mimosine, this effect being reversed by Al^{3+} (Tsai and Ling 1972).

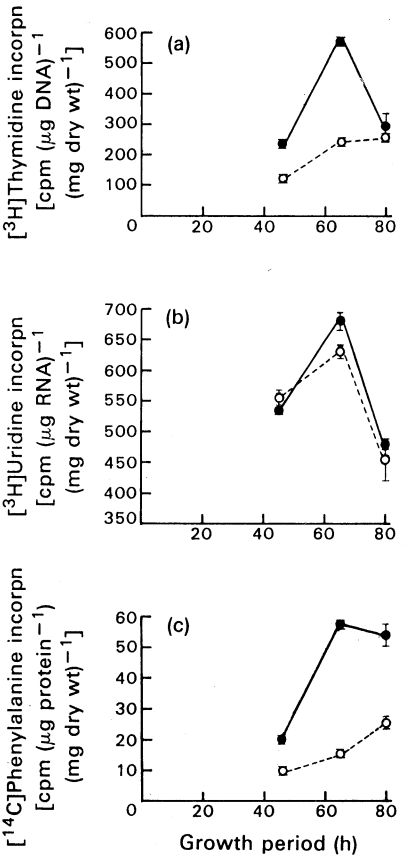


Fig. 4. Effect of 2·5 mM mimosine on [^{3}H]thymidine (*a*), [^{3}H]uridine (*b*) and [^{14}C]phenylalanine (*c*) incorporation into DNA, RNA and proteins, respectively, of *S. rolfsii*. ● Control. ○ With mimosine. Means of two replications ± standard deviation (vertical lines).

RNA synthesis in *S. rolfsii* was not significantly affected by mimosine (Fig. 4*b*). Tsai and Ling (1971) also found that mimosine did not affect RNA synthesis in human epithelial cells. It was noted, however, that the maximum uptake in both control cells and cells grown in the presence of mimosine occurred during the log phase of growth and dropped appreciably at early stationary phase possibly because of a dilution effect.

Protein synthesis was apparently inhibited by as much as 70% in cells grown with mimosine (Fig. 4*c*). However, the overall decreased [^{14}C]phenylalanine incorporation in proteins might have resulted from competitive inhibition of the label uptake by mimosine. Although Table 1 suggests little competition between mimosine and phenylalanine in terms of the former's effect on fungal growth, it cannot be ruled out that such competition does not occur at the level of amino acid uptake by cells of *S. rolfsii*. With human epithelial cells, mimosine did not inhibit [^{14}C]phenylalanine incorporation in proteins (Tsai and Ling 1971).

The toxic action of amino acid analogues like mimosine could result from repression of enzyme synthesis (this applies more particularly to lower organisms), enzyme activity inhibition through false end-product inhibition and blocking of normal amino acids to tRNA (Richmond 1962). Any of these effects could result in amino acid depletion, leading to interruption of DNA, RNA or protein synthesis.

Mimosine Degradation in vitro *by* S. rolfsii

The cell-free extract of *S. rolfsii* incubated with mimosine, on chromatography, produced spots corresponding to mimosinic acid, DHP, a large amount of undegraded mimosine and an unknown substance with an R_F of 0·10 which is lower than the R_F of mimosine (0·28). This compound cannot be mimosinamine because Tang and Ling (1977) showed that the R_F value of mimosinamine was higher than that of mimosine.

DHP, when incubated with the cell-free extract, was not degraded. No further studies on the toxicity of DHP or mimosinic acid were done. The *in vitro* experiments showed that mimosine was largely undegraded, suggesting that most of the toxic action may be attributed to mimosine.

Previous studies showed metabolism of mimosine into mimosinamine, mimosinic acid and DHP *in vivo* in rats (Takahashi and Hashiguchi 1976) and DHP in mung bean seedlings (Smith and Fowden 1966). Hegarty *et al.* (1979) had reported that DHP is a potent goitrogen which is responsible for goitre development in cattle and sheep grazing *Leucaena* sp. Hegarty *et al.* (1978) also showed that the 3-hydroxy-4-oxo function of the pyridine ring as well as α-alanine or a 2-aminoethyl side-chain were needed for inhibitory action against mouse bone marrow cells. It remains to be determined which structural features of mimosine are necessary for toxic action to *S. rolfsii*.

Acknowledgments

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