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Selective determination of mimosine and its dihydroxypyridinyl derivative in plant systems

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Summary. Our observations on the growth stimulatory nature of mimosine, (β-(3-hydroxy-4-pyridon-1-yl)-L-alanine), the toxic non-protein plant amino acid, in some model experimental systems, warranted sensitive and selective routine estimations. For the determination of both mimosine and DHP, an indirect spectrophotometric method was developed based on their individual reaction with known excess of DZSAM and by estimating the remaining DZSAM with N-(1-naphthyl)ethylene-diamine (NEDA). The resultant decrease in the secondary coupled product was measured at 540 nm. On equimolar basis, DHP had 40% of the reactivity of mimosine while interference from other relevant compounds was 15–35%. The determination of mimosine and DHP in tissue samples under different physiological conditions was effected after paper chromatographic separation of mimosine and DHP with distinctly differing R_f, from other compounds. The indirect method is superior in terms of absolute selectivity, sensitivity and ease of applicability with linear decreases in absorbance, proportional to increasing concentrations of mimosine from 0.1 to 0.75 μM or DHP from 0.2 to 1.5 μM and with recoveries of 99.2 to 100.5%.

Keywords: Mimosine – Dihydroxypyridine – *Leucaena leucocephala* – Reaction with diazotized sulfanilamide – Spectrophotometric determination – Uptake of mimosine – Plant mitochondria

Introduction

Mimosine, β-(3-hydroxy-4-pyridon-1-yl)-L-alanine, is a non-protein, toxic plant amino acid present exclusively in *Mimosa pudica* and some species of *Leucaena* (Matsumoto and Sherman, 1951; Tiwari and Spenser, 1965; Norton et al., 1994). Though *Leucaena leucocephala* is widely propagated, the use of its protein-rich leafy matter as a source of crude protein for livestock (Norton et al., 1994; Kudo et al., 1990) is limited by the toxicity of mimosine present (2–10% in the dry matter of leaves and seeds) (Dominguez-Bello and Stewart, 1990; Acamovic et al., 1982). Ingestion of mimosine and its dihydroxypyridinyl metabolite (DHP) resulted in toxicity such as hair loss, goiter, reproductive disorders, epithelial damage and ultimately death in grazing and experimental animals (Kamada et al., 1998; Hegarty et al., 1979, 1964). In studies with human lymphoblastoid and HeLa cell lines, mimosine was reported to cause reversible inhibition of cell cycle at the late G1 phase (Hughes and Cook, 1996; Watson et al., 1991; Lalande, 1990). This phytochemical inhibited some enzymes *in vitro* including tyrosinase (Hider and Lerch, 1989; Tanaka et al., 1989; and Hashiguchi and Takahashi, 1977), ribonucleotide reductase (Dai et al., 1994), serine hydroxymethyl transferase (Lin et al., 1996) and prolyl 4-hydroxylase (Ju et al., 1998). Studies demonstrated enzymatic biosynthesis of mimosine from O-acetyl serine and DHP in *L. leucocephala* seedlings (Muraskoshi et al., 1972; Ikegami et al., 1990). Results on the incorporation of radioactivity from selective amino acids into mimosine skeleton point to the physiological significance of this phytochemical (Tiwari and Spencer, 1965; Tiwari et al., 1967).

Degradation of mimosine was studied in rumen bacteria and symbiotic rhizobia (Allison et al., 1990; Jones and Megarrity, 1986; Soedarjo and Borthakur, 1998). During anaerobic digestion of *L. leucocephala* mimosine was almost completely degraded by mixed culture as revealed in our earlier investigations (Narayanaswami et al., 1986) and the resultant detoxified biomass (Vargheese and Lalitha, 1993), when fed to Japanese quails, revealed considerable growth promoting activity. Also, studies from our laboratory demonstrated, for the first time, significant growth promoting activities of mimosine at an exposure level of 100 μM in the seedlings of the plant *Trigonella foenum-graecum* (Santosh et al., 1999) and 200 ppm in the larvae of the insect *Corcyra cephalonica* (Santosh, 1999).

Further investigations related to the physiological interactions of mimosine necessitated development of methods for selective estimation of the phytochemical and its metabolite(s) at very low levels.

The procedure for the determination of mimosine based on its complexation in the concentrations of 20–500 μM with Fe^{3+} (Hegarty et al., 1964; Matsumoto and Sherman, 1951) lacks selectivity and sensitivity, while other methods based on gas-liquid chromatography (Mee and Brooks, 1971) and high-performance liquid chromatography (Puchala et al., 1996; Lowry et al., 1985; Acamovic et al., 1982; Tangendjaja and Wills, 1983) require elaborate sample preparation steps and with no appreciable improvements in sensitivity. In a sensitive spectrophotometric method earlier reported (Lalitha et al., 1993), mimosine was estimated by direct coupling with diazotized p-nitroaniline, forming a yellow colored product ($\lambda_{max} = 400$ nm) in concentrations as low as 0.25 μM and up to 10 μM. For studies on specific biological interactions of mimosine at sub-cellular levels, further improvement in terms of selectivity and sensitivity in the determination of mimosine was warranted. The coupling of diazotized aryl amines with N-(1-naphthyl)ethylenediamine (NEDA), described as the "Griess reaction", was used in the past for the estimation of both aryl amines and nitrite (Norwitz and Keliher, 1984; Anderson, 1979). Although a very sensitive indirect spectrophotometric procedure was recently proposed for the determination of mimosine in the range of 0.025–0.75 μM, based on its reaction with diazotized sulfanilamide (DZSAM) (Lalitha and Rajendra, 2004), our exploratory physiological studies revealed lack of selectivity in this method. The present paper describes the possible interference from other physiologically relevant compounds and the development of a method, suitably calibrated for selective and sensitive estimation of both mimosine and its dihydroxypyridinyl metabolite (DHP), present in plant extracts under different physiological conditions. Mimosine or DHP, in graded concentrations, were first reacted with a known excess amount of DZSAM, followed by a second coupling reaction of the remaining quantity of DZSAM with NEDA, resulting in a proportional and linear decrease in the absorbance of the pink colored product of the latter reaction measurable at 540 nm. The specificity of the reactions of DZSAM with both mimosine and DHP has been confirmed by the preparation of the respective products. Results are presented on the calibration of the method with absolute selectivity and superior sensitivity for effective estimation of both the compounds in plant extracts and ease of applicability for metabolic studies with recoveries of 99.2–100.5%.

Materials and methods

Materials

L-Mimosine, L-tyrosine, L-3,4-dihydroxyphenylalanine (DOPA), pyridoxine hydrochloride, pyridoxal 5′-phosphate (PLP), catechol, pyrogallol, N-(1-naphthyl)ethylenediamine hydrochloride (NEDA) and cation exchange resin, Dowex 50W-X4 (100–200 dry mesh, H^+ form) were from Sigma (St. Louis, USA). Sulfanilamide (SAM), analytical standard, was from Fluka (Buchs, Switzerland). Sodium nitrite and other chemicals were of analytical reagent grade from Qualigens (Mumbai, India). Whatman 1 chromatographic paper used for paper chromatography was from Whatman International Ltd. (Kent, UK). Leaves and seeds from *L. leucocephala* were freshly harvested from young trees in the local nursery maintained by the Agricultural Technology Information Center (Kancheepuram, India). Solvents used for paper chromatography were distilled and deionized water was used for all the experiments.

Preparation of DHP

Mimosine, 0.5% w/v in 0.5 M HCl, was refluxed for 8 h and the solution evaporated to dryness at 60°C under reduced pressure using a Buchi rotary evaporator model R-200 (Flawil, Switzerland) to remove the acid. The cooled residue was dissolved in 10 ml water and passed through a column of Dowex 50W-X 4 resin in the H^+ form (15 × 1.5 cm). The product was eluted from the column with 0.5 M HCl and the acid was removed from the eluate by evaporation as described above. The resulting solid DHP was further dried in a vacuum desiccator.

Preparation of the products of coupling reactions of DZSAM with mimosine and DHP and identification by NMR

The solutions used for the preparation of the coupled products between DZSAM and mimosine or DHP were: A) SAM solution (0.2 M in 0.5 M HCl); B) sodium nitrite solution (0.2 M in water); C) DZSAM, the reagent prepared just 5 min prior to reaction by mixing equal volumes of solutions A and B; D) Na_2CO_3 solution (8.8% in water), and E) solutions of mimosine and DHP, 50 mM in 0.1 M HCl.

To 1 ml of either mimosine or DHP solutions taken in test tubes, 1 ml each of DZSAM reagent and Na_2CO_3 solution were added. After 5 min at room temperature, about 0.75 ml of the red colored solutions obtained, were spotted on to silica gel G preparative TLC plates (20 × 20 cm) and the chromatograms were developed using a solvent mixture of methanol and chloroform (2:1). The plates were dried under a stream of warm air and the colored bands for mimosine and DHP products with R_f 0.15 and 0.85 respectively were scraped out, placed in test tubes and eluted with water. After filtering off the grains of silica, the solutions were dried at 50°C under vacuum and the respective residues obtained were dissolved in 0.5 ml of D_2O and ^1H NMR analysis was carried out using Bruker Avance 400 MHz spectrometer.

Interference studies

Working standard solutions of 5 μM each of pyridoxine, pyridoxal 5′-phosphate (PLP), catechol, pyrogallol, L-tyrosine and L-3,4-dihydroxyphenylalanine (DOPA) were prepared. Ascorbic acid and glutathione solutions (5 μM) were always freshly prepared. The above compounds in the concentrations of 0.25, 0.5 and 1 μM were first reacted with DZSAM in different sets in the presence or absence of added mimosine at 0.25 μM or DHP at 0.5 μM and the color reaction with NEDA was studied as per procedure described earlier (Lalitha and Rajendra, 2004). All absorption measurements were made using PerkinElmer spectrophotometer model Lambda 35 (Norwalk, USA).

Separation and detection of mimosine, DHP and interfering compounds by paper chromatography

Reagents: The reagents prepared for the detection of the compounds on paper after chromatographic separation were: (1) SAM ,10 mM in 0.05 M HCl; (2) $NaNO_2$, 10 mM in water, prepared fresh; (3) DZSAM solution, by mixing equal volumes of solutions1 and 2, prepared 5 min prior to use; (4) NEDA reagent, 10 mM, prepared fresh by dissolving 259 mg in 30 ml of ethanol and diluting to 100 ml with 0.7 M HCl; and (5) Na_2CO_3 solution (0.44% in water). Working solutions of mimosine, pyridoxine, PLP, ascorbate, glutathione, catechol, pyrogallol, DOPA and L-tyrosine, each (0.5 mM) were prepared fresh in water. The solvent system used for developing the chromatogram was 1-butanol:acetic acid:water (60:15:35).

Procedure: Suitable aliquots containing 2 to 10 nmol of different compounds along with mimosine and DHP were individually spotted on Whatman 1 chromatographic paper and the chromatograms were developed for 8 h using a solvent system of 1-butanol, acetic acid and water (60:15:35). A mixture of 5 nmol each of all the compounds was also spotted and the separation effected. The paper was dried using warm air, sprayed with DZSAM reagent and kept for 10 min at room temperature. Then, Na_2CO_3 solution (0.44%) was sprayed and after 5 min at room temperature, the paper was treated with NEDA reagent. On appearance of clear colorless spots against the pink background of the NEDA-DZSAM product, the position of the respective products was located and marked on the paper. The products corresponding to mimosine and DHP on the paper were also confirmed by spraying duplicate strips of paper with diazotized p-nitroaniline solution (Lalitha et al., 1993) and the presence of mimosine was further confirmed by spraying the paper with ninhydrin solution (Oreskes and Saifer, 1955).

Standardization of the method

Effect of different buffers and time course of the reaction: The reaction of mimosine, in concentrations ranging from 0.05 to 0.75 μM, with DZSAM under standard conditions was tested at pH 7.6 using different buffers viz. Tris-HCl, sodium phosphate and sodium borate at 0.05 M. In the procedure, Na_2CO_3 solution was replaced by 1 ml of 0.25 M buffer solutions. Time course of the reaction was studied by adding NEDA reagent after reaction period of 10, 20 and 30 min.

Calibration of the method: The reagents prepared were: (A) SAM solution (0.25 mM, in 0.025 M HCl stored at 4°C for a month); (B) sodium nitrite solution (2.5 mM, freshly prepared in water and diluted to 0.25 mM); (C) stock solutions of mimosine, 5 mM in 0.025 M of HCl (diluted in water to get a working standard of 3.75 μM); (D) stock solution of DHP, 5 mM in water (diluted in water to get a working standard of 8 μM); (E) diazotized sulfanilamide (DZSAM) solution, prepared 20 min prior to the estimation, by mixing equal volumes of solutions A and B; (F) NEDA reagent, (0.25 mM, freshly prepared in 0.5 M HCl), and (G) sodium borate buffer (0.25 M, pH 7.6).

Aliquots of either mimosine from 0.25 to 3.75 nmol or DHP in the range of 1.0–7.5 nmol were added to a series of test tubes and made up to 2 ml with water. The control tubes had 2 ml of water. Addition of 1 ml of borate buffer was followed by 1 ml of reagent E (DZSAM). After mixing and keeping for 20 min, 1 ml of reagent F (NEDA) was added and the color developed was read after 10 min at 540 nm.

Procedure for estimation after paper chromatographic separation

For detection as controls, mimosine and DHP, 5 nmol each, were spotted on the paper along with suitable aliquots containing 0.5 to 3.75 nmol of mimosine and 1 to 7.5 nmol of DHP as standards for calibration. After developing the chromatogram, the paper was first cut along the entire height of the paper in strips along the movement of the control spots, corresponding to 5 nmol of mimosine and DHP. The strips were sprayed with DZSAM reagent, followed by Na_2CO_3 solution and lastly with NEDA reagent as described earlier. Colored products for mimosine and DHP spots were clearly visible only in concentrations above 5 nmol. Based on the respective R_f, of the control spots, 2 × 2 cm strips corresponding to standard spots of known quantities of both mimosine and DHP were also cut out from the rest of the chromatogram and eluted with 0.025 M HCl and water respectively for estimation.

Preparation of tissue extracts from L. leucocephala for the estimation of mimosine and DHP

Extraction of mimosine and DHP from plant tissues was carried out as per the procedure described by Lalitha et al., 1993 and modified as follows: 1) Preparatory step: Fresh leaves of *L. leucocephala* and dry seed powder, taken in batches of 2 g each were first added directly to boiling water 20 ml in 100 ml conical flasks, and allowed to remain boiling for 10 min for inactivation of enzymes, if any. The suspensions were cooled. 2) Extraction of mimosine: To each of the suspensions from step 1, 20 ml of 0.05 M HCl was added the plant tissues were further homogenized using a mortar and pestle to a final volume of 40 ml and the extracts filtered using Whatman No. 1 filter paper. The prepared extracts were diluted 1:200 with water and used for estimation of mimosine. 3) Extraction of native DHP: From the cooled suspensions from step 1 above, DHP was extracted further by grinding the tissue suspensions using a mortar and pestle in the same water medium and filtered using Whatman No. 1 filter paper. The prepared samples were diluted 1:10 times with water for estimation of native DHP. 4) Extraction of total DHP: To 10 ml of the undiluted filtered extract from the above step, 5 ml of 0.5 M HCl was added and refluxed for 8 h, after which the mixture was diluted 1:100 with water. From all the diluted extracts, aliquots of 10, 20 and 40 μl were individually spotted on to paper for chromatographic separation and spots corresponding to mimosine and DHP were eluted for accurate determination of the compounds present.

Application of the method for studying uptake of mimosine by plant mitochondria

Seeds of *Trigonella foenum-graecum and Vigna radiata* were germinated in the dark for 72 h using deionized water. Germinated seedlings, 20 g, were ground manually using a mortar and pestle in a medium containing: 0.22 M mannitol; 0.07 M sucrose; 1 mM EDTA; 0.3% (w/v) polyvinylpyrrolidone (PVP) and 0.05% (w/v) BSA. The ratio of medium to wet weight of the seedlings was maintained at 2:1 and the pH was maintained at 7.2 by the addition of 0.1 N KOH. The homogenate, after filtering through layers of cheese cloth was centrifuged for 5 min at 800 × g to remove cell debris and the supernatant further centrifuged at 8000 × g for 20 min to collect the mitochondrial pellet. Mitochondria were suspended in the same medium without PVP (20–25 mg protein/ml). Mitochondrial uptake of mimosine at added levels of 25 or 50 μM was followed for specified time intervals from 30 sec to 10 min in medium (1 ml) containing: 2.5 mg protein freshly prepared active mitochondria; succinate, 3 mM; ADP, 0.15 mM and potassium phosphate buffer (0.01 M, pH 7.5). After incubation of the reaction mixture at 37°C for specified intervals of time, the uptake was terminated by rapidly chilling the tubes in ice. The contents were immediately filtered through 0.22 μm PVDF membrane filters using Millipore filter assembly (Millipore Inc. Billerica, USA) and the amount of mimosine remaining in the filtrate was estimated by the present method.

Results

Preparation of DZSAM coupled products of mimosine and DHP

Coupling of DZSAM individually with mimosine and DHP at 0.05 mM each was carried out at pH 7.6, adjusted with Na_2CO_3. The molar extinction coefficients for the products were 2.4 mM^{-1} cm^{-1} for mimosine at 540 nm and

$7.35\,mM^{-1}\,cm^{-1}$ for DHP at 495 nm. Though DZSAM-mimosine coupled product had absorption maximum at 540 nm, similar to that of NEDA-DZSAM product, the former's contribution to the total absorbance during the estimation was very negligible due to low level of molar absorptivity. Proton NMR analysis of the products with DZSAM revealed: coupling at C5 position, disappearance of peak corresponding to 6.4 ppm for mimosine product and 7.2 ppm for the DHP product.

Interference studies

The results on the reactivity for some of the biologically relevant compounds when tested at 0.25 μM and 0.5 μM with DZSAM are given in Table 1. The reactivity of the compounds with DZSAM, when compared with that of mimosine (0.25 μM) was: catechol, 37%; glutathione, pyridoxine and pyrogallol, 30–32%; and ascorbate, DOPA and PLP, 17–25%. The reactivity of these compounds when compared to that of DHP at 0.5 μM was: catechol, 43%; glutathione, pyridoxine and pyrogallol, 38–41%; ascorbate, 32%; and DOPA and PLP, 21–25%. Tyrosine was unreactive even at 0.5 μM. The compounds when tested at equimolar level with mimosine at 0.25 μM or DHP at 0.5 μM, revealed an additive effect on the reactivity. From the results it is evident that the aromatic hydroxyls in catechol and pyrogallol direct the electrophilic substitution favorably. A role for the ring nitrogen in accelerating the reaction can be discerned based on the reactivity of mimosine, DHP, pyridoxine and PLP. The aromatic amino acids, DOPA and tyrosine were minimally reactive. For eliminating the possible interference from some of these and related compounds during the estimation procedure under physiological conditions, a paper chromatographic procedure was developed for effective separation of different compounds.

Table 1. Interference from some biologically relevant compounds during the estimation of mimosine and DHP

Compounds tested	Additions**	Reactivity (ΔA)* at added levels of compound		
		0.25 μM	0.5 μM	1.0 μM
1. Ascorbate	–	0.053	0.130	0.242
	+ Mimosine	0.280	0.359	–
	+ DHP	–	0.320	0.442
2. Catechol	–	0.077	0.160	0.306
	+ Mimosine	0.357	0.380	–
	+ DHP	–	0.368	0.518
3. DOPA	–	0.041	0.090	0.186
	+ Mimosine	0.257	0.306	–
	+ DHP	–	0.290	0.390
4. Glutathione	–	0.073	0.151	0.327
	+ Mimosine	0.285	0.375	–
	+ DHP	–	0.340	0.484
5. Pyridoxal phosphate	–	0.035	0.089	0.140
	+ Mimosine	0.248	0.306	–
	+ DHP	–	0.289	0.347
6. Pyridoxine	–	0.063	0.128	0.263
	+ Mimosine	0.278	0.353	–
	+ DHP	–	0.296	0.475
7. Pyrogallol	–	0.068	0.135	0.224
	+ Mimosine	0.281	0.338	–
	+ DHP	–	0.310	0.394
8. Tyrosine	–	–	–	–
	+ Mimosine	0.211	0.211	–
	+ DHP	–	0.164	0.164
9. None	–			
	+ Mimosine	0.21		
	+ DHP	0.164		

* Reactivity of compounds tested at indicated levels in reaction medium, pH 7.0 and given as decrease in absorbance (ΔA) from that of the control
** Each compound tested in the absence (–), and presence (+) of added mimosine at 0.25 μM or DHP at 0.5 μM
Values are averages of triplicates from three sets of experiments. Standard deviation in absorbance <0.001

Separation and detection of the interfering compounds by paper chromatography

After the separation by paper chromatography, the respective compounds were detected on the paper in concentrations above 5 nmol, by spraying with DZSAM reagent followed by Na_2CO_3 solution and lastly with NEDA reagent, when colorless spots for the products were clearly visible. R_f values for mimosine, DHP and other compounds were: mimosine, 0.25; glutathione, 0.34; DOPA, 0.35; ascorbate, 0.36; PLP, 0.5; pyridoxine, 0.6; DHP, 0.7; pyrogallol, 0.8; and catechol, 0.9. While it was feasible to clearly discern the direct product formation on paper only at concentrations above 5 nmol, the estimation carried out in test tubes, was enabled at very low ranges of metabolite concentrations as 0.1–0.75 μM of mimosine and 0.2–1.5 μM of DHP. It was therefore decided to first separate the compounds on paper, elute them into test tubes and then carry out the reaction. It was difficult to have control over the amount of Na_2CO_3 solution sprayed on the paper and precise control over pH on the paper, the resultant elution of the product was unsatisfactory. The method was therefore tested using different buffer solutions.

Choice of buffer and time course of the reaction of DZSAM with mimosine

The time course of the reaction was followed in different buffer media. Results presented in Fig. 1 reveal: i) when

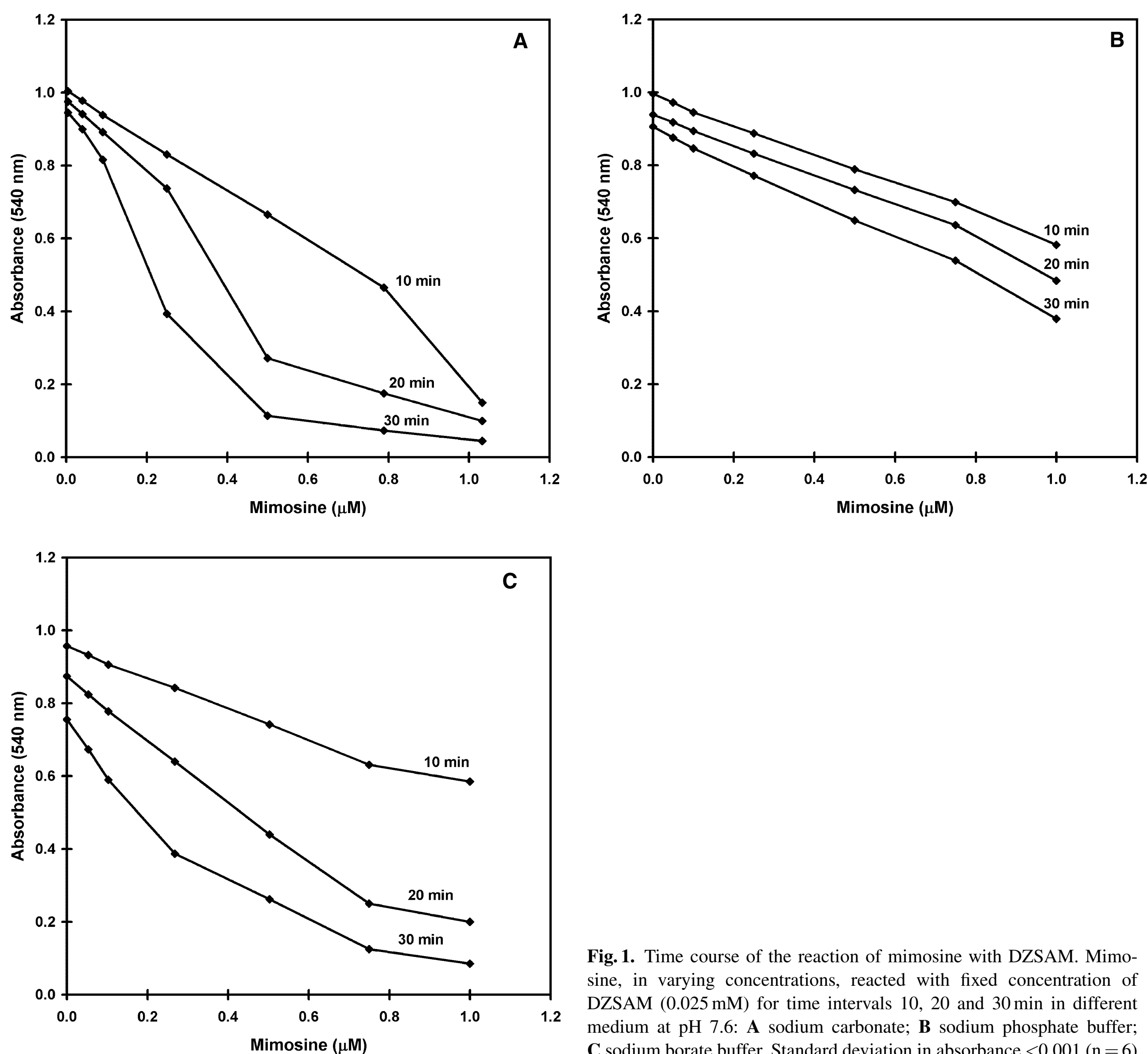

Fig. 1. Time course of the reaction of mimosine with DZSAM. Mimosine, in varying concentrations, reacted with fixed concentration of DZSAM (0.025 mM) for time intervals 10, 20 and 30 min in different medium at pH 7.6: **A** sodium carbonate; **B** sodium phosphate buffer; **C** sodium borate buffer. Standard deviation in absorbance <0.001 ($n = 6$)

pH was maintained with Tris-HCl buffer, at 10 min, the decrease in the color intensity was linear with mimosine concentration, 0.05–0.75 μM. At 20 and 30 min, the reaction occurred at faster rates with mimosine concentrations only up to 0.5 and 0.3 μM respectively. Beyond these levels, the reaction proceeded in a biphasic manner (Fig. 1A). ii) In sodium phosphate buffer, the reaction rate slowed down considerably at all time intervals (Fig. 1B). iii) In sodium borate buffer, the reaction proceeded with reasonable rate at 20 min, with linear decreases in absorbance proportional to mimosine concentrations from 0.05 to 0.75 μM. At 30 min interval, the rate was initially fast up to 0.2 μM of mimosine and proceeded in a biphasic manner with increasing levels. (Fig. 1C). These results reveal the strict requirement of conditions near neutral pH for the reaction to occur. The possibility of occurrence of some secondary reactions leading to the depletion DZSAM at faster, but varying rates controlled by the nature of the reactant can be speculated. More studies are warranted toward a better understanding of this reaction. Based on reasonable reaction rates with linearity of response in relation to concentration of mimosine, borate buffer at pH 7.6 was chosen for further calibration studies with the time interval for reaction of DZSAM with mimosine fixed at 20 min.

Calibration of the method for the determination of mimosine and DHP

The reaction rate was expressed in terms of ΔA_{Mim} or ΔA_{DHP}, determined by subtracting the value of absor-

bance, measured after reaction with each added level of mimosine or DHP, from that of the control for the total NEDA product formed under standard conditions.

Representative absorption spectra of NEDA product at varying levels of added mimosine are given in Fig 2A. Linear responses in the reactivity for mimosine in concentrations varying from 0.05 to 0.75 μM (Fig. 2B) and for DHP from 0.2 to 1.5 μM (Fig. 3) are seen when ΔA values are plotted against corresponding level of added mimosine or DHP in the medium as indicated below in equations (1) and (2) respectively.

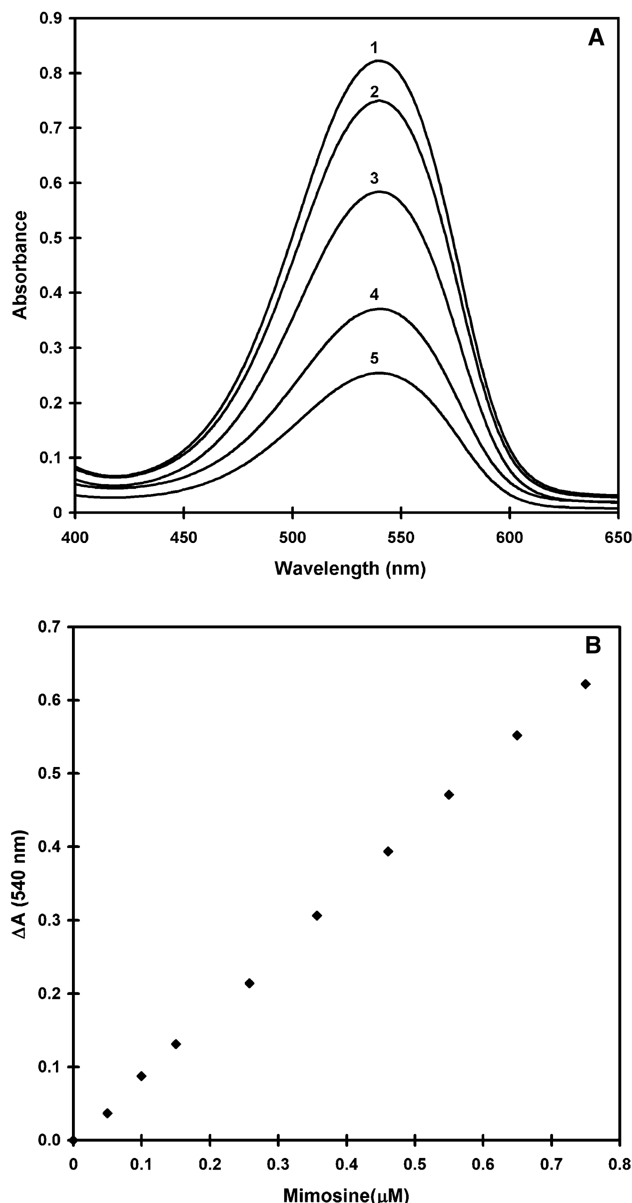

Fig. 2. **A** Absorption spectra of the DZSAM-NEDA product formed at varying concentrations of mimosine. Reaction studied in borate buffer at pH 7.6 with mimosine in concentrations: *1* Control, (no added mimosine); *2* 0.1 μM; *3* 0.25 μM; *4* 0.45 μM; and *5* 0.65 μM. **B** Calibration graph for the estimation of mimosine. Reaction carried out in borate buffer at pH 7.6 represented as plot of ΔA (calculated by subtracting absorbance measured at each added concentration of mimosine from that of the control) as a function of mimosine concentration. Standard deviation in absorbance <0.001 (n = 6). [$\Delta A_{Mim} = 0.8435\ C_{Mim}$ (r = 0.9996)]

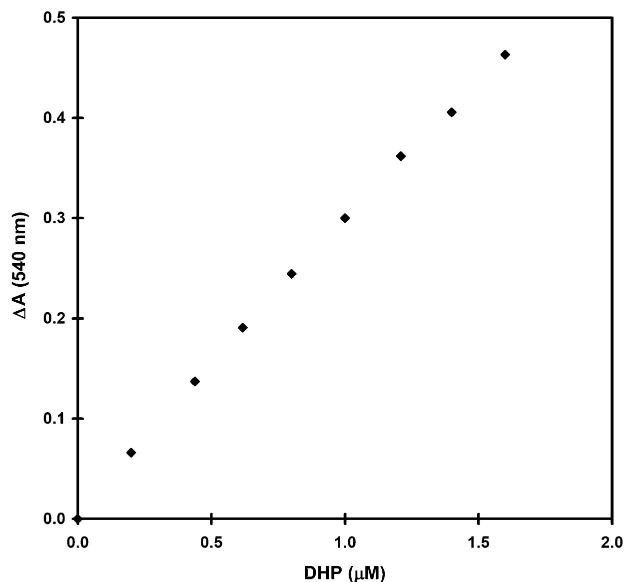

Fig. 3. Calibration graph for the estimation of DHP. Reaction with DHP carried out in borate buffer at pH 7.6, represented as plot of ΔA (calculated by subtracting absorbance measured at each added concentration of DHP from that of the control) as a function of DHP concentration. Standard deviation in absorbance <0.001 (n = 6). [$\Delta A_{DHP} = 0.2954\ C_{DHP}$ (r = 0.9993)]

$$\Delta A_{Mim} = 0.8435\ C_{Mim} (r = 0.9996) \quad (1)$$

$$\Delta A_{DHP} = 0.2954\ C_{DHP} (r = 0.9993) \quad (2)$$

After paper chromatography, the sensitivity of the method of estimation of both mimosine and DHP were comparable to the results of the direct estimation at 95%.

Determination of mimosine and DHP in the extracts of tissues from L. leucocephala *after paper chromatography*

The levels of mimosine and DHP estimated by the present method, in the leaf and seed tissue extracts of the plant *L. leucocephala* are given in Tables 2 and 3. The presence of other interfering compounds in the diluted sample extracts, after paper chromatographic separation was minimal and were not detectable. A comparison of the estimated DHP levels a) in the native extract of leaves as such, and b) the total DHP formed after conversion of mimosine present in the leaves by acid hydrolysis, also gave a measure of mimosine levels and confirmed the

Table 2. Determination of mimosine in the tissue extracts of *L. leucocephala* after paper chromatographic separation

Sample volume* (μl)	Concentration of mimosine			Recovery (%) Mean ± S.D.
	Added[a] (nmol)	Estimated[b] (nmol)	Calculated amount in plant tissue (mmol/g tissue)	
I. Leaf extract				
10	–	0.413	0.165	
	0.5	0.906		99.24 ± 0.27
	1.0	1.420		100.52 ± 0.47
20	–	0.815	0.163	
	0.5	1.317		100.21 ± 0.15
	1.0	1.821		100.32 ± 0.21
40	–	1.650	0.165	
	0.5	1.954		100.21 ± 0.37
	1.0	2.657		100.27 ± 0.69
II. Seed extract				
10	–	0.345	0.138	
	0.5	0.838		99.26 ± 0.39
	1.0	0.335		99.27 ± 0.24
20	–	0.724	0.141	
	0.5	1.228		100.34 ± 0.35
	1.0	1.731		100.41 ± 0.41
40	–	1.547	0.137	
	0.5	2.054		100.34 ± 0.26
	1.0	2.553		100.24 ± 0.55

* Volume of the diluted tissue extract, spotted for paper chromatography
[a] Mimosine added externally to the reaction mixture at indicated levels prior to estimation
[b] Estimated concentration of mimosine in 5 ml of reaction mixture adopting the present procedure
Values given are averages of triplicates in three independent sets of experiments. Standard deviation in absorbance <0.001

validity of the present method. Determined dry weight equivalent of 2 g fresh leaves in four batches averaged at 0.85 ± 0.03 g. Estimated amounts of mimosine was calculated on the average to be 7.8% dry weight for the leaves and 2.7% dry weight for seeds. The level of DHP estimated in the native extract of leaves was only 0.28%. But after hydrolytic treatment, the total measured amount averaged at 4.1% dry weight. Based on these values, the yield of DHP on molar basis, obtained after acid hydrolysis of mimosine amounts to 96%. Recovery of mimosine or DHP, added prior to paper chromatographic separation, was 99.24–100.24% and 99.73–100.57% respectively.

Uptake of mimosine by plant mitochondria

Recent studies in our laboratory (Santosh, 1999, and Lalitha and Rajendra, unpublished observations) with germinating seedlings of *T. foenum-graecum* and *V. radiata* revealed enhanced mitochondrial functional efficiency on exposure to mimosine at 0. 1 mM and the uptake of mimosine by isolated plant mitochondria was therefore explored for the first time. The mitochondrial uptake of mimosine from medium containing 25 and 50 μM level of mimosine was studied for time intervals from 30 sec to 8 min and mimosine remaining in the medium was estimated using the present method. Evidence for effective uptake of mimosine by plant mitochondria is presented in the results obtained (Fig. 4). Uptake of mimosine by active mitochondria from two different plant seedlings viz., *T. foenum-graecum* and *V. radiata* was found to be linear for 1 min, attaining steady state levels thereafter. These results reveal that effective metabolic interactions of mimosine are not exclusive to *L. leucocephala* indicative of possible biochemical significance of this interesting molecule with other plant systems.

Table 3. Determination of DHP in the leaves of *L. leucocephala* after paper chromatographic separation

Sample volume* (μl)	Concentration of DHP			Recovery (%) Mean ± S.D
	Added[a] (nmol)	Estimated[b] (nmol)	Calculated amount in plant tissue (mmol/g tissue)	
I. In the original extract				
10	–	1.093	0.011	
	2	3.075		99.43 ± 0.3
	4	5.093		100.01 ± 0.24
20	–	2.192	0.011	
	2	4.202		100.24 ± 0.24
	4	6.203		100.19 ± 0.31
40	–	4.392	0.011	
	2	6.413		100.34 ± 0.24
	4	8.439		100.56 ± 0.24
II. After acid hydrolysis of the extract				
10	–	1.158	0.157	
	2	3.348		99.73 ± 0.24
	4	5.553		99.93 ± 0.17
20	–	2.326	0.158	
	2	4.537		100.25 ± 0.47
	4	6.740		100.21 ± 0.31
40	–	4.647	0.158	
	2	6.886		100.57 ± 0.16
	4	9.063		100.17 ± 0.27

* Volume spotted for paper chromatography from the diluted tissue extracts of: I, native extract and II, hydrolyzed extract after converting all mimosine to DHP
[a] DHP added externally to the reaction mixture at indicated levels prior to estimation
[b] Estimated concentration of DHP in 5 ml of reaction mixture adopting the present procedure
Values given are averages of triplicates in three independent sets of experiments. Standard deviation in absorbance <0.001

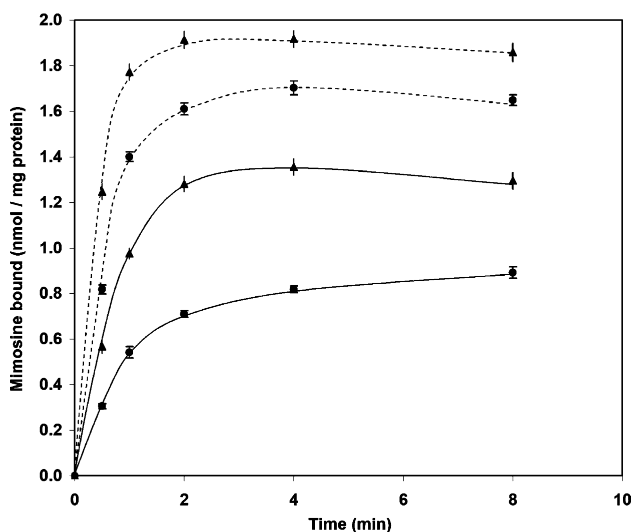

Fig. 4. Time course of uptake of mimosine by plant mitochondria. To uptake medium (1 ml) containing: succinate, 3 mM; ADP, 0.15 mM and added mimosine at (——), 25 μM or at (- - - -), 50 μM in potassium phosphate buffer (0.01 mM, pH 7.5), active mitochondria (2.5 mg protein) from seedlings of (●), *V. radiata* and (▲), *T. foenum-graecum* added at and incubated for specified time intervals. Reaction stopped by chilling the mixture in ice at chosen time intervals. Mitochondria separated by passing the mixture through 0.22 μm membrane filters and mimosine estimated in the filtrate

Discussions

The present indirect method enabled accurate and selective estimation of mimosine and emerged superior to all the other available in terms of its sensitivity (Table 4). The prepared primary derivative of mimosine, (DHP), was also sensitive to the reaction procedure with 40% reactivity as that of mimosine on equi-molar basis, while other physiologically relevant compounds such as phenols and structurally related pyridinium compounds also showed reactivity of 15–35% with DZSAM. With effective separation of the compounds by chromatography, selective and sensitive estimation of both mimosine and DHP was possible in the ranges of 0.05–0.75 μM and 0.2–1.5 μM respectively. The method was successfully applied for selective estimation of both the compounds present in plant tissue extracts with recovery of the metabolites at 99.2–100.5%. The extension of this method for determination of other reactive compounds appears feasible with application potential. Specific uptake of mimosine by mitochondria of *Trigonella foenum graecum* and *Vigna radiata* demonstrated for the first time underscores the importance of investigations on the physiological interactions of mimosine in plant systems and more detailed investigations are in progress.

Table 4. A comparison of the methods for the estimation of mimosine

Method	Range of determination (µM)	Reference
Complexation with Fe^{3+}	20–500	Hegarty et al., 1964; Matsumoto and Sherman, 1951
Reversed phase HPLC	6.25–250	Acamovic et al., 1982
HPLC	1.6–4000	Puchala et al., 1996
Diazotized p-nitroaniline	0.25–10	Lalitha et al., 1993
Present method	0.05–0.75	–

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