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Detection of Norspermidine and Norspermine in *Medicago sativa* L. (Alfalfa)[1]

Benjamin Rodriguez-Garay, Gregory C. Phillips*, and Glenn D. Kuehn

Department of Agronomy and Horticulture (B.R.G., G.C.P.), *and Department of Chemistry* (G.D.K.), *Plant Genetic Engineering Laboratory, New Mexico State University, Las Cruces, New Mexico 88003*

ABSTRACT

Shoot meristem tissues of alfalfa, *Medicago sativa* L., were found by high performance liquid chromatography analyses to contain the uncommon polyamines, norspermidine and norspermine. The chemical structures of norspermidine and norspermine, purified from alfalfa, were confirmed by comparison of mass spectra with those from authentic standards. The discovery of norspermidine and norspermine in alfalfa implicates the presence of at least two biosynthetic enzymes, a polyamine oxidase and a previously uncharacterized aminopropyltransferase.

Much research has implicated the naturally occurring diamine, putrescine,[2] and the polyamines, spermidine and spermine, in processes controlling cellular growth in prokaryotes, higher animals, and plants. They are involved in numerous aspects of plant growth, developmental processes, and in the response of plants to specific abiotic stress stimuli especially at the level of the membrane (1, 20–22). In microbial systems, polyamines have been shown to modulate membrane fusion, exhibit differential preferences for alignment of membrane phospholipids, and mediate preferential attachment or expulsion of membrane surface proteins (15). These and other observations have important implications for protection by polyamines of the structural integrity and function of both prokaryotic and eukaryotic organisms susceptible to osmotic shock (14).

Relatively little is known about the occurrence of other structurally related and uncommon polyamines such as norspermidine, norspermine, or the higher mol wt pentamines and hexamine. These uncommon polyamines were initially discovered in some thermophilic (6, 16) and halophilic (27) bacteria after growth in extreme environments. Subsequently, norspermidine and norspermine were identified in Bryophyta, lichens, certain fungi (10), and eukaryotic algae (9), but not in Pteridophyta or in higher plants (10).

The investigation of the distribution of the uncommon polyamines in biological systems is far from complete. Thus, during a study in our laboratory of the changing patterns of polyamine titers among related populations of drought tolerant and drought susceptible alfalfa in response to imposed stress conditions (17, 18), attention was given to the identification of new polyamines apart from the more common putrescine, spermidine and spermine. In this communication, we report analytical and structural evidence for the occurrence of norspermidine and norspermine in alfalfa.

MATERIALS AND METHODS

Analytical Analyses

Shoot meristem tissues of alfalfa, *Medicago sativa* L., were collected from plants grown in drought boxes (19) inside a shade house. The alfalfa materials included the cv Mesilla and the three cycles of phenotypic recurrent selection populations derived from Mesilla, selected for increasing forage productivity under limited moisture conditions (5), designated Mesilla-

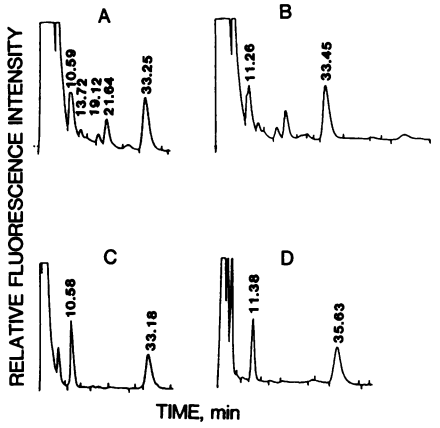

Figure 1. HPLC separations of dansylated-polyamines from shoot meristem tissues of alfalfa (tracings A, B) and comparison of retention times with authentic standards (tracings C, D). The elution peaks and corresponding retention times in min for separated, identified fractions are: (A) norspermidine, 10.59; norspermine, 33.25; (B) spermidine, 11.26; norspermine, 33.45; (C) norspermidine, 10.58; norspermine, 33.18; (D) spermidine, 11.38; spermine, 35.63. Other dansylated, polyamine-like compounds separated from plant tissues shown in tracings A and B (from two different alfalfa samples) have not been identified.

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[2] Abbreviations: putrescine, 1,4-diaminobutane [$H_2N(CH_2)_4NH_2$]; spermidine, 1,8-diamino-4-azaoctane [$H_2N(CH_2)_3NH(CH_2)_4NH_2$]; spermine, 1,12-diamino-4,9-diazadodecane [$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$]; norspermidine (caldine), 1,7-diamino-4-azaheptane [$H_2N(CH_2)_3NH(CH_2)_3NH_2$]; norspermine (thermine), 1,11-diamino-4,8-diazaundecane [$H_2N(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$]; *sym*-homospermidine, 1,9-diamino-5-azanonane [$H_2N(CH_2)_4NH(CH_2)_4NH_2$]; 1,3-diaminopropane [$H_2N(CH_2)_3NH_2$].

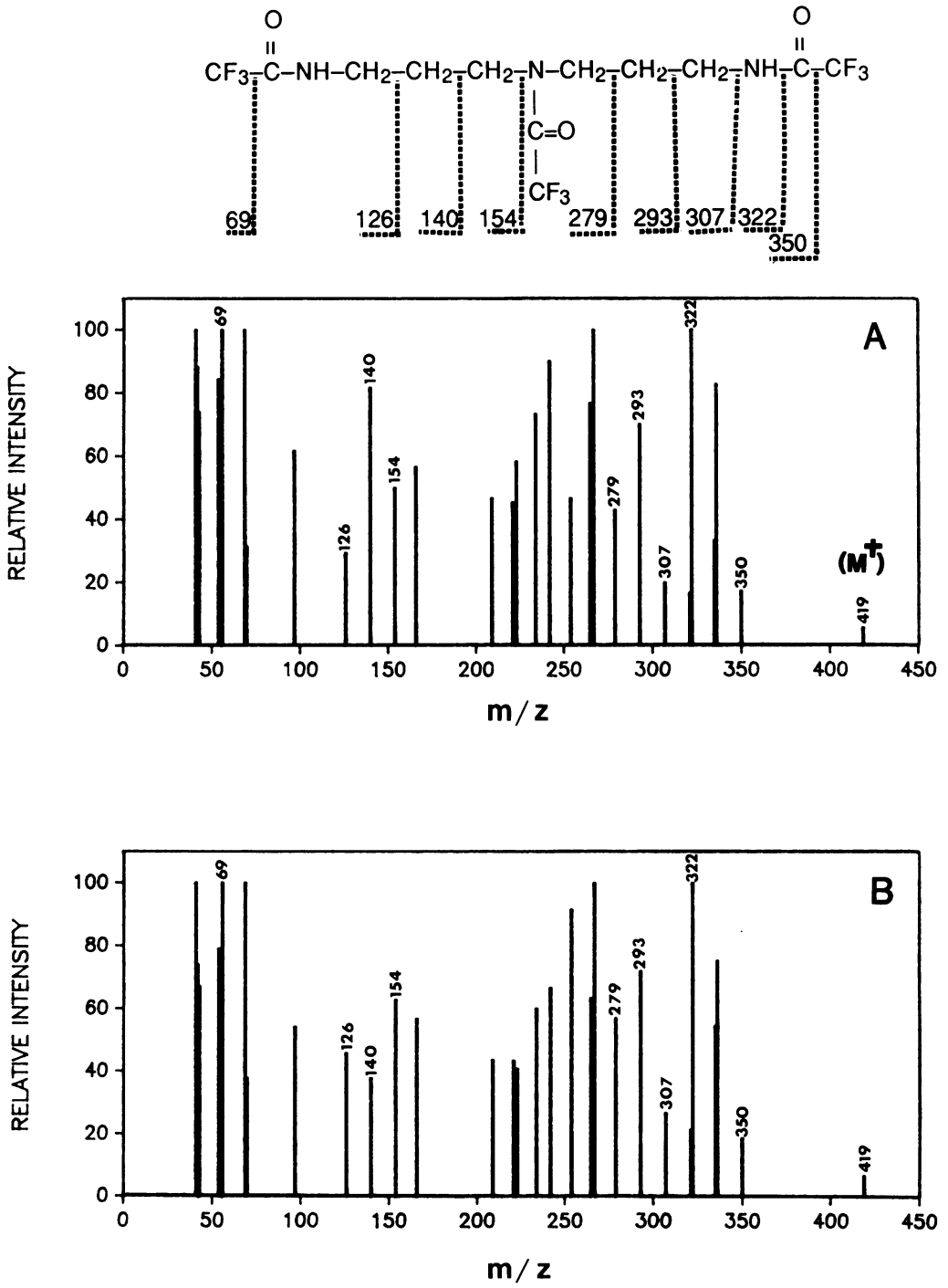
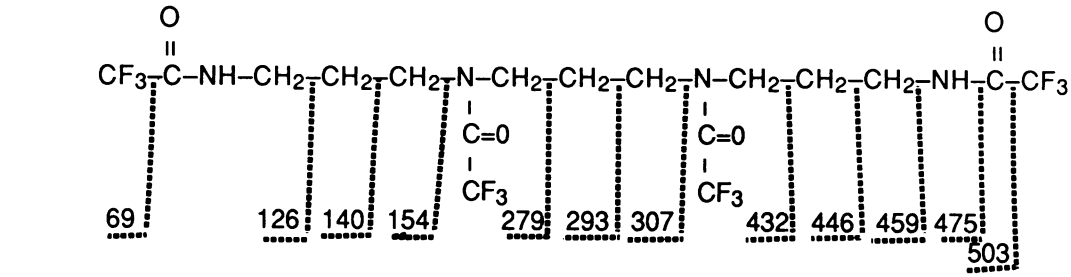
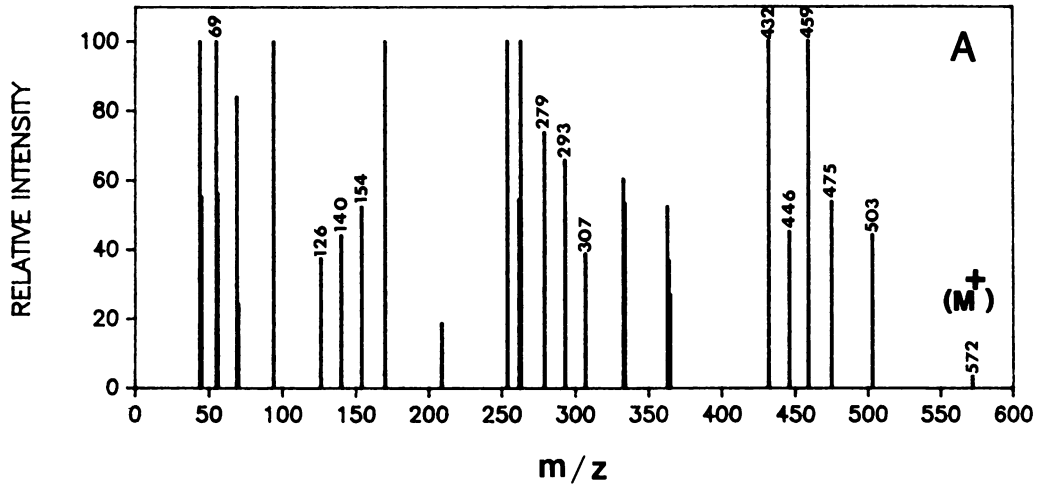


Figure 2. Normalized mass spectra of TFA derivatives of norspermidine isolated from an alfalfa plant (A) and authentic standard norspermidine (B). Analyses were performed on a Hitachi model RMU-6E mass spectrometer using an ionizing voltage of 70 eV. Intense fragment ions obtained at m/z 69, 126, 140, 154, 279, 293, 307, 322, and 350 are particularly indicative of norspermidine. A weak molecular ion (m^+) peak at m/z 419 identifies norspermidine. The identity of norspermidine from alfalfa is established by comparing the mass spectrum of the plant sample (A) with authentic derivatized norspermidine (B). Relative fluorescence intensity values for m/z between 0 and 200 were taken from a relative scale of 1× sensitivity, between 200 and 269 from a scale of 5.3× sensitivity, and between 270 and 419 from a scale of 16.3× sensitivity.

0, Mesilla-1, Mesilla-2, and Mesilla-3, respectively. These four populations appear similar agronomically with the primary difference among them being their response under limited moisture conditions (5). Mesilla-0 is the least productive population and Mesilla-3 is the most productive population under limited moisture conditions. Heretofore, biochemical parameters have not been determined for these populations that might correlate with their respective water use efficiencies. Fresh specimens were excised, immediately frozen with dry ice, and stored at −20°C. The frozen sample was ruptured by sonication-shear with a Polytron homogenizer equipped with a PT-10 probe in 10% (w/v) TCA. After centrifugation, the supernatant solution containing the free cellular polyamines was chromatographed on a 1× 7 cm column of Dowex 50W×8 (400 mesh, H^+ form) cation exchange resin (8). The polyamine fraction was then dansylated (12) and quantitated using a Pharmacia automated fast protein liquid chromatograph system equipped with a PepRPC HR5/5 bonded reverse phase C_2/C_{18} column (11). The elution program was achieved isocratically in methanol:water (4:1, v/v). Eluant peaks with their areas and retention times were recorded by a Hewlett-Packard model 3390A integrator.





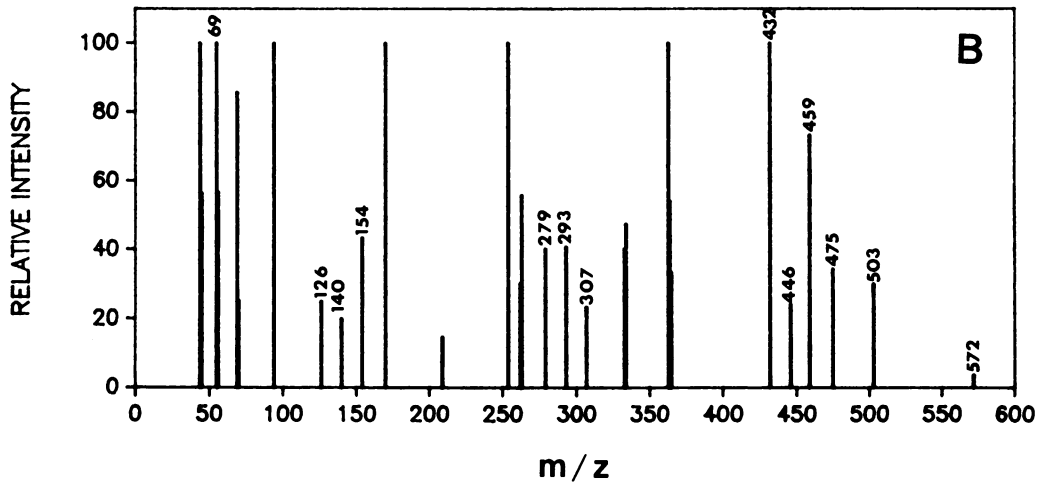


Figure 3. Normalized mass spectra of TFA derivatives of norspermine isolated from an alfalfa plant (A) and authentic standard norspermine (B). Analyses were performed on a Hitachi model RMU-6E mass spectrometer using an ionizing voltage of 70 eV. Intense fragment ions obtained at m/z 69, 126, 140, 154, 279, 293, 307, 432, 446, 459, 475, and 503 are particularly indicative of norspermine. A weak, but visible, molecular ion (M^+) at m/z 572 identifies norspermine. The identity of norspermine from alfalfa is established by comparing the mass spectrum of the plant sample (A) with authentic derivatized norspermine (B). Relative fluorescence values for m/z between 0 and 210 were taken from a relative scale of 1× sensitivity, between 211 and 335 from a scale of 2.6× sensitivity, and between 336 and 572 from a scale of 8.5× sensitivity.

MS Analyses

Approximately 2 mL of the polyamine fraction obtained from the Dowex 50W×8 column was used for MS analyses. The fractions were dried on a warm plate with flowing clean air. The dried sample was resuspended with 0.4 mL of deionized, distilled water and centrifuged for 5 min at *ca.* 11,000*g*. Plant samples and standards of spermidine, spermine (Sigma Chemical Co., St. Louis, MO), norspermidine and norspermine (Eastman Kodak Co., Rochester, NY) were further purified by electrophoresis on Whatman 3 MM paper at 250 V for 1 h, in 44 mM sodium citrate buffer, pH 4.3. The polyamines were localized with standard samples on the paper by staining with 2% (w/v) ninhydrin in butanol-saturated water. Polyamines from plant samples and standards were eluted from the paper with 1.5 mL of 0.1 N HCl, collected in plastic tubes, and evaporated to dryness with a stream of clean air. The polyamines were converted to their TFA derivatives (25). The mass spectra were obtained from a Hitachi model RMU-6E mass spectrometer.

Table I. *Representative Amounts of Uncommon Polyamines Quantitated in Shoot Meristem Tissues of Different Alfalfa Populations*

Quantities of purified, dansylated polyamines from plant samples were determined from areas of peaks derived from HPLC tracings, which were normalized by peak areas generated from authentic standards of dansylated norspermidine or norspermine, respectively. The alfalfa populations were previously selected by phenotypic recurrent selection for increasing productivity under conditions of limiting moisture (5), and represent the original, unselected cv Mesilla (Mesilla-0) and the three subsequent cycles of selection (Mesilla-1, -2, and -3, respectively). The plants were grown in drought boxes with limited irrigation water as described by others (19). Each data point represents at least 15 plants.

Population	Norspermidine	Norspermine
	nmol·g⁻¹ fresh wt of tissue	
Mesilla-0	Not detected	50.2
Mesilla-1	12.5	98.7
Mesilla-2	12.3	47.3
Mesilla-3	16.5	65.1

RESULTS

Analyses were performed by HPLC of dansylated derivatives of the polyamine fraction obtained after cation exchange chromatography of TCA extracts from alfalfa shoot meristem tissues. These fractions revealed unidentified polyamine-like components in addition to the common polyamines, spermidine and spermine (Fig. 1). Dansylated, authentic spermidine and spermine exhibited retention times of 11.38 and 35.63 min, respectively (Fig. 1D), at 20°C. Unidentified dansylated components were detected in alfalfa meristem tissues with retention times of 10.59, 13.72, 19.12, 21.64 and 33.25 min (Fig. 1A). Retention times for dansylated standards of monoacetylated putrescine, monoacetylated spermidine, monoacetylated spermine, tyramine and tryptamine did not coincide with the retention times of the unidentified components (profiles not shown). However, dansylated derivatives of authentic norspermidine and norspermine exhibited retention times of 10.58 and 33.18 min, respectively (Fig. 1C), which were nearly identical to retention times for two unidentified components observed from different alfalfa plant samples (Fig. 1, A and B).

Structural evidence for the occurrence of norspermidine and norspermine in alfalfa was obtained by MS. Quantities of each respective polyamine were purified by paper electrophoresis from eluants collected after cation exchange chromatography. TFA derivatives were prepared for the purified alfalfa polyamines and authentic standards of norspermidine and norspermine. The compound which demonstrated a retention time of 10.59 min by HPLC as a dansylated derivative (Fig. 1A), gave a mass spectrum (Fig. 2A) which was identical to standard norspermidine (Fig. 2B). Similarly, the compound whose dansylated derivative showed a retention time of 33.25 min by HPLC (Fig. 1A), yielded a mass spectrum (Fig. 3A) identical to standard norspermine (Fig. 3B). The mass spectra of TFA-derivatized spermine and spermidine standards were also compared with the alfalfa components of interest, and they were clearly different (spectra not shown).

Table I presents data representative of the amounts of the uncommon polyamines, norspermidine and norspermine, that can be quantitated in shoot meristem tissues from different alfalfa populations. Amounts of putrescine, spermidine and spermine present in the same samples were comparable to the amount of norspermine detected (data not shown). The simultaneous occurrence of the uncommon polyamines, norspermidine and norspermine, is variable. The sample profile shown in Figure 1B, for example, contained spermidine and norspermine, but not putrescine or spermine. In contrast, the sample profile shown in Figure 1A contained norspermidine and norspermine, but not the other common polyamines. Possible reasons for this variability are being investigated (17, 18).

DISCUSSION

The detection of the uncommon polyamines norspermidine and norspermine in a higher plant such as alfalfa, as presented in this paper, is unprecedented (10). Earlier identification of these polyamines has been restricted to bacteria that thrive in extreme environments, certain fungi, eukaryotic algae, lichens and mosses. Numerous studies have reported the wide distribution of the common polyamines, putrescine, spermidine and spermine, among higher plants, Also, the uncommon triamine, *sym*-homospermidine, has been found in higher plants including sandalwood (13), water hyacinth (26), *Heliotropium* (3) and grass pea (24). Thus, the current finding of norspermidine and norspermine in alfalfa extends knowledge concerning the variety of triamines and tetramines that can be synthesized by higher plants.

This finding has important implications for future investigation of polyamines and their biosynthetic enzymes in higher plants. First, the occurrence of norspermidine and norspermine in alfalfa implicates a previously unrecognized biosynthetic pathway in higher plants. These reactions could proceed through successive additions of an aminopropyl moiety from decarboxylated *S*-adenosylmethionine by the action of an aminopropyltransferase enzyme, beginning with the precursor 1,3-diaminopropane, as proposed for thermophilic bacteria (7). We speculate that a nonspecific aminopropyltransferase may exist in alfalfa that catalyzes elongation of 1,3-diaminopropane to norspermidine, and norspermidine to norspermine. Such an aminopropyltransferase with broad specificity has recently been isolated from an extreme acidothermophilic archaebacterium (4).

Second, the occurrence of norspermidine and norspermine in alfalfa implicates the existence of a polyamine oxidase in this dicotyledonous plant. The sole known origin of 1,3-diaminopropane in plant metabolism is from oxidative cleavage of spermidine or spermine through the action of polyamine oxidase (20, 23). 1,3-Diaminopropane is the essential precursor for biosynthesis of norspermidine and norspermine (10). Polyamine oxidases demonstrate specificity for spermidine and spermine, and they occur throughout the Gramineae in cereals such as barley, oats and maize (20, 23), and in other monocots such as water hyacinth (28). Its existence has also been implicated in *Helianthus tuberosus* (2) through the detection of 1,3-diaminopropane. A possible role for this enzyme in higher plants may be to generate the precursor, 1,3-

diaminopropane, for the biosynthesis of the uncommon polyamines, norspermidine and norspermine.

The uncommon polyamines have been implicated in the adaptation of thermophilic and halophilic bacteria to extreme environments (6, 16, 27). In the present study, norspermidine and norspermine were identified in alfalfa populations after growth in drought boxes under limited moisture conditions. Additional study is needed to ascertain the functional significance of their occurrence.

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