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Betaines of Alfalfa[1]

Characterization by Fast Atom Bombardment and Desorption Chemical Ionization Mass Spectrometry

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ABSTRACT

Leaf tissue of alfalfa (*Medicago sativa* L.) was found to contain prolinebetaine, pipecolatebetaine, hydroxyprolinebetaine, and glycinebetaine. As *n*-butyl esters, these chemical species exhibit molecular cations at mass/charge ratio (m/z) 200, 214, 216, and 174, respectively, when analyzed by fast atom bombardment mass spectrometry. The underivatized betaines exhibit protonated molecular ions at m/z 144, 158, 160, and 118, respectively, when analyzed by desorption chemical ionization mass spectrometry. Extensive (>45-fold) genotypic variation for hydroxyprolinebetaine level was identified in alfalfa. Because a significant inverse correlation between prolinebetaine and hydroxyprolinebetaine levels was observed among 15 alfalfa genotypes evaluated, it is possible that these compounds may be derived from a common intermediate. Birdsfoot trefoil (*Lotus corniculatus* L.) contained prolinebetaine, but only traces of glycinebetaine, pipecolatebetaine, and hydroxyprolinebetaine. Red clover (*Trifolium pratense* L.) lacked prolinebetaine, pipecolatebetaine, and hydroxyprolinebetaine, but contained appreciable levels of both glycinebetaine and trigonelline. Trigonelline was not detectable in the leaf tissue of any alfalfa genotype or cultivar evaluated.

Positive ion FAB-MS[2] has been employed to simultaneously quantify the levels and determine the stable isotope abundance of quaternary ammonium compounds such as glycinebetaine (3, 4, 9, 10, 12, 13, 16) and its precursors (*e.g.* betaine aldehyde) (3, 10, 13) in plants. Desorption ionization mass spectrometry methods, including FAB-MS, are particularly suited to analysis of complex mixtures of preformed ionic species which exhibit surface activity in liquid sample matrices (13). The FAB-MS method has proven particularly useful in screening *Zea mays* germplasm for genotypic variation in glycinebetaine levels, and has been instrumental in identifying several inbreds of maize which lack this quaternary ammonium compound (4, 12). Such betaine-deficient genotypes provide opportunities to investigate the biochemical genetics of quaternary ammonium compound synthesis (10). Glycinebetaine deficiency in maize is caused by a single nuclear recessive gene (10, 12). Glycinebetaine-deficient genotypes of maize appear to lack the capacity to oxidize choline to betaine aldehyde, but not the capacity to oxidize betaine aldehyde to glycinebetaine (10).

Prolinebetaine (stachydrine) is the major betaine accumulated by alfalfa (*Medicago sativa* L.) (1, 5, 14, 17), and is thought to be synthesized by *N*-methylation of proline via the intermediate *N*-methylproline (5, 14). Other betaines that have been reported in alfalfa include pipecolatebetaine (homostachydrine) (5, 17), trigonelline (17), and glycinebetaine (1). Because the betaine composition of alfalfa is complex, and significant genotypic variation for prolinebetaine accumulation has been reported for alfalfa (17), we were prompted to examine further the range and extent of genotypic variation for betaine composition in this species by mass spectrometry, with the view to identifying alfalfa genotypes that are deficient in one or more of these quaternary ammonium compounds. The occurrence of prolinebetaine, pipecolatebetaine, and glycinebetaine in alfalfa leaf tissues was confirmed. However, trigonelline was not found in the leaf tissue of any alfalfa genotype or cultivar evaluated. Rather, hydroxyprolinebetaine (a quaternary ammonium compound which has not previously been reported in alfalfa [17]) was found to be accumulated to high levels in leaf tissue of certain alfalfa genotypes. Genotypes of alfalfa are shown to differ substantially (>45-fold) for leaf hydroxyprolinebetaine levels.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Sampling Procedures

Alfalfa (*Medicago sativa* L.) tissues were obtained from cultivars and genotypes exhibiting extensive phenotypic variation including plants differing in rate of regrowth after defoliation ("SSER," "RSER"), plants having multifoliate leaves

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[2] Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; amu, atomic mass unit; DCI-MS, desorption chemical ionization mass spectrometry; m/z, mass/charge ratio.

("5–1" through "9–2"), and plants selected for contrasting concentrations of root starch ("LSt," "HSt") (6, 7). Other alfalfa tissues described were obtained from randomly selecting individuals from the tetraploid cultivar ("Hi-Phy") and a diploid (2×) population ("W2×iso-1") and its derived, near-isogenic tetraploid (4×) counterpart ("W4×iso-1"). Red clover (*Trifolium pratense* L.) and birdsfoot trefoil (*Lotus corniculatus* L.) tissue was obtained from randomly selected plants from the cultivars "Redland II" and "Fergus," respectively. Origin of plants and details of growth conditions in both the greenhouse and field environments have been described elsewhere (2, 6, 7). Greenhouse samples of immature, partially expanded leaves were obtained from plants at midregrowth (14–18 d after defoliation). Sample weights ranged from 0.16 to 1.3 g fresh weight and all were extracted in 15 mL methanol. Field samples were obtained from either immature, partially expanded leaves or mature, fully expanded leaves at two harvest times; early regrowth (10 d after cutting) or midregrowth (17 d after cutting). Field samples ranged from 0.81 to 5.04 g fresh weight and were extracted in 40 mL methanol.

Purification and Derivatization of Betaines

Aliquots of 10 or 15 mL of methanol extract were phase-separated with chloroform and H_2O (methanol:chloroform:H_2O ratio = 10:5:6 v/v), and the upper aqueous phase was then evaporated to dryness. Deuterium labeled ($^2H_9$) glycinebetaine (442 nmol) was added to each sample as internal standard, and the samples were then redissolved in 2 mL H_2O, and processed via two ion exchange resins (Dowex-1-OH^- and Dowex-50-H^+) as described previously (4, 12, 13). The betaine fractions (6 mL of 6 M NH_4OH eluate from the Dowex-50-H^+ column) were then concentrated to dryness under a stream of dry air. Half of the betaine fraction was derivatized with *n*-butanol:acetyl chloride as described previously (4, 12, 13) for FAB-MS analysis, and half was analyzed by DCI-MS in the underivatized form.

Chemicals

Synthesis procedures for $^2H_9$-glycinebetaine, prolinebetaine, and pipecolatebetaine have been given in a previous paper (13). (−)4-Hydroxyprolinebetaine (betonicine) was obtained from Atomergic Chemetals Corp. (Plainview, NY), and trigonelline from Sigma Chemical Co. Authentic standards were derivatized as described for the purified betaine fractions from plant tissues, either alone or in combination with $^2H_9$-glycinebetaine.

FAB-MS

The FAB-MS studies were carried out essentially as described previously (4, 12, 13), with a Kratos MS-50 mass spectrometer (Manchester, UK) interfaced to a Finnigan INCOS data system. Sample (as *n*-butyl betaine esters redissolved in methanol) was introduced into the mass spectrometer by mixing a 1 to 2 μL aliquot with a small volume (1–2 μL) of glycerol applied to the FAB probe (a solid probe with a stainless steel tip manufactured by Ion Tech). Samples were irradiated at 50°C with Xenon atoms (8 keV; 50 μA). The mass range scanned was typically 130 to 500 amu. Relative response factors with respect to $^2H_9$-glycinebetaine (*i.e.* relative abundance of $^2H_9$-glycinebetaine molecular cation/relative abundance of the molecular cation of an equimolar amount of standard) were: 1.0 for glycinebetaine, 0.363 for prolinebetaine, 2.642 for pipecolatebetaine, 1.724 for betonicine, and 18.43 for trigonelline.

DCI-MS

The DCI-MS studies were carried out using a Finnigan 4000 GC/MS with an Incos Data System (San Jose, CA) and a Vacumetrics (Ventura, CA) DCI probe. The samples (as the underivatized betaines) were placed on the DCI Rhenium filament wire from solution (methanol solvent) and evaporated prior to desorption and mass analysis. The sample was desorbed by passing 3 A of current (rise time 7.5 s) through the Rhenium filament wire. Isobutane was used as the reagent gas at an ion source gauge pressure of 0.4 torr. The ion source temperature was maintained at 250°C. The mass range scanned was usually 60 to 250 amu. Relative response factors with respect to $^2H_9$-glycinebetaine (*i.e.* relative abundance of the $^2H_9$-glycinebetaine protonated molecular ion/relative abundance of the protonated molecular ion of an equimolar amount of standard) were: 1.0 for glycinebetaine, 0.535 for prolinebetaine, 1.481 for pipecolatebetaine, 4.909 for betonicine, and 6.097 for trigonelline.

RESULTS AND DISCUSSION

The major ions observed in the FAB spectra of *n*-butyl derivatized betaine fractions of leaf tissues of three contrasting greenhouse-grown alfalfa genotypes are summarized in Table I; all include $^2H_9$-glycinebetaine-*n*-butyl ester ($[M^+]$ = m/z 183) as internal standard. All three samples contain abundant quantities of prolinebetaine -*n*-butyl ester ($[M^+]$ = m/z 200; $[M^+ - C_4H_8]$ = m/z 144). Pipecolatebetaine-*n*-butyl ester ($[M^+]$ = m/z 214; $[M^+ - C_4H_8]$ = m/z 158) and relatively low levels of glycinebetaine-*n*-butyl ester ($[M^+]$ = m/z 174) are also present in all three alfalfa samples. The identities of prolinebetaine, pipecolatebetaine and glycinebetaine were confirmed by derivatizing authentic standards, which gave strong signals from the molecular cations at m/z 200, 214, and 174, respectively, together with the corresponding fragment ions (see ref. 13 for FAB spectra of *n*-propyl esters of these compounds). Note that genotype "9–2" appears to contain a high level of a hydroxyprolinebetaine, as evidenced by the strong signals at m/z 216 and 160 ($[M^+]$ = m/z 216; $[M^+ - C_4H_8]$ = m/z 160) (Table I). Authentic (−)4-hydroxyprolinebetaine (betonicine) *n*-butyl ester gave identical signals at m/z 216 and 160 as those observed in genotype "9–2" (not shown). These results establish the presence of a hydroxyprolinebetaine in alfalfa and extensive genotypic variation for this quaternary ammonium compound within this plant species. The cultivar "Hi-Phy" and genotype "SSER" appear to contain only trace levels of this compound (Table I), as evidenced by the low signals at m/z 216 and 160 in the FAB spectra. Various isomers of hydroxyprolinebetaine [(−)4-, (+)4-, and/or 3-hydroxyprolinebetaine isomers (17)] have been reported in a number of plant species but apparently

Table I. *Summary of the Major Ions Observed in the FAB Mass Spectra of n-Butyl Esterified Betaine Fractions of Leaf Tissues of Greenhouse-Grown Alfalfa Genotypes '9-2' and 'SSER,' and Alfalfa Cultivar 'Hi-Phy'*

m/z	Chemical Species	Alfalfa Genotype or Cultivar		
		'9-2'	'SSER'	'Hi-Phy'
		relative ion intensity (%)		
144	Prolinebetaine-*n*-butyl ester [M^+-C_4H_8]	9.23	8.97	11.48
158	Pipecolatebetaine-*n*-butyl ester [M^+-C_4H_8]	2.92	1.21	2.03
160	Hydroxyprolinebetaine-*n*-butyl ester [M^+-C_4H_8]	6.96	0.09	0.32
174	Glycinebetaine-*n*-butyl ester [M^+]	3.31	0.83	1.78
183	$^2H_9$-Glycinebetaine-*n*-butyl ester [M^+]	10.84	3.28	3.21
200	Prolinebetaine-*n*-butyl ester [M^+]	100.00	100.00	100.00
214	Pipecolatebetaine-*n*-butyl ester [M^+]	11.04	4.42	4.67
216	Hydroxyprolinebetaine-*n*-butyl ester [M^+]	77.68	0.55	1.64

not previously in alfalfa (17). The precise isomer of hydroxyprolinebetaine accumulated by alfalfa genotype "9–2" has not yet been determined.

To verify further the identities of the betaines in alfalfa leaf tissues, the underivatized betaine fractions were analyzed by DCI-MS together with authentic standards. For each authentic quaternary ammonium compound (a zwitterionic species of mass M) the expected protonated molecular ion ([$M + H^+$]) and a major fragment ion corresponding to thermal loss of one of the quaternary methyl groups (*i.e.* [$M - CH_3 + H + H^+$] for unlabeled samples, and [$M - C^2H_3 + H + H^+$] for methyl-deuterium labeled samples) were observed (not shown). Authentic (−)4-hydroxyprolinebetaine (betonicine) gave the least abundant protonated molecular ion (m/z 160) relative to the internal standard ($^2H_9$-glycinebetaine; m/z 127), and a unique fragment product at m/z 142 corresponding to loss of H_2O ([$M - H_2O + H^+$]), presumably from the hydroxyl moiety, in addition to the expected fragment ion at m/z 146 corresponding to thermal loss of one of the quaternary methyl groups (not shown).

The major ions observed in DCI-MS analyses of the underivatized betaine fractions from the same three samples whose FAB-MS spectra are illustrated in Table I are summarized in Table II. The occurrence of hydroxyprolinebetaine in geno-

Table II. *Summary of the Major Ions Observed in the DCI Mass Spectra of Underivatized Betaine Fractions of Leaf Tissues of Greenhouse-Grown Alfalfa Genotypes '9-2' and 'SSER,' and Alfalfa Cultivar 'Hi-Phy'*

m/z	Chemical Species	Alfalfa Genotype or Cultivar		
		'9-2'	'SSER'	'Hi-Phy'
		relative ion intensity (%)		
118	Glycinebetaine [$M + H^+$]	1.75	0.40	0.72
127	$^3H_9$-Glycinebetaine [$M + H^+$]	5.57	1.28	1.18
130	Prolinebetaine [$M - CH_3 + H + H^+$]	11.76	10.01	7.88
142	Hydroxyprolinebetaine [$M - H_2O + H^+$]	13.06	2.69	2.09
144	Prolinebetaine [$M + H^+$][a]	100.00	100.00	100.00
146	Hydroxyprolinebetaine [$M - CH_3 + H + H^+$]	9.48	1.15	1.46
158	Pipecolatebetaine [$M + H^+$]	20.60	17.88	15.41
160	Hydroxyprolinebetaine [$M + H^+$]	25.34	0.36	0.53

[a] The ion at m/z 144 corresponds to the sum of the protonated molecular cation of prolinebetaine ([$M + H^+$]) and the [$M - CH_3 + H + H^+$] fragment ion of pipecolatebetaine.

type "9–2" is confirmed by the presence of strong signals at m/z 160, 142, and 146 in the mass spectrum (Table II). The signal at m/z 160 is relatively low but not completely absent from the genotypes identified to contain low hydroxyprolinebetaine levels by FAB-MS (Table II; *cf.* Table I). All genotypes give the expected signals at m/z 144 ($[M + H^+]$) and 130 ($[M - CH_3 + H + H^+]$) for prolinebetaine. The presence of pipecolatebetaine in all three genotypes is revealed by the strong signals at m/z 158 ($[M + H^+]$) (Table II). Because pipecolatebetaine produces a fragment ion at m/z 144, which is of the same mass:charge ratio as the protonated molecular ion of prolinebetaine, the signal at m/z 144 in the DCI mass spectra represents the sum of the protonated molecular ion of prolinebetaine ($[M + H^+]$) and the $[M - CH_3 + H + H^+]$ fragment ion of pipecolatebetaine (Table II). The presence of glycinebetaine is revealed by the signals at m/z 118 (Table II).

Results of FAB-MS analyses of the betaine fractions of leaf tissues of different maturities of five alfalfa genotypes grown under different environmental conditions (field and greenhouse) confirm that the variation for hydroxyprolinebetaine content of alfalfa illustrated in Tables I and II is likely of genotypic origin rather than of developmental or environmental origin. Thus, "SSER" always exhibited a signal at m/z 216 from the hydroxyprolinebetaine-*n*-butyl ester molecular cation which was less than 2% (and frequently less than 1%) of the prolinebetaine-*n*-butyl ester molecular cation (m/z 200), regardless of leaf maturity and environment (Table III). In contrast genotype "9–1" always exhibited a signal at m/z 216 from the hydroxyprolinebetaine-*n*-butyl ester molecular cation which was greater than 14% (and frequently greater than 20%) of the prolinebetaine-*n*-butyl ester molecular cation (m/z 200), regardless of leaf maturity and environment (Table III). The ratios of the imino acid betaines are clearly highly dependent on genotype (Table III).

Summaries of the betaine levels derived from FAB-MS analyses of *n*-butyl derivatized betaine fractions of several greenhouse-grown populations and genotypes of alfalfa and two other leguminous species (birdsfoot trefoil and red clover) are shown in Table IV, correcting for the response factors for each betaine relative to the internal standard. These results reveal a 5-fold range of prolinebetaine levels, a 4-fold range of pipecolatebetaine levels, over a 10-fold range of glycinebetaine levels, and over a 45-fold range of hydroxyprolinebetaine levels in alfalfa. It is notable that alfalfa population "W2Xiso-1" exhibited mean betaine levels that were similar to those from population "W4Xiso-1." Since the latter population is a near isogenic tetraploid population derived from the former diploid population (2), there appears to be little effect of gene dosage on accumulation of betaines in these alfalfa populations.

Birdsfoot trefoil (*Lotus corniculatus*) contained only trace levels of glycinebetaine, pipecolatebetaine, and hydroxyprolinebetaine. Only prolinebetaine was detectable at appreciable levels (>500 nmol/g fresh wt) in this species (Table IV). In red clover (*Trifolium pratense*) glycinebetaine was a major betaine (Table IV). As noted previously (16), glycinebetaine may be more widely distributed in dicotyledons than has hitherto been recognized. In the red clover samples, two additional ions at m/z 138 and 194 were abundant in the FAB mass spectra (not shown). It is likely that these ions correspond to *n*-butyl trigonelline ($[M^+]$ = m/z 194; $[M^+ - C_4H_8]$ = m/z 138) (*cf. Lycopersicon esculentum* [16]). The DCI mass spectra of the underivatized betaine fractions of red clover revealed a strong signal at m/z 140, as did authentic underivatized trigonelline (not shown). We estimate the trigonelline levels of red clover leaf tissue to be approximately 5000 nmol/g fresh wt based on FAB-MS and DCI-MS analyses of the two replicate red clover samples and calibrations of authentic trigonelline *versus* $^2H_9$-glycinebetaine by both methods. The related species *Trifolium repens* has been reported to contain trace levels of glycinebetaine (15) and trigonelline (17). Although alfalfa has been reported to contain trigonelline (17), this may be largely restricted to seeds (17).

Table III. *Relative Ion Intensities of the Molecular Cations of n-Butyl Esters of Prolinebetaine (m/z 200), Pipecolatebetaine (m/z 214), and Hydroxyprolinebetaine (m/z 216) Observed in FAB-MS Analyses of Leaf Tissues of Different Maturities of Five Diverse Alfalfa Genotypes in Different Environments*

Environment	Genotype and Leaf Maturity[a]		m/z		
			200	214	216
			relative ion intensity (%)		
Field	'SSER'	IE	100.0	6.0	0.9
Field		ME	100.0	10.4	1.9
Field		IL	100.0	7.0	1.0
Greenhouse		IL	100.0	4.9	0.5
Field		ML	100.0	6.6	1.6
Mean			100.0	7.0	1.2
Field	'5-2'	IE	100.0	17.1	12.5
Field		ME	100.0	14.6	15.7
Field		IL	100.0	11.7	9.8
Greenhouse		IL	100.0	8.5	10.3
Field		ML	100.0	15.5	14.7
Mean			100.0	13.3	12.6
Field	'7-1'	IE	100.0	4.4	10.4
Field		ME	100.0	4.7	13.2
Field		IL	100.0	9.4	12.9
Greenhouse		IL	100.0	4.4	11.7
Field		ML	100.0	10.9	26.9
Mean			100.0	6.8	15.0
Field	'7-2'	IE	100.0	8.3	19.7
Field		ME	100.0	17.1	17.4
Field		IL	100.0	12.3	13.5
Greenhouse		IL	100.0	6.5	13.0
Field		ML	100.0	12.9	20.3
Mean			100.0	11.4	16.8
Field	'9-1'	IE	100.0	12.6	24.2
Field		ME	100.0	8.3	19.7
Field		IL	100.0	7.6	14.9
Greenhouse		IL	100.0	8.1	20.1
Field		ML	100.0	4.2	22.2
Mean			100.0	8.2	20.2

[a] IE, immature leaves, early regrowth (10 d of regrowth after defoliation); ME, mature leaves, early regrowth (10 d of regrowth after defoliation); IL, immature leaves, late regrowth (17 d of regrowth after defoliation); ML, mature leaves, late regrowth (17 d of regrowth after defoliation).

Table IV. *Summaries of Betaine Levels Estimated Using FAB-MS in an Alfalfa Cultivar ('Hi-Phy'), Two Populations Differing in Nuclear Ploidy (diploid = 'W2Xiso-1', tetraploid = 'W4Xiso-1'), Several Diverse Alfalfa Genotypes, and in Two Other Leguminous Species (Red Clover and Birdsfoot Trefoil)*

All samples were from immature leaves (late regrowth, 14–18 d after defoliation) of greenhouse-grown plants. Unless otherwise indicated, analyses are based on single samples.

Plant Source	Glycine Betaine	Proline Betaine	Pipecolate Betaine	Hydroxyproline Betaine
		nmol/g fresh wt		
Alfalfa 'Hi-Phy'[a]	576	6,759	2,156	804
'W2Xiso-1'[b]	699	5,352	1,187	444
'W4Xiso-1'[c]	845	6,343	1,422	650
'SSER'[d]	275	11,304	3,915	263
'RSER'[d]	992	10,278	3,008	224
'HSt'[d]	1,459	5,380	1,813	327
'LSt'[d]	1,854	8,467	2,299	701
'5-1'	839	2,436	3,471	5,489
'5-2'	2,201	9,055	5,321	4,204
'5-3'	326	3,188	2,984	4,856
'7-1'	676	6,266	1950	3,402
'7-2'	804	5,033	2,313	3,025
'7-3'	661	3,780	2,874	5,619
'9-1'	1,262	5,902	3,429	5,536
'9-2'	249	3,770	2,199	10,099
Red Clover[d]	593	10	30	19
Birdsfoot Trefoil[d]	62	871	83	123

[a] Means of four independent samples. [b] Means of five independent samples. [c] Means of six independent samples. [d] Means of two independent samples.

Trigonelline was not detectable in leaf tissues of any alfalfa genotype or cultivar examined in these studies.

CONCLUDING DISCUSSION

The striking variation for the levels of quaternary ammonium compounds among alfalfa cultivars and genotypes and related leguminous plants illustrated here conforms to a general pattern of variability for *N*-methylated compounds among plant species (11, 15–17) and cultivars of domesticated crops (4, 8, 10, 12, 17). The present study confirms that routine screening of cultivars of domesticated crops by mass spectrometry holds significant promise for identifying genotypes that lack certain quaternary ammonium compounds. Such genotypes provide unique opportunities to begin to dissect genetically the pathways of quaternary ammonium compound synthesis in higher plants (10) and to begin to elucidate the physiological functions of these metabolites (17).

In alfalfa the pathway of prolinebetaine biosynthesis may involve sequential *N*-methylation of proline via the intermediate *N*-methylproline, hygric acid (5, 14). The precise pathways of synthesis of pipecolatebetaine and hydroxyprolinebetaine in alfalfa are presently unknown. It may be significant that alfalfa contains high levels of free pipecolic acid, as evidenced by GC-MS analysis of *N*-heptafluorobutyryl isobutyl derivatized amino acid fractions by electron ionization and chemical ionization GC-MS. For example, pipecolic acid levels ranged from 4000 to 5000 nmol/g fresh weight in leaf tissue of alfalfa genotype "SSER," from 350 to 750 nmol/g fresh weight in alfalfa genotype "RSER", and from 200 to 1400 nmol/g fresh weight in alfalfa cultivar "Hi-Phy" (D Rhodes, JJ Volenec, unpublished results). It is possible that pipecolatebetaine could be synthesized via an analogous pathway to that described for prolinebetaine (5, 14), involving sequential *N*-methylation of free pipecolic acid via the intermediate *N*-methylpipecolic acid. However, hydroxyproline was not detectable as a constituent of the free amino acid pool of alfalfa (including alfalfa genotypes with high hydroxyprolinebetaine pools), and it would therefore seem unlikely that hydroxyprolinebetaine could be synthesized by *N*-methylation of a free hydroxyproline pool. Hydroxylation of *N*-methylproline, followed by methylation of *N*-methylhydroxyproline to hydroxyprolinebetaine remains a possible alternative pathway of hydroxyprolinebetaine synthesis. In this context it is notable that there is a significant inverse correlation ($r = -0.5997$) between the levels of prolinebetaine and hydroxyprolinebetaine among the 15 alfalfa genotypes and cultivars listed in Table IV. This indicates the possibility that hydroxyprolinebetaine may accumulate at the expense of prolinebetaine, suggesting that these compounds may be derived from a common precursor. Determination of the levels of the putative intermediates involved in the synthesis of these imino acid betaines (*e.g. N*-methylproline, *N*-methylpipecolic acid, and *N*-methylhydroxyproline) (11), and their rates of labeling from supplied isotopically labeled amino acid precursors are now required to elucidate these pathways. Presumably, the pathway of hydroxyprolinebetaine synthesis in alfalfa could be explored by supplying isotopically labeled proline, hydroxyproline, *N*-methylproline, and *N*-methylhydroxyproline to high- and low-hydroxyprolinebetaine genotypes to determine if hydroxyprolinebetaine deficiency results from a lesion in the capacity to metabolize hydroxyproline to *N*-

methyhydroxyproline, a lesion in the capacity to metabolize *N*-methylhydroxyproline to hydroxyprolinebetaine, and/or a lesion in the capacity to metabolize *N*-methylproline to *N*-methylhydroxyproline.

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