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QUATERNARY AMMONIUM COMPOUNDS IN PLANTS IN RELATION TO SALT RESISTANCE

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Key Word Index—Angiosperms; glycophytes and halophytes; quaternary ammonium compounds; glycine; betaine; proline; inorganic ions; salt resistance.

Abstract—Fourteen plant species exhibiting a wide range of salt resistance as halophytes, semi-resistant glycophytes and sensitive glycophytes, have been grown in nutrient solution culture under low and high salt conditions. Inorganic analyses and shoot sap osmotic pressure values of these plants confirm that osmotic compensation at high salt levels is largely achieved by the accumulation of Na salts. Choline was found in shoots and roots in the range 1.0–0.2 μmol g fr. wt^{-1} and varied little following salt stress. Trigonelline was found in some of the sensitive glycophytes and did not increase significantly in stressed plants. Betaine levels were high (> 10 μmol g fr. wt^{-1}) in the shoot of the halophytes at low salt conditions, lower values (1–10 μmol g fr. wt^{-1}) were found in the semi-resistant glycophytes and none detected in the sensitive glycophytes. In the two resistant groups betaine accumulated to higher levels following NaCl stress. Shoot betaine levels always exceeded root levels. Proline occurred in all plants and in all cases was accumulated following NaCl stress.

INTRODUCTION

Choline is the best characterised of the quaternary ammonium compounds (QACS) found in plants and has long been recognised as a component of most plant tissues [1, 2]. It is important as a precursor of lecithin in membranes and, together with its acid derivative glycinebetaine, it can act as a methyl donor in plant systems [3]. A number of choline esters also appear to have important physiological functions in plants. Phosphoryl- and sulphurylcholine may mediate the transport of their respective bound anions [4, 5], while acetylcholine may be involved in membrane function [6] although its biochemical role is far better documented in animals [7].

Glycinebetaine does not appear to enjoy the near-universal distribution observed for choline [8] but is probably the most widely distributed of the betaines in plants [9]. It has been isolated from a number of plants, particularly Chenopodiaceae, [10, 11] but subjected to little intensive work. Choline is biosynthesised by the sequential methylation of ethanolamine and is subsequently converted to betaine by two oxidation steps [12]. Betaine has been found to be a fairly stable product of metabolism, but, apart from its possible role as a methyl donor, little is known of its function. It has been dismissed as a metabolic waste product [13], but as noted by Bowman and Rohringer [14], its structure, ease of formation and readily accessible methyl groups make this unlikely. Furthermore, it may represent up to 20% of the total plant nitrogen in some healthy young plants in the field [15].

As described in previous communications [16, 17], various reports in the literature appeared to us to associate QACS with salt resistance in plants and microbes and this led to our studying the effects of these compounds on the salt sensitivity of maize seedlings. Choline and, to a lesser extent betaine, was found to partially ameliorate sodium chloride toxicity and, in order to assess the general biological significance of this observation, a survey of the distribution of these compounds in plants has been undertaken. The results from part of this survey are reported in this paper.

Fourteen plants ranging from extreme halophytes to sensitive glycophytes have been grown in hydroponic cultures in low and high salt solutions and analysed for inorganic ions, osmotic pressure, quaternary ammonium compounds and proline. These data, some of whom were reported in a previous short communication [18], suggest that a relationship does exist between the occurrence of one of the QACS, namely glycinebetaine, in a number of plant species and salt resistance. However, only a few families are covered in this study and in a further paper the taxonomic distribution of betaine is discussed in greater detail [15].

Proline levels were recorded in view of the suggestions that the accumulation of this amino acid might be associated with salt resistance [19] and drought resistance [20]. The relationship of proline and betaine accumulation will be discussed.

RESULTS AND DISCUSSION

Growth and classification of the salt resistance of the species

The 14 species, representative of the wide range of salt resistance found in terrestrial plants, were grown in

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Table 1. Conditions for the growth and extraction of the 14 plant species

Genus and species	Variety	No. of plants per 10 l. sol.	Age at start of expt (wk)	Incremental increase in NaCl (mM)	Final NaCl level (mM)	Duration at final NaCl level (wk)	Extraction procedure
Suaeda monoica		5	4	100	1000	6	II
Atriplex spongiosa		5	13	50	500	7	II
Spartina x townsendii		10	10	50	300	7	II
Hordeum vulgare	California Mariout	30	3	50	150	3	I
	Arimar	30	3	50	150	3	I
	Chevron	30	3	50	150	3	I
Chloris gayana		30	3	50	150	3	I
Triticum vulgare	Capelle Desprez	30	3	50	100	3	I
Avena sativa		30	3	50	150	3	I
Zea mays	Crow Hybrid WF9 X M14	30	2	50	100	2	II
Lycopersicon esculentum	Marmande	5	5	50	100	3	I
Pisum sativum	Dwarf Progress 9	30	3	50	100	3	I
Daucus carota	Chanteney Red Cored		3	50	100	3	I
Raphanus sativus	Cherry Belle		2	25	75	3	I
Trifolium repens	White Clover S100, RH/70		4	25	75	3	I
Phaseolus vulgaris	Prince	5	3	25	75	3	I

*Procedures described in Experimental.

either standard Hoagland's solution or in the same medium with NaCl added incrementally to produce a significant inhibition of growth (Tables 1 and 2). In order to facilitate comparisons, the aim was to effect a 50% decrease of shoot growth in each species but this aim was not consistently fulfilled. Nevertheless, the range of salt sensitivity of the plant species was clearly demonstrated (Table 2). It should be noted that, although only a 9% inhibition of growth was recorded at 1 M NaCl with *Suaeda monoica* compared with the 'low salt' control control plants, the growth of this species was stimulated by NaCl in the range 50–500 mM and inhibited by further increments of NaCl up to 1 M [21].

Growth data, expressed as g fr. wt per plant, was not obtained in all cases as some seeds were grown as a blanket cover on gauze trays rather than as individual plants in which case the visible response was recorded photographically [21]. The information in Table 2 is therefore, of itself, insufficient to classify the plants in terms of their salt resistances although it is in general agreement with published tables of salt sensitivity. Therefore, the plants are listed in Table 1 and subsequent Tables according to information available in the literature [22]. However, it is improbable that any specific order of salt resistance is maintained under all conditions as comparative responses vary with temperature, humid-

Table 2. Growth of plants grown under high and low salt conditions

	Low salt growth conditions		High salt growth conditions			
	Shoot Yield g fr. wt per plant	Root Yield g fr. wt per plant	Shoot Yield g fr. wt/plant^{-1}	Shoot % low salt control	Root Yield g fr. wt plant^{-1}	Root % low salt control
S. monoica	23	26	21	91	9	36
A. spongiosa	N.D.†	N.D.	N.D.	—	N.D.	—
S. x townsendii	89	22	66	74	23	104
H. vulgare						
Calif. Mar.	10	8	7	69	4	49
Arimar	4	3	2	47	2	64
Chevron	25	7	8	31	4	56
C. gayana	15	11	7	50	9	83
T. vulgare	10	4	3	32	1	36
A. sativa	8	3	5	63	1	37
Z. mays	4	2	2	38	1	41
L. esculentum	274	52	159	58	68	130
D. carota	N.D.	N.D.	N.D.	—	N.D.	—
P. sativum	1	1	1	81	1	87
R. sativus	N.D.	N.D.	N.D.	—	N.D.	—
T. repens	N.D.	N.D.	N.D.	—	N.D.	—
P. vulgaris	11	2	9	83	3	129

*Growth conditions are described in Table 1 and Experimental. †N.D.—not determined.

ity, salt balance and concentration and the particular cultivar employed (see barley cultivars, Table 2). It is more appropriate to consider the plants as falling into 3 broad, rather poorly deliniated, physiological groups; halophytes, (*Suaeda monoica*, *Atriplex spongiosa* and *Spartina x townsendii*), semi-resistant glycophytes (barley, *Chloris gayana*, wheat, oats) and sensitive glycophytes (french bean, clover, carrot, radish, pea) while corn and tomato occupy an intermediate position between the two latter groups.

Osmotic potentials and inorganic analyses

The osmotic potentials of the plant species grown under low salt conditions averaged around 350 mOsm although there appeared to be a small, but not unambiguous tendency for the three halophytes and three most sensitive glycophytes to have rather high and low values respectively (Table 3). As would be anticipated, there was a substantial increase in osmotic pressure when plants were exposed to NaCl and in all cases except *S. monoica* and *A. spongiosa* osmotic adjustment was complete; that is $\Delta\pi$ between sap and external solution was approximately constant (Table 3).

The shoots were analysed for K^+, Na^+ and Cl^- and the contents of these ions are presented in Table 4 on a tissue water basis to facilitate inter-species comparison. The species varied considerably in the fresh weight: dry weight ratio (fr. wt:dry wt), and this ratio decreased in all plants except the extreme halophytic succulent, *S. monoica*, at the high salt levels. Therefore, it is considered that although the concentrations of water soluble, non-structural ions K^+, Na^+ and Cl^- could be expressed either on a dry weight or on a tissue water basis, the latter afforded a number of advantages in this study.

On the basis of the inorganic ion contents the approximate contribution of K^+, Na^+ salts to the osmotic potential could be calculated (Table 3). These salts appear to be dominant in the halophytes and barleys under low and high salt conditions but other molecules and ions make a major contribution to the potential in the more sensitive plants. In agreement with previous work [22] these figures also show that in all cases the uptake of Na^+ salts played a major role in osmotic adjustment at high salt levels. It should be noted that this simplified calculation of osmotic pressure does not take account of any change of osmotic coefficient with concentration nor the effect of anions on published coefficients [23]. In *S. monoica* and *S. townsendii* there was a major discrepancy as the calculated π exceed the measured π sap. This problem is explored further in another paper [24].

The inorganic analyses (Table 4) show that the cellular K^+ concentrations of the sensitive glycophytes were in the range 80–120 mol m^{-3} while those of the semi-resistant glycophyte and halophytes were somewhat higher. Cl^- values appeared to reflect a similar pattern,

Table 4. Inorganic analyses on shoots of plants grown under high and low salt conditions

Plant species	Low salt growth conditions*			High salt growth conditions		
	K^+	Na^+ (mM plant water)	Cl^-	K^+	Na^+ (mM plant water)	Cl^-
S. monoica	280†	46	12	63	1523	796
A. spongiosa	139	24	5	25	598	364
S. x townsendii	168	49	29	92	438	251
H. vulgare						
Calif. Mar.	176	10	24	122	237	128
Arimar	232	4	29	158	230	150
Chevron	123	10	17	47	298	197
T. vulgare	166	14	14	170	126	120
A. sativa	181	2	17	108	222	134
Z. mays	82	6	10	80	235	224
D. carota	124	14	10	95	284	172
P. sativum	82	16	7	66	280	256
R. sativus	90	7	5	38	128	68
T. repens	118	9	3	107	170	97
P. vulgaris	82	11	3	104	51	117

*Growth conditions are described in Table 1. †All values are the mean of four determinations.

Table 3. Osmotic potentials of plants grown under high and low salt conditions

Plant species	Low salt growth conditions*		High salt growth conditions*				
	π_{sap} (mOsm)	π_{calc}	π of high salt medium	π_{sap}	π_{calc} (mOsm)	$\Delta\pi_{sap}^{H-L}$	$\Delta\pi_{calc}^{H-L}$
S. monoica	680	587	1840	2140	2855	1460	2270
A. spongiosa	380	293	920	1400	1121	1020	828
S. x townsendii	470	391	552	816	954	346	563
H. vulgare							
Calif. Mar.	300	335	276	614	646	314	311
Arimar	367	425	276	681	698	314	274
Chevron	284	239	276	708	621	424	382
T. vulgare	540	326	184	732	545	192	211
A. sativa	331	329	276	674	594	343	265
Z. mays	328	155	184	512	567	184	412
D. carota	472	248	184	720	682	248	434
P. sativum	312	176	184	736	623	424	446
R. sativus	250	175	138	396	299	146	124
T. repens	228	229	138	456	499	228	270
P. vulgaris	232	167	138	456	279	224	111

*Growth conditions are described in Table 1. π_{calc} Values calculated from inorganic K^+ and Na^+ values assuming osmotic coefficients of 0.9. $\Delta\pi_{sap}^{H-L}$ change in measured osmotic potential of sap from low to high salt conditions; $\Delta\pi_{calc}^{H-L}$ change in calculated osmotic potential of sap from low to high salt conditions, i.e. contribution of K and Na salts to osmotic adjustment.

except in the case of *A. spongiosa* but this plant is known to be a massive oxalate accumulator [25]. As previously established by Collander [26], the halophytes absorbed more Na^+ than the glycophytes even from low salt solutions.

Few valid comparisons may be made of inorganic ion concentrations under the high salt conditions as the growth conditions varied widely. However, within the barley cultivars the most salt sensitive variety, Chevron had the lowest affinity for K^+ and was least able to exclude Na^+ and Cl^-. More detailed comparative studies on the salt resistance of the barley cultivars have been carried out and will be reported elsewhere [27].

Organic analyses

Although a number of Dragendorff-positive compounds were detected on chromatograms of shoot and root extracts of the 14 species, only three of them were unambiguously identified: choline, trigonelline and betaine. Similar difficulties in identifying these compounds have been experienced by other authors [28]. These uncharacterised compounds were minor components but tended to occur more frequently and in greater abundance in the salt resistant species.

The quantitative distributions of the three major QACS were obtained by the use of both the specific but semiquantitative TLC method and the sensitive quantitative but less specific periodide assay. Both analytical methods gave essentially identical choline values and only the periodide values are shown in Table 5. Similar choline values were obtained for the shoot and roots of all the species grown at low and high salt although the root values were often lower than those of the shoots. The rather high level found in low-salt grown *S. monoica* was, at least in part, a reflection of the low fr. wt:dry wt ratio of this plant grown without NaCl, since the discrepancy was not apparent when the results were expressed on a dry weight basis.

With betaine the agreement between the two assay methods was sometimes less satisfactory principally because trigonelline (in tomato, pea, bean and possibly radish) or other uncharacterised compounds (e.g. in Arimar, barley) reacted in the low pH periodide assay (Table 6). The specific but somewhat less sensitive TLC method detected little or no betaine in the salt-sensitive species, several of whom contained trigonelline. Hence in some species the periodide assay could be adapted to measure the trigonelline levels (Table 7). This compound did not appear to change significantly with salt stress.

The betaine distribution in these 14 plants fell into a distinctive pattern. The three halophytes contained 10–50 μmol g fr. wt^{-1} in shoots and 3–20 μmol g fr. wt^{-1} in roots; the semi-resistant glycophytes, 1–5 μmol g fr. wt^{-1} and about 0.5 μmol g fr. wt^{-1} in the two tissues respectively, while little or no betaine was found in the sensitive glycophytes. In the two salt resistant classes, stress increased the shoot and, less markedly, the root betaine levels. In both groups of resistant plants the betaine content appeared to be correlated with the magnitude of the sap osmotic pressure (cf. Tables 3 and 6). The rank orders being *S. monoica* > *S.* x *townsendii* > *A. spongiosa* in the halophytes and *T. vulgare* > Arimar > Calif. Mar., *A. sativa* > Chevron in the more resistant glycophytes.

The species distribution and salt response of proline (Table 5) differed markedly from that described for betaine. The proline levels in low salt plants showed no obvious relationship to their genetic salt resistance. Proline levels increased in all species in response to salt stress so that the per cent change was much greater than that of betaine. Quantitatively, however, betaine levels

Table 5. Choline and proline contents of plants grown under high and low salt conditions

	Low salt growth conditions*				High salt growth conditions*			
	Choline content† (μmol g fr. wt^{-1})		Proline content (μmol g fr. wt^{-1})		Choline content (μmol g fr. wt^{-1})		Proline content (μmol g fr. wt^{-1})	
Plant species	shoot	root	shoot	root	shoot	root	shoot	root
S. monoica	1.7‡	0.2	0.6	0.1	0.8	0.1	4.2	2.7
A. spongiosa	0.2	0.3	0.3	0.2	0.2	0.2	1.3	0.4
S. x *townsendii*	0.5	0.3	0.4	0.1	1.0	0.5	6.8	0.8
H. vulgare								
Calif. Mar.	0.2	0.2	0.1	0.1	0.2	0.2	1.3	0.4
Arimar	0.4	0.3	0.1	<0.1	0.6	0.2	1.9	0.4
Chevron	0.3	0.5	0.1	<0.1	0.6	0.2	0.5	0.2
C. gayana	0.6	0.5	0.1	<0.1	0.5	0.3	0.6	0.3
T. vulgare	0.8	0.4	0.1	<0.1	1.0	0.3	1.5	0.1
A. sativa	0.3	0.2	0.1	0.1	0.2	0.1	2.4	1.3
Z. mays	0.6	0.2	0.1	0.1	0.7	0.3	0.6	0.1
L. esculentum	0.4	0.3	0.6	0.3	0.3	0.2	6.3	4.7
D. carota	0.5	0.3	0.1	<0.1	0.6	0.3	0.9	0.1
P. sativum	1.0	0.4	0.5	0.1	0.8	0.5	5.0	0.2
R. sativus	0.5	0.3	0.2	0.1	0.5	0.2	1.3	0.2
T. repens	0.5	0.4	0.4	0.1	0.6	0.4	4.2	0.5
P. vulgaris	0.7	0.2	0.1	<0.1	0.7	0.3	0.2	0.1

*Experimental growth conditions described in Table 1. †Choline determined by periodide method at pH 8.0. ‡All values are the mean of two determinations.

Table 6. Betaine content of plants grown under high and low salt conditions

Plant species	Low salt growth conditions*				High salt growth conditions*			
	Betaine content (μmol g fr. wt^{-1})				Betaine content (μmol g fr. wt^{-1})			
	Shoot		Root		Shoot		Root	
	I†	II‡	I†	II‡	I†	II‡	I†	II‡
S. monoica	49	58	18	16	54	58	26	18
A. spongiosa	11	13	5	3	28	34	8	5
S. x townsendii	31	26	7	9	68	59	15	12
H. vulgare								
Calif. Mar.	2	2	0.5	0.5	4	N.D.	0.5	N.D.
Arimar	3	4	<0.2	0.7	13	15	2	2
Chevron	1	2	<0.5	0.9	9	13	<0.4	2
C. gayana	2	2	0.5	0.3	4	4	0.5	0.5
T. vulgare	5	8	<0.4	0.6	9	14	<0.4	0.7
A. sativa	2	2	<0.1	<0.1	2	2	<0.1	<0.1
Z. mays	<0.5	1	<0.5	<0.4	0.6	2	<0.5	<0.4
L. esculentum	<0.1	0.4§	<0.1	<0.1	<0.1	0.6§	<0.1	0.2
D. carota	<0.3	<0.2	<0.3	<0.1	<0.3	0.2	<0.3	<0.1
P. sativum	<0.4	0.7§	<0.3	<0.2	<0.4	0.8§	<0.3	0.5
R. sativus	<0.3	0.2	<0.4	<0.1	<0.3	0.3	<0.4	0.3
T. repens	<0.3	0.2	<0.4	<0.2	<0.3	0.4	<0.4	0.2
P. vulgaris	<0.4	0.6§	<0.3	<0.1	<0.4	0.8§	<0.3	0.1

*Experimental growth conditions are shown in Table 1. †Betaine determined by TLC (mean of two determinations). ‡Betaine determined by periodide colorimetric method (mean of two determinations). §Major contamination due to trigonelline.

greatly exceeded those of proline in the more resistant plants even at high salt levels but in the sensitive plants the proline levels were the greater. As in the case of betaine, the shoot proline levels exceeded those in the roots.

It appears, therefore, that in these species betaine accumulation is related to salt resistance. The rather sparse data in the literature also confirm the association of high betaine content with halophytic character [10, 11]. However, the data are restricted to a few families, principally the Chenopodiaceae and require further expansion [15].

It is also quite apparent that other factors are also involved in salt resistance; for example, the more resistant barley cultivar, California Mariout contained less betaine than the more sensitive Arimar. It would appear from our own [24] and other studies [29] that avoidance of salt accumulation and maintenance of the cellular K^+ level in the growing shoot is of fundamental importance in conferring resistance in barley cultivars. Nevertheless, the massive accumulation of betaine in some halophytes, to comprise up to 20% of total cell nitrogen [15], is clearly a very important aspect of their metabolism.

Table 7. Trigonelline content of plants grown under high and low salt conditions

Plant species	Low salt growth conditions*		High salt growth conditions*	
	Trigonelline content (μmol g fr. wt^{-1})		Trigonelline content (μmol g fr. wt^{-1})	
	Shoot	Root	Shoot	Root
L. esculentum	04.	<0.1	0.5	0.1
P. sativum	0.6	0.1	0.7	0.4
P. vulgaris	0.6	0.1	0.7	0.1

*Growth conditions as described in Table 1.

The relation between betaine content and osmotic potential was noted previously. Further evidence has been produced to support this association and together with other data, this has led to our proposing that betaine acts as a cytoplasmic non-toxic osmoticum in plant cell operating at osmotic pressures above about 350 mOsm [30, 31].

EXPERIMENTAL

Growth conditions. Seeds were germinated in either moist vermiculite or on gauze trays suspended over aerated water and transferred initially to × 0.5 strength Hoagland's solution [32] and then finally to full strength medium. Clover, radish and carrot plants were grown on metal gauze trays for the period shown in Table 1. Seedlings of other species were transferred to bakelite tops suspended over 10 l Hoagland's soln in plastic bowls. Nutrient soln was replaced fortnightly. Further experimental details are given in Table 1.

Inorganic analyses

Preparation. Shoot tissue was washed for 5 min in running H_2O, blotted dry with tissue paper (fresh weight: fr. wt) and oven dried at 100–110° for 48 hr (dry weight: dry wt). Following a 5 min rinse in H_2O, roots were desorbed for 20 min in 5 mM $Ca(NO_3)_2$ and finally washed again in running H_2O for 5 min prior to oven drying.

Osmotic pressure. Tissue sap was extracted, using a French pressure cell and a hydraulic press, from 5–10 g chopped plant material, which had been washed and blotted dry as described above. Osmotic pressure of clarified sap was measured in a cryoscopic osmometer (Advanced Instruments Inc., MA).

Inorganic ions. Oven-dried tissue was dry ashed at 450–470° for 12 hr and dissolved in 0.1—mM HNO_3. Na and K were determined by flame emission spectrophotometry and Cl with an automated Corning-Eel chloride meter.

Organic analyses

Extraction. The method I was based upon procedure described

by Toyosawa and Nishimoto [2]. Washed, chopped plant material (100 g) was homogenised with a Waring blender in *i*-PrOH (750 ml) for 5 min and filtered. Residue was homogenised with further vols of *i*-PrOH (500 ml), MeOH (250 ml) and refluxing MeOH (250 ml) for 3.3 and 10 mih respectively. The 4 combined vols were taken to dryness *in vacuo* at 30–40°. The flask was washed successively with $CHCl_3$ (75 ml); MeOH (37.5 ml); dist. H_2O (22.5 ml); $CHCl_3$ (50 ml); MeOH (25 ml); and dist. H_2O (15 ml). The 2 phases of the 6 bulked washings were partitioned by bench centrifugation and the upper aq. layer removed, and the remaining lipid phase was washed 2 × with 2 vols (60 ml) of MeOH–H_2O (1:1). The 3 combined aq. phases were reduced to 10 ml, washed with Et_2O (3 × 30 ml), evaporated to dryness, redissolved in H_2O and stored at −10°. More than 95% of standard betaine added to biological samples was recovered by this extraction method. The second extraction method II was employed for some samples (Table 1) as described by Singh *et al.* [33].

Colorimetric assays. Betaine and choline were both determined by the non-specific periodide method in which choline and betaine are selectively precipitated at different pHs; so that the following expression: Total Quaternary Ammonium Compounds (QACS) (pH 2.0) − Choline (pH 8.0) = Betaine was found, by comparison with the results obtained by thin layer photodensitometry, to be a reliable estimate of the betaine content of some species. The modified periodide method published by Speed and Richardson [34] for the determination of QACS was developed further on the basis of the findings by Wall *et al.* [35]. The acid potassium triiodide soln (for total QACS) was prepared by dissolving 7.5 g I_2 and 10 g KI in 1 M HCl and filtering, while the same reagents dissolved in a 0.4 M KH_2PO_4–NaOH buffer (pH 8.0) provided the alkaline reagent to determine choline. Precisely 0.2 ml of either the acid or alkaline potassium triiodide reagent was added to a sample containing between 10–150 μg of QACS in H_2O. The mixture was shaken and left for at least 90 min in an ice bath with intermittent shaking. 2 ml ice-cooled H_2O was added rapidly to the mixture to reduce the absorbance of the blank and improve replication. This was quickly followed by 20 ml 1,2-dichloroethane, at −10°, and the 2 layers mixed by a constant stream of air bubbles for 5 min while the temperature was maintained at 4°. The absorbance of the lower organic layer was measured at 365 nm. Coefficients of variation of 2.6% were found for standard choline and betaine samples. Proline was determined by the method of Singh *et al.* [33].

Chromatography. The identification and chromatography of other QACS in the 14 species was investigated by TLC and thin layer electrophoresis (TLE). This also provided a means of assessing the specificity of the periodide assay for both choline and betaine in each species. To achieve a satisfactory separation of these compounds by TLC it was necessary to remove interfering compounds, e.g. sugars by ion exchange chromatography, unless the betaine concentration was greater than 10 μmol g fr. wt^{-1}. A 10 × 3 cm column of Zeo-karb 225 SRC 14 (standard resin, 52–100 mesh) was prepared, washed with 3 cycles of NaOH and HCl (6 M). The column was brought to pH 14 with NaOH and washed with H_2O, HCl and H_2O to bring the effluent pH to 5–6. The sample extract, pH 5–6, from 25 g fr.wt of plant material was applied to the column and washed with 20 ml H_2O. The QACS were eluted from the column with HCl (4 M) and rotor-evaporated *in vacuo* at 30–40° to dryness, redissolved in H_2O and stored at −10°. The QACS were separated by TLC using: I. MeOH–Me_2CO–conc HCl (90:10:4), II. MeOH: 0.88 NH_4OH (3:1), III. MeOH 0.88 NH_4OH-: 1.4-Dioxan (6:9:5) or TLE using a citrate–phosphate buffer (200 mM citric acid and 400 mM Na_2HPO_4 adjusted to pH 2.6). Purified extracts were applied to the Si gel G plates for both TLC and TLE, with an A.I.S. TLC multi-spotter. Prior to TLE, the plate was sprayed evenly with the citrate–phosphate buffer and then subjected to an electrophoretic current of 350 V approximately 50 mA, for 3–9 hr using a Shandon TLE tank with a water-cooled block. All TLC and TLE plates were sprayed with Dragendorff's reagent [36]. More satisfactory resolution of the QACS was achieved by cooling the TLE plate to 40° just prior to spraying. Betaine, choline and trigonelline were determined by direct reflectance densitometry in the visible range after TLC and TLE as described by Radecka *et al.* [36] The method was both rapid and highly specific but lacked the precision of the periodide colorimetric assay. Coefficients of variation as high as 20% were found between chromatography plates. This error was substantially reduced by standardization of the analytical techniques wherever possible, e.g. automated spotting of extracts onto TLC plates, and running reference standards alongside the plant extracts on the same plate for comparison with the corresponding values of a calibration curve run on a second TL plate. By these methods coefficients of variation of only about 5% could be achieved but we would consider that greater variation occurs with biological samples. Duplicate plant extract samples or standard solutions containing 10–100 μg betaine hydrochloride, or 5–50 μg choline chloride or 2.5–30 μg trigonelline hydrochloride were chromatographed as described above. The TLC or TLE plates were scanned (120 mm min^{-1}) in the direction of solvent flow with a Zeiss TLC spectrophotometer coupled to a chart recorder (30 mm min^{-1}). The densitometer was set up for direct reflectance measurements using a wavelength of 550 nm (0.075 mm wave band width). The plate was scanned with the slit shaped test area with the maximum height of 14 mm. The clearance distance between the measuring head and the thin layer was 4–5 mm as a second TLC plate was used to cover the Si gel layer in order to protect the optics of the instrument from the corrosive spray reagent. The covering TLC plate did not interfere with the measurements at 550 nm. The corresponding peak area from each spot was expressed as a wt of the chart paper enclosed within the peak. By employing the sensitive but non-specific periodide assay with the highly specific but semiquantitative TL photodensitometry, betaine and choline levels in plant extracts were determined by a relatively routine and rapid procedure.

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