

Gossypium betaines and some of their potential roles

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ABSTRACT

From extracts of the leaves of *Gossypium barbadense* and *G. armourianum*, glycinebetaine has been isolated and characterized by ^1H NMR spectroscopy and fast atom bombardment mass spectrometry. Trigonelline was also isolated from *G. barbadense*, but was not detected in *G. armourianum*. The contents of each betaine were estimated using a ^1H NMR spectroscopic method. Glycinebetaine yields were 3.1% for *G. barbadense* and 0.7% for *G. armourianum*. The trigonelline yield from *G. barbadense* was 0.006%. The presence of these betaines in such yields is consistent with those recorded for other species of Malvaceae, from the leaves of which yields of glycinebetaine, when present, ranged from 0.5% (*Modiola caroliniana*) to 4.6% (*Hibiscus rosa-sinensis*) and trigonelline, when present, from 0.005% (for example, *Hoheria sexstylosa*) to 0.1% (*Hibiscus rosa-sinensis*). *G. barbadense* and *G. armourianum* are both betaine-accumulating species in which the concentrations of glycinebetaine could behave as compatible cytoplasmic solutes in facilitating adaptation to saline and dry conditions, and to have a role in vacuolar osmotic adjustment. However, other potential functions of betaines need to be considered. Application of these compounds in very low amounts to plants has resulted in significant enhancement of the ability of these treated plants to resist stress conditions. In particular, application of glycinebetaine, α -aminobutyric acid betaine and α -aminovaleric acid betaine led to significantly higher leaf chlorophyll levels in the treated plants in comparison to the controls. Application of the same betaines to tomato plants also resulted in significantly reduced invasion of the roots of the plants by second stage juveniles of the root knot nematodes *Meloidogyne javanica* and *M. incognita*.

Introduction

Betaines are derivatives of amino acids containing a fully methylated quaternary nitrogen moiety. Compounds of this type have been recorded for many animals, plants, algae, fungi and bacteria (Blunden and Gordon, 1986). For plants, Rhodes and Hanson (1993) divided the betaine-yielding families and genera into those that accumulate betaines and those that do not. The most investigated role of betaines is that of cytoplasmic solutes aiding adaptation to saline and dry conditions. For this use, however, the concentrations of the compounds must be high, as in the betaine ac-

cumulating species, for example those in the families Chenopodiaceae (Adrian-Romero *et al.*, 1998) and Amaranthaceae (Blunden *et al.*, 1999). However, in the non-betaine accumulating species, the levels of these compounds are low, as in the Polygonaceae (Adrian-Romero *et al.*, 1998) and the Bromeliaceae (Adrian-Romero and Blunden, 2001). The role of the betaines in these cases has not been fully elucidated, but in recent years, it has been demonstrated that low concentrations of betaines can have a significant role in aiding plants to resist stress conditions, such as attack by pathogens (for example Wu *et al.*, 1997; Tyihak *et al.*, 2002) and frost damage (Allard *et al.*, 1998). Evidence for these claims is presented in this communication.

Experimental procedure

The species examined and their places of collection (all in the UK) are given in Table 1. The plant materials were oven dried at 4 °C, powdered and extracted for 6 h with 80% methanol. The methods of extraction, purification of the extracts by passage through a column of cation exchange resin, two-way thin-layer chromatography (TLC) examination and isolation of the Dragendorff-positive compounds by preparative TLC have been published previously (Blunden *et al.*, 1981, 1985). The isolated compounds were identified by TLC, using appropriate reference compounds, ^1H NMR spectroscopy (D_2O ; 400 MHz) and fast atom bombardment mass spectrometry (FAB MS).

Estimation of the content of each betaine present in the extracts was achieved using the ^1H NMR spectroscopy assay procedure of Blunden *et al.* (1986).

Results and Discussion

The leaves of *Gossypium barbadense* and *G. armourianum* were extracted and the extracts examined for the presence of betaines, which were detected by TLC in both extracts. The betaines were isolated by preparative TLC and characterized from TLC, ^1H NMR spectroscopic and FAB MS data. Glycinebetaine was found in the extracts of both species, and trigonelline in that of *G. barbadense*. High yields of glycinebetaine were recorded for both species, but the content of trigonelline in *G. barbadense* was only 0.006% (Table 1). These yields are in line with those recorded earlier by us for many other species of Malvaceae (Blunden *et al.*, 2001); some of these values are included in Table 1 for comparative purposes. Gorham (1996) had earlier reported large amounts of glycinebetaine in *Gossypium* species.

The concentrations of glycinebetaine present in the leaves of *G. barbadense* and *G. armourianum* are certainly high enough for them to act as cytoplasmic solutes to counteract salinity and water stresses. However, glycinebetaine and other betaines can also act as

antistressors in other biotic as well as abiotic stress conditions. However, most of the evidence for these effects has been obtained after application of the betaines to the plants, either by spraying on the leaves or application to the roots via the soil.

It has been demonstrated by several research groups that when glycinebetaine is applied to plants it is rapidly absorbed. Pocard *et al.* (1991) applied [¹⁴C]-glycinebetaine to alfalfa plants and detected radioactivity in the roots and nodules of both stressed and unstressed plants less than two hours after application. Mäkelä *et al.* (1996) applied [¹⁴C]-glycinebetaine to the leaves of turnip rape plants and monitored them autoradiographically. They detected the compound in the roots within two hours of application and, one day after treatment, the labeled glycinebetaine had been translocated to all plant parts. However, Shafi and Gorham (this volume) found that, although [¹⁴C]-glycinebetaine was rapidly absorbed by cotton leaves, there was no significant translocation of the compound. They also found that the applied compound did not undergo any detectable degradation over fourteen days. These results were in agreement with earlier observations by Hanson and Hitz (1982) and Weretilnyk *et al.* (1989). However, Baburina and Shevyakova (1995) reported that betaines acted as methyl group donors in transmethylation reactions in alfalfa shoots exposed to saline media.

Glycinebetaine was shown to be involved in frost resistance of plants by Bokarev and Ivanova (1971). Allard *et al.* (1998) reported that treatment of wheat plants with glycinebetaine resulted in large increases in the tolerance of the plants to freezing stress. A four-day exposure to a 250 mM solution resulted in improvement in the freezing tolerance by more than 5°C. Of particular interest was the finding that the treatment induced a subset of low temperature responsive genes that are also induced by salinity and drought stresses. The data suggest that glycinebetaine improved the freezing tolerance of the plants by eliciting some of the genetic and physiological responses associated with cold acclimation.

Several studies have been conducted on the role of betaines and other quaternary ammonium compounds, such as choline, in increasing the resistance of plants to attack by fungi. Application of betaines in very low amounts has resulted in significant enhancement of the treated plants to resist fungal attack (Kraska and Schönbeck, 1992). Tyihák *et al.* (1988) linked the resistance of tomato plants to *Fusarium oxysporum* to their content of quaternary ammonium compounds. Tyihák *et al.* (2002) have shown that application of very low amounts of betaines and other methylated compounds to plants induces an immune response to fungal attack. The compounds act within two concentration ranges. For example, in the case of an induction time of two days between application of the betaine solution and inoculation with the fungal spore

suspension, the effective concentration ranges of the inducer to produce low infection rates were 10⁻⁵ to 10⁻⁶ and 10⁻¹² to 10⁻¹³ mol/liter.

Many of the experimental results that have advanced the knowledge of the effects produced when betaines are applied to plants have come from studies made using seaweed extracts and suspensions. The extracts are prepared by extraction of the dried algal material with either water or aqueous alkali, whereas the suspensions are produced from fresh algae. The most commonly utilized species in Europe and North America is *Ascophyllum nodosum*, although small quantities of *Fucus* and *Laminaria* species are also used. In South Africa, an *Ecklonia maximum* is employed.

The use of seaweed extracts has led to many claims, but of particular interest are those that relate to increased resistance of the treated plants to stress conditions (Blunden *et al.*, 1994). In 1984, Blunden *et al.* had suggested that betaines present in the extracts might be responsible for some of the reported effects. From *A. nodosum*, λ-aminobutyric acid betaine, δ-aminovaleric acid betaine and laminine were isolated, and from *Fucus* and *Laminaria* species, γ-aminobutyric acid betaine, glycinebetaine and laminine (Blunden *et al.*, 1986). The commercial seaweed extracts contain the same betaines as those present in the seaweed species from which they have been prepared.

Enhanced chlorophyll levels

Application of an *A. nodosum* based extract, either to the soil or to the foliage of tomato plants, produced leaves that, after 34 days, were visually greener than those of the control plants. The possible role of betaines in producing this result was considered and the effect on leaf chlorophyll content was investigated using a cucumber bioassay procedure devised for cytokinins (Fletcher, 1982). The seaweed extract was demonstrated to increase the chlorophyll levels of the cucumber cotyledons. The betaines in the seaweed extract, when tested separately, also produced significantly enhanced chlorophyll concentrations in the cotyledons and it was concluded that the effects of enhancing chlorophyll levels was due, at least in part, to the betaines it contains (Whapham *et al.*, 1993). This work was extended by examining the effects on leaf chlorophyll of various species due to either foliar or soil application of either seaweed extract or a mixture of the betaines in the same concentrations as those present in the extract. Application of the seaweed extract resulted in higher concentrations of chlorophyll in the leaves of the treated plants (tomato, dwarf French bean, wheat, barley, maize) in comparison to the controls. When the betaines were applied, very similar leaf chlorophyll levels were recorded for the seaweed extract and betaine treated plants. This suggests strongly that the enhanced chlorophyll content of plants treated with seaweed extract is dependent on the betaines it contains. Table 2 shows the leaf chlorophyll levels of maize

leaves after soil application of either seaweed extract or betaine solution. More detailed results and the full methodology can be found in Blunden *et al.* (1997). It has to be stressed that these effects were produced with a total betaine application rate to the soil around each plant of about 0.17 mG.

Effect on root knot nematodes

Soil application to the roots of tomato plants of an *A. nodosum* based extract resulted in a significant reduction in the number of second-stage juveniles of both *Meloidogyne javanica* and *M. incognita* invading the roots, compared to those of plants treated with water alone. Egg recovery from the seaweed extract treated plants was also significantly lower. The three major betaines found in the extract, when applied at concentrations equivalent to those present in the extract, also led to significant reductions in both the nematode invasion profile and egg recovery. This led to the conclusion that the betaines present in the extract play a major role in bringing about the observed effects. Detailed results can be found in Wu *et al.* (1997 and 1998) and Jenkins *et al.* (1998). Table 3 summarizes some of the data.

Conclusions

It has been demonstrated by several workers that application of betaines to plants leads to them having increased resistance to several stress conditions. Evidence has been published to show that application of glycinebetaine to wheat plants led to the induction of a subset of low temperature responsive genes; these are also induced by salinity and drought stresses. It thus suggests that application of betaines stimulates the plant to produce its own defense mechanisms. This is consistent with the finding that betaines induce an immune response to fungal attack. Therefore, with *Gossypium* species, the betaines present, as well as their potential role in adapting the plants to saline and arid conditions, may have an additional function in producing resistance to other stress conditions.

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Table 1. Betaines in the Malvaceae.

Species	Place of collection	Date of collection	Glycinebetaine yield (%, dry wt)	Trigonelline yield (%, dry wt)
<i>Abelmoschus moschatus</i>	Havant, Hampshire	July 2000	nd*	0.07
<i>Abutilon vitifolium</i>	Greenway Gardens Nursery, Cherston Ferrers, Devon	February 2000	0.9	nd
<i>Alcea rosea</i>	Havant, Hampshire	January 2000	1.9	0.03
<i>Athaea officinalis</i>	Mill Hill Plants, East Stoke, Newark, Nottinghamshire	July 2000	0.8	nd
<i>Callirhoë involucrata</i>	A. & A. Thorp, Theddingworth, Leicestershire	July 2002	0.7	0.02
<i>Gossypium armourianum</i>	Bangor, Gwynedd	July 2000	0.7	nd
<i>G. barbadense</i>	Bangor, Gwynedd	September 2000	3.1	0.006
<i>Hibiscus rosa-sinensis</i>	Havant, Hampshire	May 2000	4.6	0.1
<i>Hoheria sexstylosa</i>	Greenway Gardens Nursery, Cherston Ferrers, Devon	February 2000	3.2	0.005
<i>Kitaibela vitifolia</i>	Chipchase Castle Nursery, Wark, Hexham, Northumberland	July 2000	1.1	0.009
<i>Malva sylvestris</i>	Havant, Hampshire	July 2000	1.6	0.01
<i>Modiola caroliniana</i>	Cambridge University Botanic Gardens	September 2000	0.5	nd
<i>Plagianthus regius</i>	Greenway Gardens Nursery, Cherston Ferrers, Devon	February 2000	0.7	nd
<i>Sidalcea candida</i>	Chipchase Castle Nursery, Wark, Hexham, Northumberland	July 2000	1.6	0.01

*(nd=not detected)

Table 2. Leaf chlorophyll concentrations (mean SPAD units) of maize plants after soil application of betaine solution.

Time after application (days)		Control	Seaweed extract	Betaine solution
0	MSU ¹	22.71	22.66	22.42
	SE ²	0.81	0.36	0.85
	P		0.53	0.60
7	MSU	30.64	33.38	33.88
	SE	0.98	0.60	0.58
	P		0.028*	0.015*
14	MSU	22.48	25.70	26.58
	SE	1.2	0.90	0.58
	P		0.033*	0.013*
21	MSU	23.64	26.82	27.44
	SE	1.4	0.16	0.61
	P		0.046*	0.03*
28	MSU	22.42	24.14	24.00
	SE	0.36	0.13	0.29
	P		0.0032*	0.0056*

¹Mean SPAD units

²Standard error

*Statistically significant difference ($p=0.05$) between control and test plants; $n=5$. Each plant treated with γ -aminobutyric acid betaine 0.096 mg, δ -aminovaleric acid betaine 0.043 mg, glycinebetaine 0.034 mg

Table 3. Mean number (+ standard error) of second stage juveniles (J2s) of *Meloidogyne incognita* and *M. javanica* per tomato plant 14 days post-inoculation (values for the mean number of eggs per plant 36 (for *M. incognita*) and 49 (for *M. javanica*) days post-inoculation are given in brackets).

Nematode species	Mean number of J2s and (eggs) / plant		
	Control	Seaweed extract	Betaine mixture
<i>M. incognita</i>	106.2 ± 15.0	10.4 ± 6.4	23.4 ± 4.9
	(11400 ± 1800)	$p < 0.001$	$p < 0.001$
		(660 ± 157)	(1460 ± 448)
		$p < 0.05$	$p < 0.05$
<i>M. javanica</i>	89.2 ± 11.0	9.6 ± 3.5	68.0 ± 9.2
	(18540 ± 1569)	$p < 0.01$	$p < 0.096$
		(4600 ± 899)	(3260 ± 773)
		$p < 0.001$	$p < 0.001$