VARIATION IN TROPANE ALKALOID ACCUMULATION WITHIN THE SOLANACEAE AND STRATEGIES FOR ITS EXPLOITATION

Adrian J. Parr, John Payne, John Eagles,* Bridget T. Chapman, Richard J. Robins and Michael J. C. Rhodes

Plant Biotechnology Group, Department of Genetics and Microbiology; *Mass Spectrometry, Department of Chemical Physics, AFRC Institute of Food Research, Norwich Laboratory, Colney Lane, Norwich NR4 7UA, U.K.

Abstract—Petioles from over 1000 individual plants of the genera *Datura*, *Scopolia* and *Hyoscyamus* were analysed to establish the variation in the levels and patterns of tropane alkaloids which occur between plants. Transformed root cultures were initiated from plants of which analyses suggested extremely high or low alkaloid biosynthetic capacities, or favourable alkaloid patterns. These root cultures were then studied in detail by HPLC, GC and GC-MS. Root cultures showed substantial differences in alkaloid patterns between different species, but the quantitative differences in tropoyl esters of α-tropine (hyoscyamine plus scopolamine) observed between different lines were less than those seen between plants. This difference is interpreted as to some extent reflecting the influence of transport and storage effects, as well as biosynthetic capacity, in determining alkaloid levels in plant petioles. In addition, there is some indication that the full biosynthetic capacity may not always be expressed in culture. Despite these limitations, the identification of plants showing favourable characteristics still, however, proved a useful first step in the development of scientifically and potentially biotechnologically interesting root cultures.

INTRODUCTION

In recent years the application of differentiated organ cultures has led to significant advances in the *in vitro* production of plant-derived secondary products. Root cultures such as those obtained by the genetic transformation of dicots by the bacterium *Agrobacterium rhizogenes* [1] have attracted considerable interest. It is a feature of such cultures that they show a stable production of (root-derived) secondary products which typically both qualitatively and quantitatively resemble that shown by roots of the parent plant [2, 3].

The production of tropane alkaloids, notably hyoscyamine and scopolamine, by root cultures of a variety of solanaceous species has been investigated within the last few years by a number of groups [4] attracted by the considerably higher productivity of such cultures in comparison to the equivalent cell suspension cultures. Knopp *et al.* [5] have examined the production of tropane alkaloids by over 100 transformed root cultures derived from 18 species, mostly within the genera *Datura* and *Hyoscyamus*. They have identified considerable variation in the levels of hyoscyamine and scopolamine accumulated. In view of the apparent predictable relationship between the biosynthetic properties of transformed roots and those of the parent plants [3], it should ultimately prove possible to understand the variations seen in root cultures in terms of the properties of the different genera, species and individuals. Screening plant populations for individuals showing particularly favourable characteristics—such as unusually high alkaloid productivity, or the production of a particular alkaloid without significant accumulation of other unwanted alkaloids—should thus be an important step in the development of commercially and scientifically useful transformed cultures. In the present paper we report on tropane alkaloid production by over 1000 individual plants from species within the genera *Datura*, *Hyoscyamus* and *Scopolia*, and on the properties of transformed root cultures initiated from selected plants within this population. A preliminary report of some of the early work has appeared elsewhere [6].

RESULTS

Variation amongst plants

In the Solanaceae, tropane alkaloids are synthesized in the root, whence large amounts may be transported to the aerial parts—sometimes with concomitant modification (see for example [7]). This means it is impossible to estimate a plant's biosynthetic potential without analysing the whole plant, a situation which is not completely satisfactory when it is also intended to establish cultures from plants identified as being of interest. As a compromise we chose to measure the alkaloid content of leaf petioles as an index of alkaloid productivity. Although high-producing plants that transport little alkaloid to the aerial parts will not be detected by this approach, it should in other respects be suitable for identifying plants producing enhanced levels of hyoscyamine and scopolamine, which are typically readily mobilized. The petiole analysed was standardized as that of the fourth leaf, and was harvested when this leaf had just fully expanded, in

order to allow for effects of leaf maturity on alkaloid content [7].

Datura

The alkaloid profile of the petioles of most *Datura* species was dominated by hyoscyamine, scopolamine and an alkaloid provisionally identified as meteloidine by HPLC comparison with reference material. *Datura ceratocaula* was atypical in containing apparently major amounts of several other unidentified alkaloids [6], an observation in line with its other unusual taxonomic features. Even within the more typical *Datura* species, substantial variation was however observed in the levels and relative proportions of the major alkaloids (Table 1). This variation operated at all levels, i.e. between species, between different accessions of the same species, and between individuals.

Amongst the variation observed two features are of particular biotechnological interest. Firstly, the absolute level of the tropic acid esters hyoscyamine and scopolamine should in part reflect the biosynthetic capacity of the roots of the plant. Alkaloid levels (hyoscyamine and scopolamine combined) were found to range from 52 to 1350 $\mu g\,g^{-1}$ fr. wt and even within the single species *D. stramonium* a 20-fold difference in petiolar alkaloid levels was observed. Overall the highest alkaloid occurred in *D. stramonium* (though this to some extent may reflect the larger number of accessions and individuals studied) and *D. fastuosa* (Table 1). Plants with a particularly high petiolar alkaloid level are good candidates for generating high-producing root cultures, for high levels require a high biosynthetic capacity.

The other feature of interest is the variation in the scopolamine to hyoscyamine ratio. Both hyoscyamine and scopolamine are of commercial interest, and it would be an advantage to have different cultures producing either hyoscyamine or scopolamine as major products. In *Datura*, hyoscyamine is synthesized in the roots, then epoxidized to scopolamine. This epoxidation can occur in the root in some species, but in others it occurs only in the aerial parts (e.g. [7, 8]). Plants with high scopolamine to hyoscyamine ratios in the petiole are likely to result from either particularly efficient epoxidation during alkaloid transport in the shoot, or from epoxidation already having begun in the root. They are thus candidates for the generation of root cultures producing high levels of scopolamine. Conversely, plants with low petiolar scopolamine to hyoscyamine ratios should include cases where there is no epoxidation occurring in the roots, and they are thus candidates for the generation of root lines producing only hyoscyamine. Within the *Datura* species we examined, the petiolar scopolamine to hyoscyamine ratio was found to show at least a 20-fold variation (Table 1). The lowest values were found in *D. leichhardtii*, where Hashimoto and Yamada [8] have already found untransformed root cultures to be unable to synthesize scopolamine. Some species exhibited wide variations between different accessions, although plants within each accession were more constant (Table 1); this suggests the presence of several physiologically distinct chemotypes.

Hyoscyamus

The alkaloid profiles of *Hyoscyamus* petioles were rather simple, being dominated by hyoscyamine and/or scopolamine. Alkaloid levels varied in much the same manner as for *Datura*, with standard deviations for results from a single accession typically being 50% of the mean, and with a seven-fold difference in the means being observed between species with the highest and lowest alkaloid content (Table 2). The absolute alkaloid levels were, however, generally only one-tenth of those seen in *Datura*—which hints at a difference in alkaloid partitioning between the two genera, since the biosynthetic capacities are not generally thought to differ so greatly. Petiolar scopolamine to hyoscyamine ratios varied widely from less than 0.01 in *H. muticus* to over 10 in some individuals of *H. desertorum*. In general, though, ratios were low (Table 2). This too indicates a difference in the behaviour of the genera *Datura* and *Hyoscyamus*, for these low ratios seen in *Hyoscyamus* are not associated with any inability to synthesize scopolamine in the roots (see Table 4). Perhaps in *Hyoscyamus* the role of aerial tissue in epoxidation is minor, whereas in *Datura* it seems more significant.

Table 1. The combined levels of scopolamine and hyoscyamine, and the ratios of these two alkaloids, in petioles of various *Datura* species

	Scopolamine + hyoscyamine ($\mu g\,g^{-1}$ fr. wt)	Scopolamine/hyoscyamine ratio
Datura alba	387 ± 296	1.4 ± 0.3
D. ceratocaula	ca 215 ± 72	ca 3.0 ± 1.2
D. discolor	219 ± 112	9.4 ± 3.8
D. fastuosa	481 ± 173	1.6 ± 0.3
D. ferox (5)	$242 \pm 115 - 320 \pm 148$	$1.7 \pm 0.7 - 11.4 \pm 4.3$
D. innoxia (3)	$119 \pm 25 - 286 \pm 131$	$2.7 \pm 0.6 - 5.6 \pm 1.0$
D. leichhardtii	240 ± 100	0.65 ± 0.15
D. metel (2)	$110 \pm 47 - 127 \pm 35$	$2.5 \pm 0.6 - 8.8 \pm 2.6$
D. stramonium (19)	$174 \pm 67 - 762 \pm 250$	$1.3 \pm 0.3 - 2.4 \pm 0.6$
D. wrightii	315 ± 117	22.6 ± 9.1

Figures quoted are means ± standard deviation for plants from a single accession. Where more than one accession of a particular species was studied that number is shown in parentheses, and the observed range of mean values for each parameter is shown.

Table 2. The combined levels of scopolamine and hyoscyamine, and the ratios of these two
alkaloids, in petioles of various *Hyoscyamus* species

	Scopolamine ± hyoscyamine ($\mu g\,g^{-1}$ fr. wt)	Scopolamine/hyoscyamine ratio
Hyoscyamus albus	28 ± 14	0.63 ± 0.35
H. aureus	5 ± 2	0.52 ± 0.94
H. canariensis	33 ± 8	0.72 ± 0.43
H. desertorum	21 ± 11	5.0 ± 4.8
H. × gyoerffyi	27 ± 15	0.15 ± 0.09
H. muticus	37 ± 30	0.03 ± 0.05
H. pusillus	24 ± 6	0.07 ± 0.12
H. turcomanicus	14 ± 17	0.35 ± 0.85

Figures quoted are means ± standard deviation for plants from a single accession.

Scopolia

Interpretation of the HPLC analyses of *Scopolia* petioles proved difficult, because both *S. lurida* and *S. stramonifolia* contained unknown alkaloids which ran very close to hyoscyamine and scopolamine and interfered with their quantitation. Petiolar alkaloid levels were however closer to those observed for *Datura* than for *Hyoscyamus*, with a level of variation similar to that observed in both these genera.

Variation amongst root cultures

Transformed root cultures were obtained from *ca* 40 plants chosen from the screened population on the basis of their showing extremes in petiolar alkaloid composition. Several lines were also initiated from species which were unavailable at the time of the initial screenings. The level and pattern of tropane alkaloids in these cultures were then determined, with emphasis being placed on the later stages of the culture cycle (typically 30–35 days), where alkaloid content is known to be at its greatest in several lines [9].

Datura

Plants of *D. stramonium* selected for their high petiolar alkaloid levels gave rise to transformed root cultures producing typically 600–1 000 $\mu g\,g^{-1}$ fr. wt hyoscyamine (Table 3). On one occasion a level as high as 1180 $\mu g\,g^{-1}$ was recorded for the culture derived from the plant with the highest petiolar alkaloid level. This is one of the highest hyoscyamine contents reported in the literature. It should, however, be noted that while there was a rough correlation between petiolar alkaloid content and levels of hyoscyamine in root cultures for the very best plants, plants showing particularly low alkaloid levels, which included representatives from varieties *inermis* and *tatula*, produced cultures which also contained substantial levels of hyoscyamine (Table 3). This indicates that a significant proportion of the variation seen in petiolar alkaloid levels may reflect transport and partitioning effects, rather than inherent variation in biosynthetic capacities. All *D. stramonium* cultures examined produced a similar spectrum of alkaloids, with essentially no scopolamine and with hyoscyamine accounting for approximately 80% of the total alkaloid (Table 5). Comparable levels and patterns of alkaloids were also observed in *D. fastuosa* cultures, with

the exception that small amounts of scopolamine were present (Tables 4, 5). *Datura quercifolia* cultures were also broadly similar, differing in having lower hyoscyamine levels (Tables 4, 5), and substantial amounts of acetoxytropane derivatives (Table 5).

Within those species showing relatively high petiolar scopolamine to hyoscyamine ratios, root cultures were produced from *D. ferox*, *D. innoxia* and *D. wrightii*. All showed the production of some scopolamine in addition to hyoscyamine, with the highest relative amount being found in *D. innoxia*, although it is not clear whether this would still hold if further root cultures of each species were to be analysed. In addition to hyoscyamine and scopolamine, 6-hydroxyhyoscyamine was also identified in *D. ferox* and *D. innoxia* (Table 5). The combined productivities of these alkaloids in cultures derived from plants of each species which also had high petiolar alkaloid levels were broadly comparable to the hyoscyamine production seen in *D. stramonium* (Table 4). It should be noted, however, that in *D. wrightii* cultures the major alkaloid is not hyoscyamine or related tropoyl esters, but 3α-acetoxytropane (Table 5). The flux into tropane alkaloids is thus particularly high in this culture, though substantially less than half goes into the alkaloids of commercial interest.

Although plants and petioles were not examined, root cultures were also produced from two 'tree daturas', *D. sanguinea* and the *D. candida* × *aurea* hybrid 'B5' of El-Dabbas and Evans [10]. These produced the highest levels of scopolamine seen in all the *Datura* cultures we examined (Tables 4, 5), under some conditions considerably exceeding that of hyoscyamine [11]. Their alkaloid spectrum was found to be particularly complex. Cultures of the 'B5' hybrid contain 13 alkaloids present at levels exceeding 1% of the total alkaloid, and no less than 50 components with levels exceeding 0.1% (Table 5, [11] and unpublished observations).

Hyoscyamus

Cultures were obtained from *H. albus*, *H. desertorum*, *H. muticus* and *H. pusillus*. Those from the first two species produced substantial amounts of scopolamine in addition to hyoscyamine, with a combined productivity similar to that observed in *Datura* (Table 4). This is in accord with the presence of significant amounts of scopolamine in the petioles of these two species (Table 2).

Table 3. The hyoscyamine and scopolamine content of transformed root cultures generated from *Datura stramonium* plants showing a range of petiolar alkaloid levels

Petioles Hyoscyamine plus scopolamine ($\mu g\,g^{-1}$ fr. wt)	Number of plants	Cultures	
		Hyoscyamine	Scopolamine ($\mu g\,g^{-1}$ fr. wt)
1001–1350	2	870–1053	0
801–1000	3	887–896	0
600–800	9	617–847	0–trace?
80–200	4	663–901	0–trace?

Values for the root cultures are the sum of intracellular and extracellular alkaloid, and are averaged over two culture cycles.

Table 4. The hyoscyamine and scopolamine content of transformed root cultures from a variety of species

Species	Hyoscyamine	Scopolamine ($\mu g\,g^{-1}$ fr. wt)
Datura stramonium	1053	0
D. ferox	849	43
D. innoxia	486	110
D. wrightii	820	15
(*D. fastuosa*)*	(561)	(9)
(*D. quercifolia*)*	(419)	(trace?)
(*D. sanguinea*)*	(268)	(165)
(*D. candida × aurea*)*	(216)	(276)
Hyoscyamus albus	727	337
H. desertorum	265	160
H. muticus	581	18
Scopolia stramonifolia	56	31

Cultures were generated from plants showing particularly high petiolar alkaloid levels relative to other plants within the individual species studied. Values quoted are the sum of intracellular and extracellular akaloid.

*Cultures obtained from plants of unknown petiolar alkaloid level.

Indeed the culture of *H. desertorum*, which was produced from a plant with a particularly high petiolar scopolamine to hyoscyamine ratio, produced a greater proportion of scopolamine than the culture of *H. albus*, which was derived from a plant with a more intermediate petiolar scopolamine/hyoscyamine ratio. *Hyoscyamus muticus* and *H. pusillus*, our accessions of which had very little petiolar scopolamine, produced only small amounts of scopolamine in culture (Tables 4, 5). It should, however, be noted that one uncloned isolate of *H. muticus*, generated from a plant with an unusually high petiolar scopolamine to hyoscyamine ratio for the accession studied (0.17), initially showed rather slow growth and marked scopolamine accumulation [6]. After a few subcultures—which inevitably involves the selection of root tips showing good morphological characteristics and higher growth rates from the mixed population—the growth rate increased and scopolamine production declined (data not shown). Despite the differences in scopolamine levels, all four *Hyoscyamus* species however showed many similarities in alkaloid production, and clearly differed from *Datura*. For example the variety of tigloyl tropine derivatives typical of *Datura* was absent. *Hyoscyamus* species also contained norhygrine (the *N*-demethylated analogue of hygrine, and conceivably a precursor of the *nor*-tropanes), as well as unusually high proportions of pseudotropine and the derived ester 3-β-acetoxytropane—which in *H. albus* accounted for as much as 62% of the total 3-acetoxytropane pool (Table 5 and unpublished data). The alkaloid profile of *H. pusillus* showed some additional unique features, for example substantial levels of 3-acetoxy and 3-tigloyl nortropanes (Table 5).

Scopolia

Root cultures were obtained from both *S. lurida* and *S. stramonifolia*. Such cultures grew slowly (not shown) and, despite being initiated from plants with high petiolar alkaloid levels, produced little alkaloid (Table 4 and unpublished data). This was somewhat unexpected, as Mano *et al.* [12] obtained hyoscyamine plus scopolamine levels of approximately 0.5% of the dry weight in cultures of *S. japonica*. The poor characteristics of our root cultures suggests that the culture conditions and/or *Agrobacterium* strain used may be less suitable for *Scopolia* than for *Datura* and *Hyoscyamus*.

DISCUSSION

Screening a large number of plants for their aerial tropane alkaloid content and composition has revealed a very considerable amount of variation. Both qualitative and quantitative differences were observed between the genera *Scopolia*, *Hyoscyamus* and *Datura*, although within each genus the petiolar alkaloid patterns observed by HPLC were fairly uniform, the one exception being *D. ceratocaula*—which was clearly different from the other *Datura* species examined. Quantitative differences within a genus were however still marked, and operated at all levels,—i.e. between species, accessions within a species, and individuals within an accession. Differences between individuals proved to be a major source of variation in the absolute levels of alkaloids and, by also taking into account inter-accession variation, up to a 20-fold difference in the petiolar levels of hyoscyamine plus scopolamine could be observed in some species. In terms of

Table 5. The alkaloid composition of transformed roots of a variety of solanaceous species as determined by gas chromatography*

	D. stramonium	D. fastuosa	D. quercifolia	D. ferox	D. innoxia	D. wrightii	D. sanguinea	D. candida × aurea	H. albus	H. desertorum	H. muticus	H. pusillus	S. stramonifolia
Norhygrine†	—	—	—	—	—	—	—	—	4	1	2	5	—
Hygrine	2	1	1	5	5	8	2	5	4	7	2	3	4
Tropinone	—	—	—	—	—	—	—	—	7	—	—	—	—
Tropine	4	1	3	5	2	2	8	3	3	2	3	2	15
Pseudotropine	—	—	—	—	—	—	—	—	4	—	1	—	1
3-Acetoxynortropane‡	—	—	—	—	—	1	—	—	—	—	—	8	—
3-α-Acetoxytropane	1	—	8	1	3	37	2	—	1	1	—	11	—
3-β-Acetoxytropane	—	—	—	—	—	1	—	—	1	—	—	6	—
3-Acetoxy-6-hydroxytropane	—	—	—	—	1	4	—	—	—	—	—	—	—
3-Hydroxy-6-acetoxytropane§	1	—	1	—	—	—	—	—	—	—	—	—	—
3,6-Diacetoxytropane‖	2	2	12	4	—	—	1	—	—	—	—	—	—
3-Tigloyloxynortropane¶	—	—	—	—	—	—	—	—	—	—	—	6	—
3-Tigloyloxytropane	—	—	—	1	—	4	4	2	—	—	—	—	—
Cuscohygrine	—	—	—	1	10	2	1	1	2	—	2	7	1
3-Tigloyloxy-6-hydroxytropane	1	—	—	2	2	4	2	4	—	—	—	—	—
3-Hydroxy-6-tigloyloxytropane	—	—	—	—	4	—	—	2	—	—	—	—	—
3-Tigloyloxy-6-acetoxytropane	—	—	—	—	—	—	—	2	—	—	—	—	—
(M_r) 325**	1	2	—	2	—	—	—	—	—	—	—	—	—
Hyoscyamine	85	82	70	60	55	24	39	26	52	59	81	34	49
3,6-Ditigloyloxytropane	1	—	—	1	1	1	—	1	—	—	—	—	?
Scopolamine	—	1	—	3	10	1	18	25	9	16	2	—	14
3,6-Ditigloyloxy-7-hydroxytropane	2	3	1	4	1	3	—	1	—	—	—	—	?
6-Hydroxyhyoscyamine	—	—	—	3	3	—	—	3	1	1	—	1	—
(M_r) 414††	—	—	—	—	—	—	8	3	—	—	—	—	—

Figures indicate the percentage contribution from each alkaloid at a level of 1% or more of the total alkaloid.

*A small amount of decomposition of tropoyl esters occurs during extraction and chromatography; any of the apo-derivatives detected have been included in with the parent compounds for quantitation, since broadly similar amounts were also seen in chromatograms obtained from the purified parent alkaloids. No values are quoted for the low levels of 3-phenylacetoxytropane and 3-phenylacetoxy-6,7-epoxytropane found since a proportion of these too may be artifactual.

†–†† Mass spectral data for new or unusual alkaloids. GC-MS 70 eV, m/z (rel. int.):

†: 127 (10) 84 (15) 70 (100) 56 (21)

‡: 169 (10) 126 (3) 110 (100) 80 (23)

§: 199 (30) 156 (6) 140 (10) 122 (12) 113 (100) 96 (43)

‖: 241 (30) 198 (5) 182 (24) 155 (13) 138 (11) 122 (58) 94 (100)

¶: 209 (19) 194 (10) 126 (2) 110 (100) 80 (20) Two isomers (α, β?) detected

**: 325 (1) 307 (15) 237 (6) 220 (11) 138 (65) 137 (52) 94 (100)

††: 414 (2) 292 (2) 290 (2) 142 (3) 124 (48) 84 (100)

variation in the ratio of hyoscyamine to scopolamine, differences between species proved to be the dominant factor.

Clearly a large pool of genetic variation exists which can be exploited in the production of tropane alkaloids. It has been found that *Agrobacterium rhizogenes*-transformed root cultures tend to reflect the genetic characteristics of the parent plants [2, 3], so one way potentially to exploit this variation is to develop transformed root lines. Our evidence indicates that substantial variation is indeed carried over to the *in vitro* transformed root system, as for example shown by the characteristic differences in the patterns of tropane alkaloids found in different genera and species. On a quantitative level, the amounts of hyoscyamine and/or scopolamine found in root cultures do not, however, show such marked variation as is observed in the aerial parts of intact plants. While such differences in plants no doubt to some extent reflect the influence of transport and partitioning on alkaloid levels, additional factors probably act to cause the decreased variation seen in root cultures. For instance in many root cultures there is a substantial utilisation of the tropane nucleus in the formation of simple acetoxy esters rather than hyoscyamine or scopolamine. This is particularly true in *D. wrightii* where almost 50% of the root alkaloid is in the form of 3-acetoxy derivatives. A similar accumulation of 3-acetoxytropane is also seen in transformed roots of *Withania somnifera* Dunal (unpublished observations). On the other hand, although small amounts were detected in *D. wrightii* (data not shown), in intact plants acetoxytropanes are rarely observed in substantial amounts (J. G. Woolley, personal communication) and some of the compounds identified in root cultures, e.g. 3,6-diacetoxytropane, have yet to be found [13]. It would

appear that in culture 'excess' tropine is being generated and, rather than being left as the free alcohol, is acetylated. Why it is not esterified to form the normal tropane alkaloids has yet to be elucidated. This effect might occur because sub-culturing disturbs the normal patterns of tropine and esterifying acid production, or perhaps because the presence of the growth medium perturbs metabolite compartmentation by providing an alternative storage pool. In contrast to the situation in many systems [2, 3], in the case of tropane alkaloid production by solanaceous species the conditions in culture may thus sometimes be unfavourable for the full expression of a line's potential biosynthetic capacity. Nevertheless, we have found that many features identified in selected plants are carried over into culture. The initial screening of plant populations, perhaps followed by a further round of screening on the cultures produced, thus appears to be valuable in the generation of scientifically and biotechnologically useful root cultures.

EXPERIMENTAL

Plants. Seeds were obtained from a variety of Botanic Gardens and commercial sources throughout Europe. The following material was investigated: *Datura stramonium* L. (including varieties *stramonium*, *inermis*, *tatula* and *godronii*), *D. innoxia* Mill., *D. ferox* L., *D. fastuosa* L., *D. alba* Nees, *D. metel* L., *D. discolor* Bernh., *D. quercifolia* H.B.K., *D. leichhardtii* F. Muell. ex Benth. subsp. *pruinosa*, *D. sanguinea* Ruiz et Pav., *D. wrightii* Regel, *D. ceratocaula* Ort., *Scopolia stramonifolia* Semenova, *S. lurida* Dunal, *Hyoscyamus aureus* L., *H. albus* L., *H. muticus* L., *H. canariensis* Ker., *H. desertorum* Täckh., *H. turcomanicus* Pojark., *H. pusillus* L. and the hybrid *H × gyoerffyi* Hammer et Melchers. In addition cuttings of the tree *Datura* hybrid *D. candida* Pasq. × *aurea* Lagerh. 'B5' described by El-Dabbas and Evans [10] were also available.

Seeds were treated overnight with 250 mg l^{-1} gibberellin GA$_3$ (Sigma) to facilitate germination, then planted in soil. The resulting plants—10 to 40 per accession—were grown under greenhouse conditions, during June and July. When the fourth true leaf had expanded to equal the size of the third (typically by 3–4 weeks post-germination), this fourth leaf was excised and the alkaloid content of the petiole determined by HPLC.

Root cultures. Surface-sterilized leaves or stems were inoculated with *Agrobacterium rhizogenes* LBA9402, in order to generate transformed root cultures [1]. Such cultures were maintained on a medium based upon Gamborg's B5 nutrients (Flow Labs Inc., Irvine, Scotland) plus 30 g l^{-1} sucrose as described in ref. [14], who also give details of the other culture conditions employed. While roots of most species grew well in media containing full strength salts, *Hyoscyamus albus* and *H. pusillus* required the B5 nutrients at half strength for best growth. Alkaloids were extracted from the roots and culture medium after 30–35 days and prepared for quantitation of hyoscyamine and scopolamine by HPLC. Subsequently a full analysis of tropane alkaloids in these samples was performed by GC and GC/MS.

Analytical methods. Procedures for alkaloid extraction and HPLC analysis were described in ref. [14]. GC and GC-MS analysis were essentially as described in refs [15, 16]. Alkaloids were identified on GC traces principally on the basis of their fragmentation patterns observed in parallel GC-MS runs, but in the case of some more minor components it was sometimes necessary to base identification on similarity of retention times with compounds positively identified in other cultures. Quantitation of alkaloids on GC was possible by assuming all compounds containing one nitrogen atom to respond similarly on the nitrogen detector. Where standards were available to test this assumption, it was found to be substantially correct. It should be noted that because the last step in the extraction of the alkaloids involves a partition into chloroform under alkaline conditions, highly polar alkaloids such as tropine will be slightly under-represented in the final sample. No effort was made to correct for this, because the possibility of artifactual production of tropine by hydrolysis of alkaloid during the extraction process means that there are already other uncertainties in the pool size of this particular alkaloid.

REFERENCES

1. Hamill, J. D., Parr, A. J., Rhodes, M. J. C., Robins, R. J. and Walton, N. J. (1987). *Bio/technology* **5**, 800.
2. Hamill, J. D., Parr, A. J., Rhodes, M. J. C. and Robins, R. J. (1986). *Plant Cell Reports* **5**, 111.
3. Parr, A. J. and Hamill, J. D. (1987). *Phytochemistry* **26**, 3241.
4. Parr, A. J. (1989) *J. Biotechnol.* **10**, 1.
5. Knopp, E., Strauss,. A. and Wehrli, W. (1988). *Plant Cell Reports* 7, 590.
6. Parr, A. J., Hamill, J. D., Payne, J., Rhodes, M. J. C., Robins, R. J. and Walton, N. J. (1988). in *Manipulating Secondary Metabolism in Culture* (Robins, R. J. and Rhodes, M. J. C., (eds), p. 101. Cambridge University Press, Cambridge.
7. Plank, K.-H. and Wagner, K. G. (1986) *Z. Naturforsch.* **41C**, 391.
8. Hashimoto, T. and Yamada, Y. (1986) *Plant Physiol.* **81**, 619.
9. Rhodes, M. J. C., Robins, R. J., Aird, E. L. H., Payne, J., Parr, A. J. and Walton, N. J. (1989) in *Primary and Secondary Metabolism of Plant Cell Cultures*, II (Kurz, W. J. W., ed.), p. 58. Springer, Berlin.
10. El-Dabbas, S. W. and Evans, W. C. (1982) *Planta Med.* **44**, 184.
11. Robins, R. J., Parr, A. J., Payne, J., Walton, N. J. and Rhodes, M. J. C. (1990). *Planta* (in press).
12. Mano, Y, Nabeshima, S., Matsui, C. and Ohkawa, H. (1986) *Agric. Biol. Chem.* **50**, 2715.
13. Lounasmaa, M. (1988) in *The Alkaloids* vol. 33 (Brossi, A., ed.), p. 1. Academic Press, San Diego.
14. Payne, J., Hamill, J. D., Robins, R. J. and Rhodes, M. J. C. (1987) *Planta Med.* **53**, 474.
15. Hartmann, T., Witte, L., Oprach, G. and Toppel, G. (1986) *Planta Med.* 390.
16. Witte, L., Müller, K. and Arfmann, H.-A. (1987) *Planta Med.* **53**, 192.