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INTERGENERIC CO-CULTURE OF GENETICALLY TRANSFORMED ORGANS FOR THE PRODUCTION OF SCOPOLAMINE

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Key Word Index—*Atropa belladonna*; *Duboisia* hybrid; Solanaceae; plant tissue culture; organ co-culture; transformed roots; shooty teratomas; alkaloids; scopolamine; hyoscyamine.

Abstract—This work demonstrates the effectiveness of interspecies and intergenus organ co-culture for the production of plant secondary metabolites. Co-cultures of *Atropa belladonna* transformed roots and *Duboisia* hybrid shooty teratomas produced significant levels of scopolamine, whereas no scopolamine could be detected in separate root and shoot cultures. Maximum scopolamine levels found in co-cultured shoots were 4.8 mg g^{-1} dry weight in a 2×2.5-l dual bioreactor system, and 3.7 ± 0.9 mg g^{-1} in dual 1-l shake flasks. Periodic crushing of the roots in shake flasks was effective in releasing hyoscyamine, but impaired growth and overall alkaloid synthesis. Less severe root damaging in the bioreactor was more successful in improving scopolamine accumulation. Uptake of hyoscyamine by shooty teratomas was inhibited at high hyoscyamine concentrations, was reduced in the presence of ATPase inhibitors, and could be significantly enhanced by pre-incubating the shoots on medium containing hyoscyamine. © 1997 Elsevier Science Ltd

INTRODUCTION

Transformed roots induced by infection of plants with *Agrobacterium rhizogenes* have been investigated for several years as a means of producing plant secondary metabolites *in vitro*. The range of metabolites synthesized by transformed roots is usually very similar to that synthesized by roots of the parent plant [1], although absence of secondary growth in cultured roots may create some differences in relative product levels [2]. Thus, a potential limitation in application of transformed roots is that metabolites formed in the aerial tissues of plants cannot be produced [3, 4]. In recent years, shooty teratomas of several plant species have been developed using particular strains of *A. tumefaciens* [5–13]. Like transformed roots, shooty teratomas are autotrophic in plant growth regulators. Several studies have indicated that the range of secondary metabolites synthesized by shooty teratomas is generally limited to those produced by shoots of the whole plant. For example, *A. belladonna* and *Duboisia* hybrid shooty teratomas do not synthesize significant levels of hyoscyamine [7] which is known to be formed in roots [14, 15]; similarly, nicotine, which is associated with the roots of tobacco [15], is not produced by *N. tabacum* shooty teratomas [5].

In plants, complete synthesis of many substances requires the participation of both the roots and the shoots. A precursor might be produced in the roots then translocated to the leaves for conversion to another compound or, *vice versa*, precursors may be transported from the shoots to the roots for further reaction. In such cases, if enzyme expression remains organ-specific in tissue culture, neither transformed roots nor shooty teratomas by themselves would be capable of synthesizing the final product. Recently, a strategy with potential for large-scale application was developed to overcome this problem using transformed roots and shooty teratomas co-cultured in the same medium [16]. Co-culture provides the opportunity for metabolites produced by one organ to be excreted into the medium and taken up by the other organ for further biochemical conversion. The availability of plant organs transformed with agrobacteria makes co-culture feasible, as transformed roots and shooty teratomas do not need different exogenous hormones and often share the same medium requirements. Although genetic engineering could be used to provide plant tissues with enzymes they do not otherwise possess, co-culture offers significant advantages over molecular methods if the conversion requires multiple enzymes or if the pathway is highly branched or regulated. Another alternative is *in vitro* culture of whole plants; e.g. some transformed root cultures have been found to spontaneously produce shoots [17]. However, the ease with which plants can be regenerated from these organs varies considerably;

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regeneration ability has been demonstrated for only *ca* 20% of transformed root species [17], while the regeneration capacity of shooty teratomas is at present largely unknown. Co-culture overcomes all of these potential difficulties and maintains other advantages, such as autotrophy in exogenous hormones and rapid growth, which may not apply to *in vitro* culture of whole plants [18].

Application of root–shoot co-culture is extended in this work to intergeneric systems in which the roots and shoots are of different plant species. The biotransformation investigated is the conversion of hyoscyamine to scopolamine. Scopolamine produces fewer side effects than hyoscyamine in medical applications and its market value is greater [19]. In scopolamine-producing plants, hyoscyamine is synthesized in the roots, then converted to scopolamine by the enzyme hyoscyamine 6β-hydroxylase (H6H) via a single intermediate, 6β-hydroxyhyoscyamine [20]. H6H has been localized in the pericycle of several species of cultured root [2]; however, the level of H6H activity in roots varies markedly between species and in many scopolamine-producing plants the principal site of scopolamine conversion is the leaves [4, 21]. Therefore, the need for co-culture of roots and shoots applies to scopolamine production to some extent, but is species dependent. As shooty teratomas of only *ca* 10 plant species have been developed so far, thus limiting the range of co-cultures that can be tested at present, the hyoscyamine–scopolamine conversion was adopted as a model system in this work for investigation of co-culture properties and performance.

In this study, *A. belladonna* transformed roots and *Duboisia* hybrid shooty teratomas were co-cultured in dual shake flasks and bioreactors for the production of scopolamine. As well as the co-culture itself, the individual processes considered necessary for co-culture to be successful, such as hyoscyamine release from the transformed roots and hyoscyamine uptake by the shoots, were investigated separately to test their effectiveness and to study the factors affecting their kinetics under various culture conditions.

RESULTS AND DISCUSSION

Hyoscyamine release from transformed roots by physical damage

Transformed roots of *A. belladonna* are known to produce high levels of hyoscyamine similar to roots of the whole plant but, at most, only a small fraction is excreted into the medium [22, 23]. Attempts in previous work to promote hyoscyamine release using medium additives such as Pluronic F-68 and copper sulphate and manipulating the medium pH were unsuccessful [16]. Periodic crushing of the roots in shake flasks considerably increased the concentration of hyoscyamine in the medium compared with the control culture without damage (Fig. 1); however, there was relatively little advantage in crushing the roots every day compared with every 2 or 3 days. Damaging released a relatively large proportion of the hyoscyamine produced: after 12 days treatment, 58, 50 and 43%, respectively, of the total hyoscyamine present in the cultures damaged every 1, 2 or 3 days, was found in the medium, compared with *ca* 1% in the controls. Although effective in promoting the release of hyoscyamine, damaging reduced root growth and hyoscyamine synthesis (Fig. 2). The more frequent the damaging, the lower was the biomass and total hyoscyamine present in the cultures.

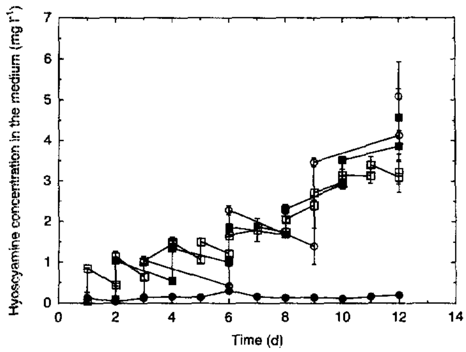

Fig. 1. Effect of physically damaging *A. belladonna* transformed roots in shake flasks on the release of hyoscyamine into the culture medium. The damaging treatments were started on Day 0, 7 days after inoculation. ●, Control without damage; □, damaged every day; ■, damaged every 2 days; ○, damaged every 3 days. Samples were taken immediately before and after damage, giving two measurements for each treatment at each time. The lower value is the measurement before damaging; the upper value was obtained after damaging. Error bars represent standard errors from triplicate flasks.

Uptake and biotransformation of exogenous hyoscyamine by shooty teratomas

Untransformed and transformed shoots of the *Duboisia* hybrid used in this work have been shown in

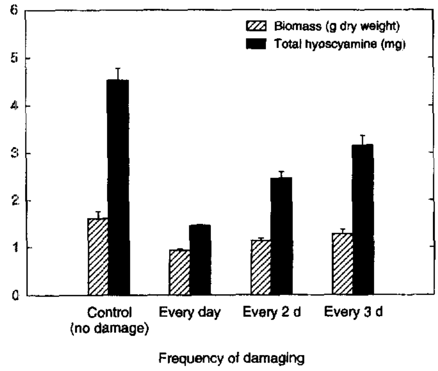

Fig. 2. Effect of damaging frequency on growth of *A. belladonna* transformed roots and total hyoscyamine production. The data were measured 12 days after beginning the damage treatments. Damaging had a detrimental effect on root growth and overall hyoscyamine production. Error bars represent standard errors from triplicate flasks.

other studies to take up exogenous hyoscyamine [16, 24]; however, the uptake kinetics and transport mechanism involved were not previously investigated. Hyoscyamine added to Murashige–Skoog medium in shake flasks without shoots was stable, the concentration remaining constant within 2% during incubation for 28 days at 25° as verified by HPLC analysis. In the presence of shooty teratomas, the amount of hyoscyamine removed from the medium was assumed to be equal to the amount taken up by the shoots. Shoot growth did not vary significantly at different exogenous hyoscyamine levels. The initial rate of hyoscyamine uptake expressed on a dry weight basis increased with exogenous concentration up to *ca* 300 mg l^{-1} [Fig. 3(a)]; above this concentration the uptake rate decreased, indicating substrate inhibition of hyoscyamine uptake at high concentrations. In each experiment, hyoscyamine partitioned between the medium and biomass to produce an equilibrium specific hyoscyamine concentration in the medium after 1–3 days which was directly proportional to the initial hyoscyamine concentration [Fig. 3(b)].

Addition of the ATPase inhibitor, diethylstilbestrol, to the medium reduced the amount of exogenous hyoscyamine taken up by the shooty teratomas (Fig. 4). Similar results have been reported for uptake of berberine by cultured cells of *Coptis japonica*, *Datura innoxia* and *Thalictrum flavum* [25, 26], uptake of vindoline by vacuoles of *Catharanthus roseus* [27], and uptake of (*S*)-reticuline by *Fumaria capreolata* vacuoles [28]. Substrate inhibition kinetics and sensitivity to ATPase inhibitor suggest that hyoscyamine uptake by *Duboisia* hybrid shooty teratomas is an enzyme-mediated, energy-requiring process.

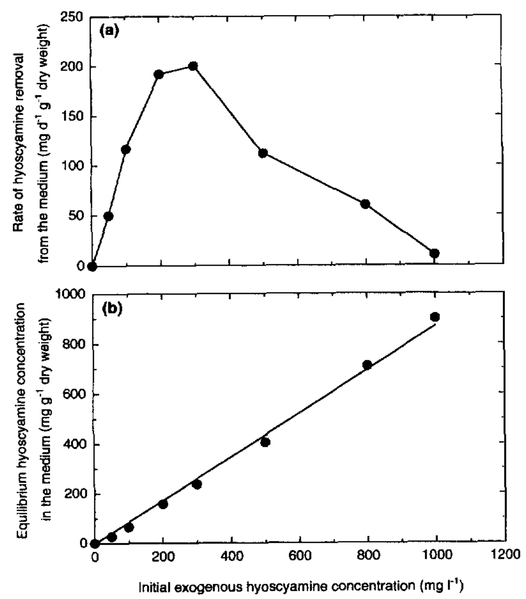

Fig. 3. (a) Initial specific rates of hyoscyamine removal from the medium by *Duboisia* hybrid shooty teratomas at different exogenous hyoscyamine concentrations. Hyoscyamine uptake was inhibited at hyoscyamine concentrations above *ca* 300 mg l^{-1}. (b) Relationship between the equilibrium specific exogenous hyoscyamine concentration in the medium and the initial exogenous hyoscyamine concentration provided to *Duboisia* hybrid shooty teratomas.

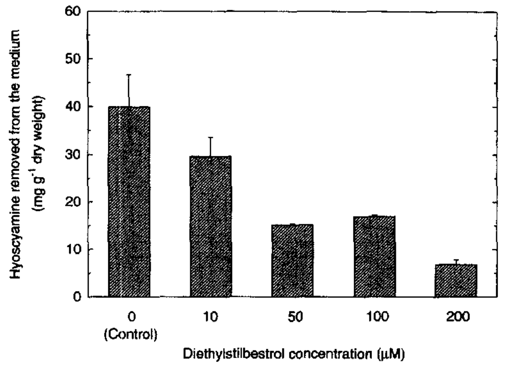

Fig. 4. Effect of the ATPase inhibitor, diethylstilbestrol, on removal of hyoscyamine from the medium by *Duboisia* hybrid shooty teratomas. The data were measured 2 days after addition of 200 mg l^{-1} hyoscyamine to the cultures. Error bars represent standard errors from triplicate flasks.

Growth of shooty teratomas in shake flasks with exogenous hyoscyamine was not affected by pre-incubating the shoots on solid medium containing 100 mg l^{-1} hyoscyamine [Fig. 5(a)]. The initial rate of hyoscyamine removal from the medium in the flasks was considerably higher in the pre-incubated cultures compared with the control [Fig. 5(b)]; however, as the biomass grew, the specific levels of hyoscyamine uptake in the two cultures became virtually identical. Of the 60 mg hyoscyamine initially provided in the medium, all but less than 1 mg was taken up by the shoots within *ca* 28 days. Pre-incubation produced no significant benefit in terms of biotransformation ability; scopolamine levels were greater in the control cultures during most of the monitoring period. The presence of scopolamine in the shoots after 35 days culture was verified by GC mass spectrometry. The average final yield of scopolamine from added hyoscyamine was *ca* 2.7% (w/w) in both cultures; this can be compared with 4.9–10% from hyoscyamine feeding experiments with regenerating shoot callus of the same *Duboisia* hybrid [24], 2.4–4.1% from shooty callus of *D. leichhardtii* [29], and 0.12–0.25% from shooty teratomas of *Atropa belladonna* [6, 16] fed with exogenous hyoscyamine. Despite very high levels, i.e *ca* 300 mg g^{-1} dry weight, of hyoscyamine being present in the pre-incubated shoots after only 2 days, the concentration of scopolamine in these tissues remained very low at less than 1 mg g^{-1} throughout the culture period. The *Duboisia* hybrid shooty teratomas tended to vitrify and some started forming callus after *ca* 14 days in liquid medium. Because of this, shooty teratomas in replicate flasks were in different stages of de-differentiation, thus producing the observed scatter in the data particularly towards the end of the culture period.

As scopolamine production was not improved even though the rate of hyoscyamine uptake and levels of hyoscyamine in the shoots were much greater after

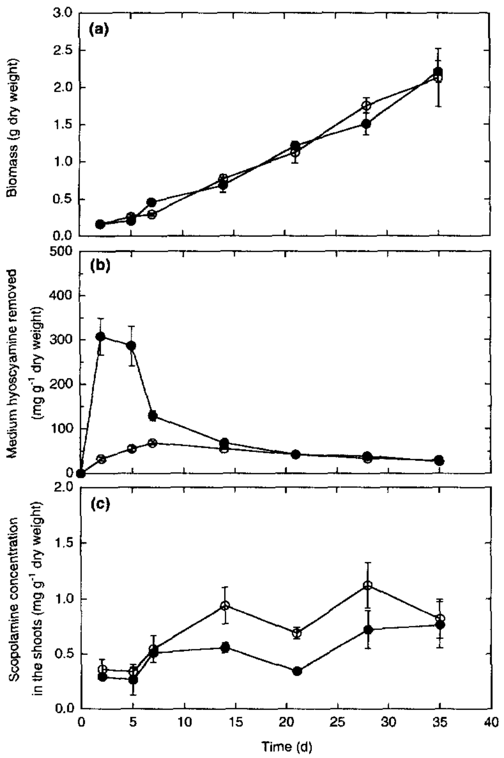

Fig. 5. Effect of pre-incubating *Duboisia* hybrid shooty teratomas with hyoscyamine prior to exposure to 200 mg l^{-1} exogenous hyoscyamine in shake flasks on Day 0. ●, Shoots pre-incubated with hyoscyamine; ○, control shoots pre-incubated without hyoscyamine. Error bars represent standard errors from triplicate flasks. (a) Shoot growth in an initial liquid volume of 300 ml. Pre-incubation with hyoscyamine did not affect growth. (b) Removal of hyoscyamine from the medium. Pre-incubation with hyoscyamine significantly increased the initial specific rate of hyoscyamine uptake. (c) Accumulation of scopolamine in the shoots. Pre-incubation with hyoscyamine produced no significant improvement in scopolamine production.

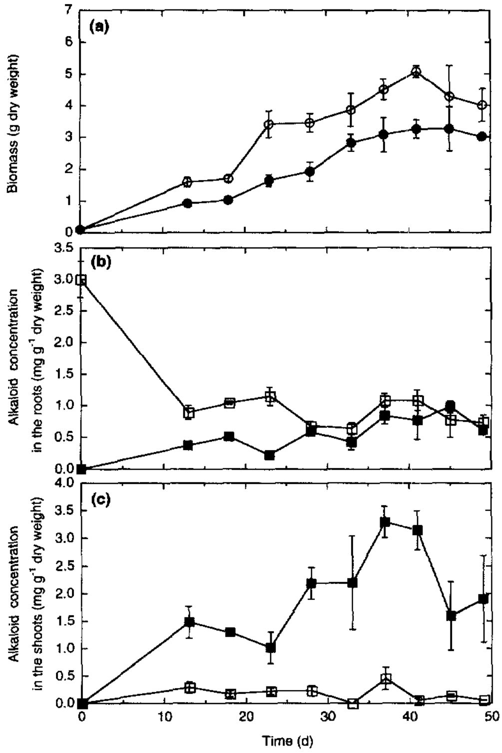

Fig. 6. Results from co-culture of *A. belladonna* transformed roots and *Duboisia* hybrid shooty teratomas in dual 1-l shake flasks. Error bars represent standard errors from triplicate flasks. (a) Growth of roots (○) and shoots (●); (b) concentrations of hyoscyamine (□) and scopolamine (■) in the transformed roots; (c) concentrations of hyoscyamine (□) and scopolamine (■) in the shooty teratomas.

pre-incubation, the results indicate that the hyoscyamine–scopolamine biotransformation is relatively slow and could be the rate-limiting process in co-culture, and that the H6H enzyme cannot be induced by prior exposure to hyoscyamine. However, it is also possible that the very high levels of intracellular hyoscyamine present in the pre-incubated shoots [Fig. 5(b)] inhibited H6H activity in this experiment.

Co-culture in dual shake flasks with and without root damage

A. belladonna transformed roots in dual-flask co-culture grew faster than the *Duboisia* hybrid shooty teratomas [Fig. 6(a)]. Levels of hyoscyamine in co-cultured transformed roots were reduced to *ca* one-third the value in roots cultured without shoots, i.e. the inoculum roots [Fig. 6(b)]; the average hyoscyamine concentration in the roots between Days 13 and 49 was 0.9±0.1 mg g^{-1} dry weight. The transformed roots also contained low levels of scopolamine, less than 1 mg g^{-1}. Accumulation of scopolamine in the shoots reached a maximum level of 3.3±0.3 mg g^{-1} after 37 days co-culture [Fig. 6(c)]. The concentration of hyoscyamine in the medium varied after Day 0 within a relatively narrow range around an average of 0.56 mg l^{-1}; no extracellular scopolamine was detected.

Results for growth and alkaloid production after 49 days co-culture in dual shake flasks are summarized in Table 1 for comparison with separate cultures of transformed roots and shooty teratomas in single flasks. Shooty teratomas cultured without roots produced no detectable scopolamine as confirmed by GC mass spectrometry. Lower hyoscyamine levels but significant amounts of scopolamine were found in co-culture compared with the separate root and shoot cultures, which is consistent with a greater conversion of hyoscyamine to scopolamine in the co-culture system. After 49 days, shooty teratomas grown in single flasks and in co-culture displayed a high level of callusing and production of suspended cells.

We consider the most likely explanation for scopolamine synthesis in the co-cultures is that hyoscyamine synthesized by the roots was released into the medium and taken up by the shoots, where it was enzymatically converted to scopolamine. This

Table 1. Growth and alkaloid data from separate root and shoot cultures and co-cultures in shake flasks. The data were measured 49 days after inoculation. ± represents standard error from triplicate flasks

	Single flask culture (controls)		Co-culture	
Parameter measured	Transformed roots	Shooty teratomas	Transformed roots	Shooty teratomas
Biomass (g dry wt)	3.0±0.2	3.4±0.7	4.0±0.5	3.0±0.02
Hyoscyamine content (mg g^{-1} dry wt)	1.1±0.03	Trace	0.7±0.1	0.07±0.0
Scopolamine content (mg g^{-1} dry wt)	None detected	None detected	0.6±0.2	1.9±0.8
Hyoscyamine concentration in the medium (mg l^{-1})	2.6±0.9	None detected	0.62±0.36	
Scopolamine concentration in the medium (mg l^{-1})	None detected	None detected	None detected	
Total hyoscyamine (mg)	3.6	Trace	3.1	
Total scopolamine (mg)	None detected	None detected	8.1	

interpretation is based on observations in this and previous work that the *A. belladonna* root line used in this study does not produce detectable levels of scopolamine [16, 22], and that shoots of *Duboisia* spp. are able to produce scopolamine after taking up exogenous hyoscyamine [16, 24, 29]. Yet, the possibility that co-culture with *Duboisia* shoots enhanced by some unknown mechanism the activity of endogenous H6H in the *A. belladonna* transformed roots cannot be entirely disregarded without further investigation. Indeed, co-cultured roots were found to accumulate low concentrations of scopolamine whereas single-organ root cultures did not [Table 1; Figs 6(b) and 9(a)], although the low levels of scopolamine measured were near the detection limit of the analytical procedure used and could have been released from the shoots and taken up from the medium. Levels of scopolamine in co-cultured shoots were always much greater than in the co-cultured roots.

As indicated in Fig. 6(c), high scopolamine concentrations in the shoots were produced when the corresponding hyoscyamine level was very low (less than 0.5 mg g^{-1}). This is consistent with the results of previous studies [16], which showed that co-culture of roots and shoots providing continuous low levels of hyoscyamine to the shoots allowed accumulation of higher levels of scopolamine than the feeding of high exogenous hyoscyamine concentrations. Less efficient scopolamine conversion in the presence of large amounts of hyoscyamine has also been found with recombinant *Escherichia coli* expressing the H6H gene from *Hyoscyamus niger*; similarly, scopolamine synthesis was more effective in transgenic tobacco leaves when the supply of hyoscyamine was limited by vascular transport from the root [30].

The maximum level of scopolamine in the shooty teratomas was reached after 37 days culture [Fig. 6(c)], even though concentrations of hyoscyamine in the roots, medium and shoots remained roughly constant after *ca* Day 13. This is the reason the co-cultures were continued beyond 14–20 days despite callusing and deterioration of the shoots. Delays of several weeks before the appearance of scopolamine have also been reported for *Anisodus tanguticus* plant cell suspensions supplied with 100 mg l^{-1} exogenous atropine (D,L-hyoscyamine), although the intermediate, 6β-hydroxyhyoscyamine, was detected 1–2 weeks before scopolamine [31].

Physical damage of the roots in co-culture had little effect on the levels of root cell biomass [Fig. 7(a)], although, after *ca* 20 days, much of the damaged biomass in the root flasks was in the form of suspended cells. The tendency of *Duboisia* hybrid shoots to vitrify and produce callus in liquid medium was exacerbated when root damaging was carried out, possibly due to the release of plant growth regulators or other substances from the roots that induced shoot de-

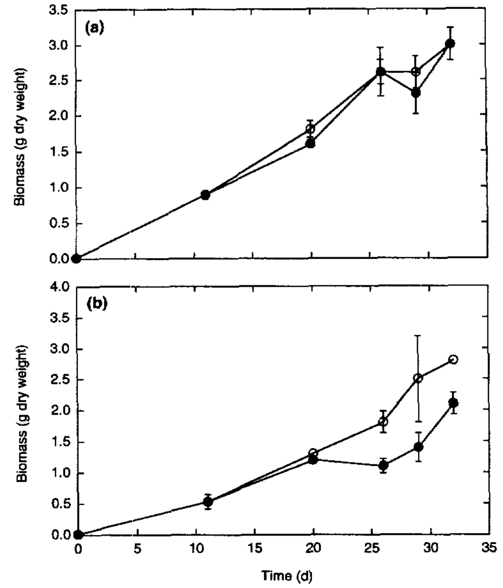

Fig. 7. Growth of (a) *A. belladonna* transformed roots, and (b) *Duboisia* hybrid shooty teratomas, in co-culture with (●) and without (○) damaging of the roots every 3 days. The damage treatment was started on Day 11. Error bars represent standard errors from triplicate flasks.

differentiation. Growth of shooty teratomas in the co-cultures with damaged roots was reduced after *ca* 20 days compared with the undamaged controls [Fig. 7(b)]. At this time, a considerable fraction of the biomass in all the shoot flasks had de-differentiated into callus and cell suspensions; the extent of shoot de-differentiation was more severe in the cultures with root damage. Alkaloid concentrations in the roots and shoots were measured after harvesting all the differentiated and undifferentiated cells from the respective flasks, so that the results represent an average from both the organized and unorganized tissues. After the start of damaging on Day 11, undamaged roots maintained higher levels of intracellular hyoscyamine than the damaged roots [Fig. 8(a)]; correspondingly, the concentration of medium hyoscyamine in damaged cultures was significantly greater than in the controls [Fig. 8(c)]. Roots in both co-cultures accumulated lower hyoscyamine levels compared with the inoculum roots cultured without shooty teratomas. There was little difference in hyoscyamine levels in shooty teratomas co-cultured with and without root damage [Fig. 8(b)]. Scopolamine was detected in the co-cultured roots [Fig. 9(a)], but remained at relatively low levels (less than 1 mg g^{-1} dry wt). Scopolamine concentration in the co-cultured shoots with root damage declined progressively after the start of damaging from a maximum of 3.1 mg g^{-1} on Day 11 [Fig. 9(b)]. In contrast, in the control cultures without damage, average scopolamine levels increased to 3.7 mg g^{-1} after 35 days, although replicates towards the end of the culture showed a high degree of scatter because of callusing. Taking into account growth and the scopolamine content in the biomass, the average final amount of scopolamine in the co-cultures without root damage (8.0 mg) was twice that in the co-cultures with damage (4.0 mg). No scopolamine was detected in the medium in either co-culture.

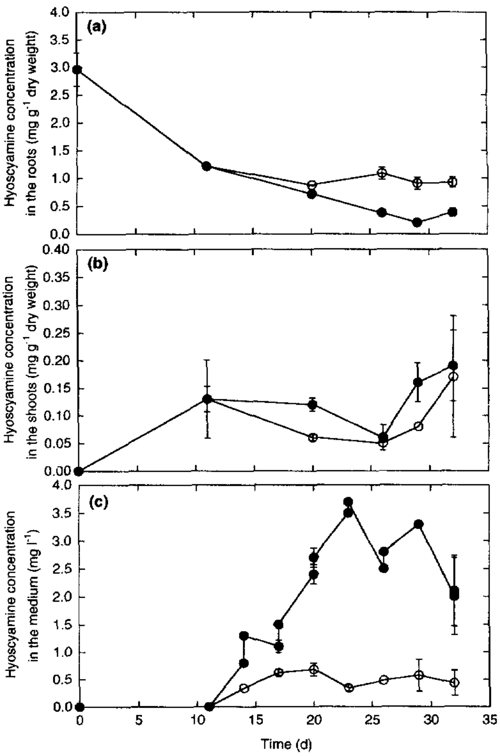

Fig. 8. (a) Hyoscyamine levels in *A. belladonna* transformed roots, (b) hyoscyamine levels in *Duboisia* hybrid shooty teratomas, and (c) hyoscyamine concentrations in the medium, during co-culture with (●) and without (○) root damage. The damaging treatment was started on Day 11 and performed every 3 days. Medium samples were taken before and after damage, giving two measurements in (c) at each time. The lower value is the measurement before damaging; the upper value was obtained after damaging. Error bars represent standard errors from triplicate flasks.

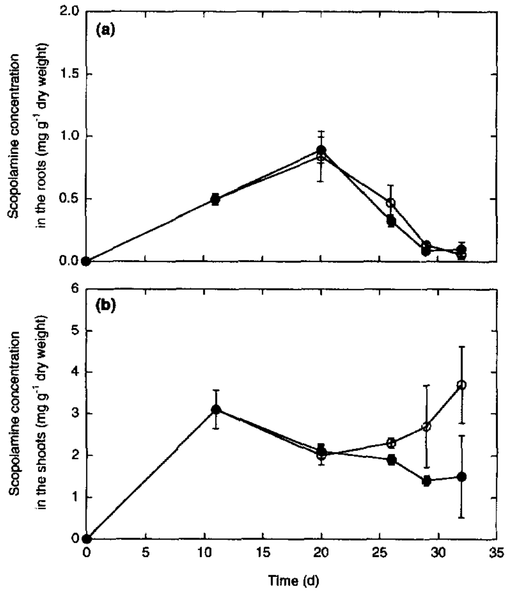

Fig. 9. Scopolamine levels in (a) *A. belladonna* transformed roots, and (b) *Duboisia* hybrid shooty teratomas, during co-culture with (●) and without (○) root damage. The damaging treatment was started on Day 11 and performed every 3 days. Error bars represent standard errors from triplicate flasks.

The maximum scopolamine contents in co-cultured shooty teratomas measured in this work correspond to 24–31% those found in leaves of the whole plant [32]. However, de-differentiation of the shoots is likely to have had a pronounced negative effect on alkaloid accumulation in co-culture; tropane alkaloid synthesis has been found in many studies to be confined to differentiated organs [29, 33–35]. Manipulation of the medium composition and/or culture conditions is needed to reduce callusing and vitrification. As well as raising scopolamine levels, an improvement in the reliability of the experimental results could also be expected. The maximum scopolamine contents in the co-cultured *Duboisia* hybrid shoots were 4.4–5.7 times higher than the maximum of 0.84 ± 0.08 mg g^{-1} measured previously in *A. belladonna* shooty teratomas after 28 days co-culture with *A. belladonna* roots [16].

Table 2. Growth and alkaloid data from dual-bioreactor co-cultures, 29 days after inoculation

	Without root damage (control)		With root damage	
Parameter measured	Transformed roots	Shooty teratomas	Transformed roots	Shooty teratomas
Biomass (g dry wt)	19	7.1	8.9	16
Hyoscyamine content (mg g^{-1} dry wt)	3.1	None detected	2.4	None detected
Scopolamine content (mg g^{-1} dry wt)	0.21	1.5	0.25	4.8
Hyoscyamine concentration in the medium (mg l^{-1})	0.62		3.5	
Scopolamine concentration in the medium (mg l^{-1})	None detected		None detected	
Total hyoscyamine (mg)	61		30	
Total scopolamine (mg)	15		80	

This result reflects the scopolamine-rich nature of *Duboisia* species compared with *Atropa*, and demonstrates the ability of interspecies root–shoot co-culture to broaden the range of alkaloid productivities attainable *in vitro*.

Co-culture in dual bioreactors with and without root damage

Damaging of roots in the dual bioreactor experiment was less severe than in the shake flasks and caused little root de-differentiation; the impeller usually only clipped the edges of the mass of roots growing in the vessel. The co-culture without root damage produced more root biomass than with damage (Table 2), while the mass of shoots was greater in the damaged system. The total amount of scopolamine produced with damaging of the roots was 5.4 times greater than in the control culture; the scopolamine content in the shoots with damage was also greater by a factor of 3.2. These results indicate that the dual bioreactor system was effective for production and conversion of hyoscyamine to scopolamine, and that damaging the roots by periodic operation of the reactor impeller improved overall scopolamine production. In contrast with the results from the shake-flask cultures, mild damaging of the roots to release hyoscyamine in the reactor was beneficial for the biotransformation of hyoscyamine to scopolamine, indicating that damaging intensity is an important factor affecting the overall success of this procedure.

The present work represents the first attempt at interspecies and intergenus co-culture, and demonstrates the effectiveness of such systems for the production of scopolamine. Interspecies co-culture has the potential to increase considerably the range of products that can be synthesized using plant tissue culture. By analogy with grafting, it may also allow production *in vitro* of completely new compounds which are not found *in vivo* because of the limited opportunity for the exchange of metabolites between individual whole plants.

EXPERIMENTAL

Plant materials and culture maintenance. Initiation and maintenance of *Duboisia* hybrid shooty teratomas and *A. belladonna* transformed roots are described elsewhere [7, 22]. Murashige–Skoog (MS) medium (ICN, USA) was used with 3% sucrose and pH adjusted to 6.0 before autoclaving.

Hyoscyamine release from transformed roots by physical damage. Roots (1.0 g fr. wt) were inoculated into 300 ml medium in 1-l conical flasks and incubated in the dark at 25° on an orbital shaker at 100 rpm. Starting 7 days after inoculation, the roots were damaged by lightly crushing them × 80 with a metal rod (contact surface diameter 2.5 cm). This treatment was performed once every day, every 2 days or every 3 days over a period of 12 days; no damaging was carried out in the control cultures. Liquid samples were taken before and after each damaging treatment for hyoscyamine determination; control culture samples were taken every day. Roots harvested at the end of the experiment were analysed for biomass dry wt and hyoscyamine content.

Effect of exogenous hyoscyamine concentration on hyoscyamine uptake by shooty teratomas. Hyoscyamine was added to liquid Murashige–Skoog medium to produce concs between 0 and 1000 mg l^{-1}. The pH was adjusted to 6.0 and the media filter-sterilized. Shooty teratomas (0.3 g fr. wt) were inoculated into 50 ml medium in 250-ml conical flasks and incubated at 25° and 100 rpm under continuous 1500–2000 lux fluorescent light. Liquid samples were removed periodically over 5 days for hyoscyamine determination; flasks were also harvested daily to determine the biomass dry wt and liquid vol.

Effect of ATPase inhibitor on hyoscyamine uptake by shooty teratomas. Shooty teratomas (0.3 g fr. wt) were inoculated into 50 ml medium in 250-ml shake flasks containing 0–200 μM diethylstilbestrol [25]. The shoots were incubated at 25° and 100 rpm under continuous fluorescent light for 24 hr prior to addition of 1 ml filter-sterilized hyoscyamine soln to give a conc. of 200 mg l^{-1}. After a further 2 days, the flasks were harvested for analysis of shoot biomass, residual hyoscyamine conc in the medium, and liquid vol.

Uptake and biotransformation of exogenous hyoscyamine by shooty teratomas after pre-incubation with hyoscyamine. In an attempt to induce earlier expression of enzymes for uptake and/or bioconversion of hyoscyamine, *Duboisia* hybrid shooty teratomas were grown on solid Murashige–Skoog medium containing 100 mg l^{-1} hyoscyamine for 21 days prior to uptake and biotransformation experiments in liquid medium. Shoots (1.0 g fr. wt) pre-incubated in this way were inoculated into 1-l conical flasks containing 300 ml medium and 200 mg l^{-1} hyoscyamine and incubated at 25° and 100 rpm under continuous light. Flasks were harvested periodically over 35 days for analysis of hyoscyamine in the medium, shoot dry wt and scopolamine content, and liquid vol. Control cultures were carried out using shoots pre-incubated on solid Murashige–Skoog medium without added hyoscyamine. To confirm the presence of scopolamine, biomass samples harvested from the control cultures after 35 days were analysed by GC-MS.

Co-culture of transformed roots and shooty teratomas in shake flasks. Dual 1-l shake flasks were used as described previously [16]. One flask was inoculated with 1.0 g fr. wt roots; the other was inoculated with 1.0 g fr. wt shoots. The flasks incubated at 25° and 100 rpm under continuous light were harvested periodically over 49 days for analysis of growth and hyoscyamine and scopolamine contents. Control cultures of roots and shoots grown separately in single 1-l flasks were also carried out. Shoots from the control culture after 49 days were analysed for scopolamine by GC-MS.

Effect of root damage on co-culture in shake flasks. A time-course co-culture experiment was carried out in dual shake flasks using the inoculation and incubation procedures described above. Root damaging began after 11 days and was performed once every 3 days for a period of 21 days using the procedures already described. A control co-culture was carried out without damage. Before and after each damaging treatment, liquid samples were removed for hyoscyamine analysis. Flasks were harvested on Day 11 (i.e. before the first damage), then periodically over the next 21 days for the measurement of root and shoot dry wts, hyoscyamine and scopolamine contents in the biomass, and liquid vol.

Co-culture in dual bioreactors. The dual bioreactor system used for co-culture of roots and shoots consisted of 2 × 2.5-l flat-bottom bioreactors. A Rushton turbine impeller of diameter 50 mm was fitted 45 mm above the floor of the root reactor; operation of the impellor was used to periodically damage the roots. The two bioreactors were connected with silicone tubing; interchange of medium between the vessels was effected using peristaltic pumps operated for 4 hr each day at a flow rate of 36 ml min^{-1}. A stainless steel mesh was fixed to the outlet of each bioreactor to prevent transfer of tissues between the vessels. Both reactors were sparged with air at a flow rate of 400 ml min^{-1} using air pumps and porous steel spargers. Medium in the shoot bioreactor was mixed using a magnetic stirrer. The bioreactors were incubated under fluorescent light (1500–2000 lux) in a clean room at *ca* 25°. Each bioreactor initially contained 2 l medium was inoculated with either 7.0 g fr. wt shoots or 7.0 g fr. wt roots. Roots in the root reactor were first damaged 11 days after inoculation by operating the impeller at 80 rpm for 3 min; thereafter, the roots were damaged every 3 days. After 29 days, the roots and shoots were harvested and analysed for alkaloid content and dry wt; extracellular alkaloids and the residual liquid vol. were also measured. A separate 29-day control experiment was carried out using the dual bioreactor system without root damage.

Biomass analysis. Dry wt was determined by freeze-drying filtered biomass at −30° and 0.5 mbar to constant wt.

Alkaloid analysis. Hyoscyamine and scopolamine were quantified using HPLC with an Alltima C8 column (5 μm, 250 mm × 4.6 mm) at ambient temp. with UV detection at 205 nm. The mobile phase was MeOH–H_2O (21:29) containing 0.005 M low-UV PIC-B7 (heptane sulphonic acid; pH 4.1) at a flow rate of 1.00 ml min^{-1}. Samples of liquid medium were analysed directly after dilution with HPLC solvent. For analysis of alkaloids in the biomass, dried tissues were ground to a powder using a mortar and pestle or grinder, and extracted × 2 using basic EtOH (19:1 EtOH–28% NH_4OH) with sonication. The supernatants were pooled and evapd to dryness in a fume hood. The residue was redissolved in HPLC solvent and analysed as described above. Hyoscyamine and its racemic mixt., atropine, cannot be distinguished by HPLC; in this work the results are referred to as hyoscyamine.

GC-MS. Me_2CO extracts of shooty teratomas were analysed using a 25 m × 0.1 mm HP5 column operated with He at a flow rate of *ca* 1 ml min^{-1}. The mass spectrometer was operated in full scan mode across the mass range 50–500 amu (Da). The temp. programme used was as follows: initial temp of 50° held for 1 min, ramped to 220° at 50° min^{-1}, ramped to 300° at 20° min^{-1}, then held for 5 min. The injection port temp. was 280°.

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