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Effect of *Agrobacterium rhizogenes* T-DNA on alkaloid production in *Solanaceae* plants

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Abstract

Inoculation of leaf sections of tobacco, *Duboisia* hybrid and *Datura metel Solanaceae* plants with A4 strain of *Agrobacterium rhizogenes*, induced transformed roots with the capacity to produce putrescine-derived alkaloids. In general, the hairy roots obtained showed two morphologies: typical hairy roots with high capacity to produce alkaloids and callus-like roots with faster growth capacity and lower alkaloid production. The *aux*1 gene of *A. rhizogenes* was detected by PCR analyses in all roots showing callus-like morphology. However, this gene was only detected in 25–60% of the root cultures established showing typical hairy morphology. This fact suggests a significant role of *aux* genes in the morphology of transformed roots. Inoculation of leaf sections with *A. tumefaciens* strain C58 C1 carrying the pRiA4TR- (deletion of *aux* genes) did not produce roots with callus-like morphology. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Agrobacterium rhizogenes*; *Datura metel*; *Duboisia* hybrid; *Nicotiana tabacum*; Transformed roots; Tobacco alkaloids; Tropane alkaloids

1. Introduction

Hairy root system arising from inoculation with *Agrobacterium rhizogenes* became popular in the last decade as a system to produce secondary metabolites synthesized in plant roots (Toivonen, 1993; Palazón, Piñol, Cusidó, Morales & Bonfill, 1997b). However, unorganized plant tissue cultures are frequently not able to produce secondary metabolites at the same level as the intact plant. This is the case of tropane and tobacco alkaloids, which have as a common precursor the diamine putrescine (Rhodes, Robins, Hamill, Parr, Hilton & Walton, 1990), and the first steps of their biosynthesis take place in the plant roots.

Hairy roots result from the transfer of genes located on the root-inducing plasmid Ri to plant cells and their expression therein (White & Nester, 1980). Two sets of pRi genes are involved in the root induction process: *aux* genes located in the TR region and the *rol* (root *loci*) genes of TL region (Jouanin, 1984). The *ags* genes responsible for opine biosynthesis in the transformed tissues are also located in the TR region (Binns & Tomashow, 1988). Opines are synthesized by plant transformed cells and are only used by *Agrobacterium* as a source of nitrogen and carbon.

Because of the similarities of the *A. rhizogenes* and *A. tumefaciens* infection process, and because both microorganism are very closely related, it has been suggested that the most important *A. rhizogenes* oncogenes, encode proteins involved in the regulation of plant hormone metabolism. *Aux* genes provide transformed cells with an additional source of auxin (Morris, 1986; Chriqui, Guivarch, Dewitte, Prinsen & van Onkelen, 1996), but these genes do not seem essen-

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Table 1
Study of development and alkaloid production of transformed root cultures established after 4 weeks of growth[a]

	Datura metel	*Duboisia hybrid*	*Nicotiana tabacum*
% of rooting explants[b]	34	80	98
% of roots with callus-like morphology[b]	13	5	3
Average of FW (g) of callus-like roots lines[c]	11.524 ± 2.661	3.228 ± 0.623	5.836 ± 0.399
Average of FW (g) of hairy roots[c]	5.779 ± 0.756	1.522 ± 0.497	3.131 ± 0.419
Average of total alkaloid content (mg/g DW) of callus-like roots[c]	1.720 ± 0.223	2.496 ± 0.415	6.938 ± 0.980
Average of total alkaloid content (mg/g DW) of hairy roots[c]	4.586 ± 0.339	9.374 ± 1.041	52.369 ± 9.008

[a] Alkaloids considered: in *D. metel* and *Duboisia* (hyoscyamine + scopolamine), in *N. tabacum* (nicotine). The high s.d. values were due to the inherent differences of the transformed root lines belonging to the same plant species.
[b] % obtained over 50–100 experiments.
[c] Average of 10–50 determinations ± s.d.

tial for developing hairy root disease (Palazón, Cusidó, Roig & Piñol, 1997a). However, recent data (Nilsson & Olsson, 1997) indicate that this is not the case of *rol* genes. These genes have functions that are most likely other than that of producing mere alterations in plant hormone concentrations.

Several authors have investigated the effect of TR and TL regions of *A. rhizogenes* on growth and morphology of transformed roots and plants, but until now, there have been few studies of the direct effects of oncogenes on secondary metabolism. As has been previously reported, a correlation exists between the expression of *rolC* gene and tropane alkaloids (Piñol, Palazón, Cusidó & Serrano, 1996), *Catharanthus roseus* alkaloids (Palazón, Cusidó, Gonzalo, Bonfill, Morales & Piñol, 1998), and ginsenoside production (Bulgakov, Khodakovskaya, Labetskaya, Chernoded & Zhuravlev, 1998). These authors did not find any correlation between *rolA* and *rolB* expression and secondary metabolism.

With these considerations in mind, this work reports the establishment of different phenotypes of *Datura metel*, *Duboisia* hybrid and *Nicotiana tabacum* transformed roots whose alkaloid pattern and productivity is dependent on root morphology. The effect of the different complements of *A. rhizogenes* T-DNA integrated in their genome is discussed.

2. Results and discussion

2.1. Morphology, growth and alkaloid production of transformed root cultures established

In all plant species tested (*D. metel*, *Duboisia* hybrid and tobacco), leaf segments inoculated with *A. rhizogenes* developed transformed roots after 2–6 weeks of culture. The effectiveness of agroinfection depended on the plant species. Table 1 shows that 98% of tobacco explants infected developed transformed roots, while only 80% and 34% of *Duboisia* and *D. metel* explants, respectively, developed the hairy root syndrome. Moreover, while tobacco transformed roots began to appear 2 weeks after the infection with *Agrobacterium*, in *Datura* or *Duboisia* transformed roots did not develop until 6 weeks after the agroinfection. In order to establish root line cultures, root tips appearing at the wound sites of leaf segments were picked off and separately cultured in hormone-free solid medium for successive subcultures of 4 weeks. As previously men-

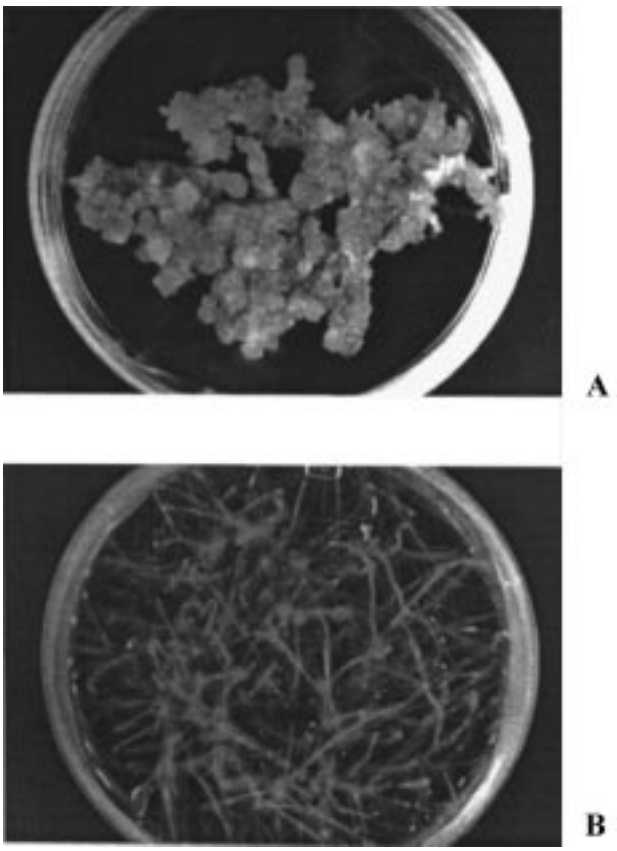

Fig. 1. Four-week-old *D. metel* transformed root cultures on B5/2 solid medium. (A) Root exhibiting callus-like morphology. (B) Root exhibiting hairy root morphology.

tioned, tobacco transformed roots were grown on MS medium and *D. metel* and *Duboisia* roots in B5/2.

When cultured on solid medium, transformed roots grew actively and showed two different morphologies. Most of the transformed root cultures showed the characteristic traits of hairy roots described previously by David, Chilton and Tempé, 1984 (fast growing, highly branched and plagiotropic). On the contrary, the remaining root lines (among 3–13%) showed the denominated callus-like morphology (see Fig. 1), characterized for its high capacity to dedifferentiate and produce callus tissues in the hormone-free culture medium. Root morphology was maintained for successive subcultures.

Regarding the growth ability of transformed root lines (see Table 1), *D. metel* transformed roots grew faster than tobacco and *Duboisia* roots. The growth of root cultures measured as fr. wt. varied according to root morphology. After 4 weeks of growth, callus-like roots on average achieved a fr. wt. approximately two-fold higher than hairy roots, in all plant species assayed.

However, as can be seen in Table 1, after 4 weeks of culture, callus-like roots achieved lower alkaloid contents than roots with typical hairy root morphology. In *D. metel* hairy roots the total alkaloid content achieved was 2.7-fold higher than callus-like roots, and 3.8- and 7.5-fold higher, respectively, in *Duboisia* and tobacco roots. Obviously, when transformed roots lose their morphology and develop callus tissues, the alkaloid content of roots decreases dramatically. These results showed a clear relationship between root morphology and alkaloid production. Previously, we have reported that in tobacco callus cultures, organisation of tissues increased nicotine production (Piñol, Palazón & Serrano, 1984), and Ondrej and Protiva, (1987) showed similar results working with *Atropa belladona* crown gall and hairy root cultures.

2.2. Study of the fragments of Agrobacterium T-DNA integrated into the transformed root genome

In this paper, we have studied the relationships among the presence of different *Agrobacterium* genes in the plant genome with the capacity of roots to grow and biosynthesize tropane or tobacco alkaloids. Two genes located in the TR-DNA of *A. rhizogenes* were considered: *ags* gene encoding a protein responsible for agropine biosynthesis and *aux1* gene which is one of two genes involved in the additional route for indole-3-acetic acid in transformed cells (Nilsson & Olsson, 1997). At the same time, the *rolC* gene located in the segment TL of the T-DNA of *A. rhizogenes*, which plays a significant role in developing hairy root syndrome, was also studied.

According to the root morphologies, the genome of roots tested showing callus-like appearance all harboured the three genes mentioned above, whereas only 25–60% of hairy roots harboured the *aux1* gene (see Table 2). These results reflect the role of this gene, which provides the transformed root cell with an additional source of auxin (Morris, 1986; Chriqui et al., 1996). In transformed roots, auxin excess could induce disorganisation of root tissues (Jung, Kwah, Choi & Liu, 1995). Our results agree with previous data (Robins, Bent & Rhodes, 1991; Palazón, Altabella, Cusidó, Ribó & Piñol, 1995), which reported that in *D. stramonium* hairy root cultures, the addition of the synthetic auxin 2,4-dichlorophenoxyacetic acid to the culture medium had a positive effect on callus biomass production, although it inhibited alkaloid biosynthesis. The capacity to biosynthesize nicotine was also lost, when root cultures of *N. rustica* were treated with auxin (Robins, 1998 and references therein). Moreover, when considering the percentage of roots

Table 2
PCR study of the presence of different fragments of *Agrobacterium rhizogenes* T-DNA inserted in the genome of transformed roots, showing callus-like or typical hairy root morphology

Plant species	Morphology	Gene		
		ags	*aux1*	*rol*
D. metel	Callus-like	100%	100%	100%
	Hairy root	20%	25%	100%
Duboisia	Callus-like	100%	100%	100%
	Hairy root	28%	30%	100%
Tobacco	Callus-like	100%	100%	100%
	Hairy root	71%	60%	100%

Table 3
Study of the presence of *mas1* and *ags* genes responsible for opine biosynthesis and opine detection, in randomly selected *Duboisia* root lines[a]

Root line	Subcultures	*Mas1* gene	*Ags* gene	Agropine	Manopine
Control	> 15	–	–	–	–
Du 3	> 15	+	+	–	–
Du 14	> 15	–	–	–	–
Du 18	> 15	+	+	–	–
Du 27	> 15	–	–	–	–
Du 35	> 15	–	–	–	–
Du 36	> 15	–	–	–	–
Du 38	> 15	–	–	–	–
Du 51	< 10	+	+	+	+
Du 54	< 10	–	–	–	–
Du 56	< 10	+	+	+	+
Du 62	< 10	–	–	–	–
Du 68	< 10	–	–	–	–
Du 72	< 10	+	+	+	+
Du 73	< 10	–	–	–	–

[a] Genes were detected by PCR and opines were detected by paper electrophoresis, as described in Section 3.

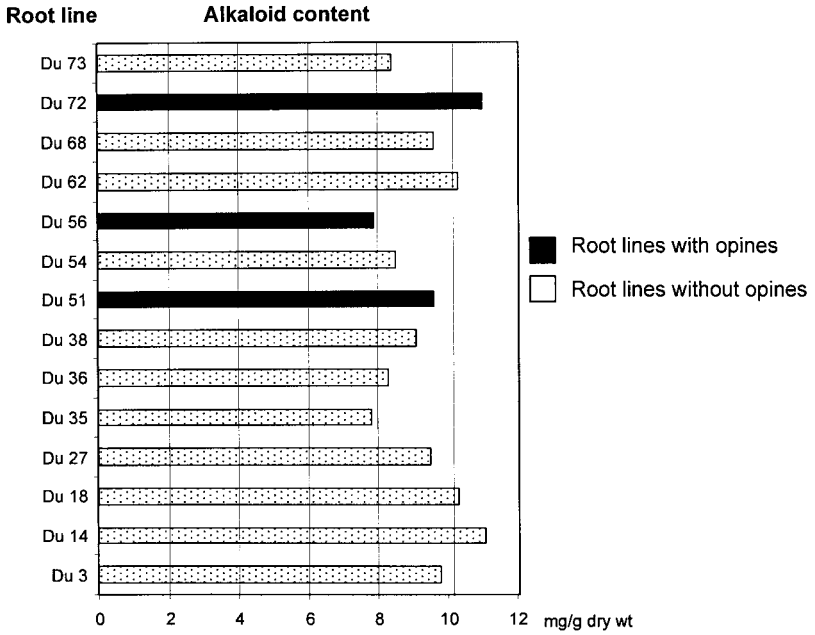

Fig. 2. Comparative study of the alkaloid production (expressed as mg/g dry weight) among randomly selected *Duboisia* hairy root lines with or without opines. The alkaloids considered were hyoscyamine and scopolamine. Each value is the average of six determinations.

showing callus-like morphology (13% in *D. metel*, 5% in *Duboisia* and 3% in tobacco) (see Table 1) and hairy root carrying the *aux1* gene (see Table 2), our results would seem to suggest that this gene was more effective for developing callus-like morphology in *D. metel* roots, since in this species, only 25% of the hairy roots tested carried the *aux1* gene.

2.3. Opine production

Randomly selected root lines were analyzed in order to investigate opine presence in transformed roots. Manopine and agropine, the opines considered, were qualitatively determined according to Dessaux and Petit (1994). The presence of these compounds in transformed roots was related to *mas1* (responsible for mannopine biosynthesis) and *ags* genes (responsible for agropine biosynthesis). It can be hypothesized that transformed roots, which synthesized opines, show limited alkaloid biosynthesis because they have an additional consumption of nitrogen and carbon.

As can be observed in Table 3, in most of the *Duboisia* transformed root lines considered, opines were not detected and even in some root lines carrying genes responsible for opine biosynthesis, those compounds were hardly detected. The low integration of *ags* gene in the transformed roots can be due to the frequent occurrence of incomplete insertion of TR-DNA into the transformed root genome (Gaudin, Vrain & Jouanin, 1994). Table 3 also shows the number of successive subcultures of transformed roots, from which a relationship between root age and their capacity to produce opines could be inferred. Previously, Petit & Dessaux (personal communication) observed the loss of capacity to synthesize opines in transgenic *Solanum nigrum* plants when cultured for a long period. On the other hand, and discarding callus-like roots, in the roots showing hairy root morphology no correlation between opine biosynthesis and alkaloid production can be established (see Fig. 2). These results suggest that the elimination of the genes responsible for opine biosynthesis does not constitute an efficient system to improve the alkaloid production in the transformed roots of the plant species considered.

Table 4
Effects of the inoculation of pRiA4TR- into leaf sections of *Datura metel*, *Duboisia* hybrid and *Nicotiana tabacum* plant species

Plant species	% of rooting explants	% of hairy roots	% of callus-like roots
Datura metel	11	100	0
Duboisia hybrid	17	100	0
Nicotiana tabacum	78	100	0

2.4. Effect of transformation with A. tumefaciens strain C58 C1 carrying the pRiA4TR- on root morphology and alkaloid production

In order to clarify the role of *aux* genes on the transformed root morphology, leaf segments of the species studied were inoculated with *A. tumefaciens* strain C58 C1 carrying the pRiA4TR-. In this plasmid *aux* genes were deleted and consequently the transformed roots obtained did not express these genes. Transformed roots began to appear 4–6 weeks after the inoculation and were subcultured independently on MS solid medium for tobacco roots and B5/2 for *D. metel* and *Duboisia* hybrid. Due to the fact that pRiA4TR- also carried the reporter gene *nptII*, culture media were supplemented with kanamycin in order to realize a previous selection of the transformed material.

As shown in Table 4, the inoculation of *A. tumefaciens* C58 C1 carrying the pRiA4TR- was able to transform plant material and develop the hairy root syndrome, but the number of explants with roots (6 weeks after the infection) was significantly lower than that obtained with the A4 strain of *A. rhizogenes*. As it is known (Chriqui et al., 1996) the TR-DNA auxin synthetic genes *aux* play a rather accessory role, being useful merely when endogenous plant auxin is insufficient to trigger differentiation of cells made competent to respond to the hormone by the expression of the TL-DNA genes. On the other hand, our results indicate that all the roots obtained showed a root morphology like the hairy root one, and the growth and alkaloid levels of these root cultures were also very similar to those achieved by A4 transformed roots (data not shown).

3. Experimental

3.1. Hairy root cultures

Hairy roots of *D. metel*, *Duboisia* hybrid and *N. tabacum* cv Xanthi, were obtained by transformation with *A. rhizogenes* A4 and with *A. tumefaciens* strain C58 C1 carrying the pRiA4TR- (constructed by White, Taylor, Huffman, Gordon & Nester, 1985). *Aux* genes were deleted in this plasmid. The transformed roots developed at wound sites were excised and cultured individually on solid, half-strength B5 medium (Gamborg, Miller & Ojima, 1986) (B5/2, medium diluted to half that of normal concentration of salts) supplemented with 30 g/l sucrose (in the case of *D. metel* and *Duboisia* hairy roots) or MS (Murashige & Skoog, 1962) hormone-free medium supplemented with 30 g/l sucrose (in the case of *N. tabacum* hairy roots). Root clones were kept in the dark at 26°C and routinely subcultured every 25–30 days.

3.2. Genomic DNA extraction and analysis

Total DNA was isolated from hairy root culture clones by using "Genomic Prep™ Cells and Tissue DNA isolation Kit" (Pharmacia Biotech). PCR analysis was performed using the pre-formulated, pre-dispensed single-dose reaction beads "Ready to Go™" (Pharmacia Biotech). The complete PCR mixture contained 200 ng of total DNA, 12.5 pmol/μl of each oligonucleotide primer, 200 μM dNTPs, 1.5 u *Taq* polymerase, and buffer supplied by the enzyme manufacturer (1/10 V) in a total volume of 25 μl. The used oligonucleotide primers that would anneal to the *rolC* gene, produced a DNA fragment of 534 bp, the ones for the *ags* and *mas1* genes a fragment of 347 and 343 bp, respectively, and the ones for the *aux1* gene a fragment of 350 bp.

PCR amplification was performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C (for *rolC* gene) or 62°C (for *ags* and *mas1* genes) or 55.9°C (for *aux1* gene) for 1 min and extension at 72°C for 1.5 min, with a final extension at 72°C for 5 min.

The PCR reaction mixtures (10 μl) were then loaded directly onto 1.5% agarose gel for electrophoretic analysis. A 100 Base-Pair Ladder (Pharmacia Biotech) was used as a molecular weight marker for the PCR-amplified double-stranded DNA fragment.

3.3. Opine analysis

Opine analysis was performed by High Voltage Paper Electrophoresis according to a modification of the method described by Petit et al. (1983). Samples were immersed in distilled water and then incubated at 100°C for 10 min. After a brief centrifugation (13,000 g for 5 min), supernatant was vacuum-evaporated at 40°C in order to yield a dry plant extract. This extract was then resuspended in distilled water and kept frozen until analyzed. One to 6 μl of plant extracts were spotted on high quality chromatography paper and allowed to dry.

Electrophoresis was performed in 1.1 M acetic acid/0.7 M formic acid (pH 1.9) at 50–100 V/cm; electrophoretograms were then dried in a stream of hot air and processed with the staining solutions to detect the presence of opines as described by Dessaux and Petit (1994).

3.4. Extraction and determination of alkaloids

The extraction and determination of scopolamine

and hyoscyamine in the transformed roots were carried out as reported earlier by Piñol et al. (1996). The nicotine content was determined according to the method described by Saunders and Blume (1981) with some modifications (see Palazón et al., 1995).

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References

Binns, A. N., & Tomashow, J. V. (1988). Cell biology of *Agrobacterium* infection and transformation of plants. *Annu. Rev. Microbiol*, *42*, 575–606.

Bulgakov, V. P., Khodakovskaya, M. V., Labetskaya, N. V., Chernoded, G. K., & Zhuravlev, Y. N. (1998). The impact of plant *rolC* oncogene on ginsenoside production by ginseng hairy root cultures. *Phytochemistry*, *49*(7), 1929–1934.

Chriqui, D., Guivarch, A., Dewitte, W., Prinsen, E., & van Onkelen, H. (1996). *Rol* genes and root initiation and development. *Plant and Soil*, *187*, 47–55.

David, C., Chilton, M. D., & Tempé, J. (1984). Conservation of T-DNA in plants regenerated from hairy root cultures. *Bio/Technology*, *2*, 73–76.

Dessaux, Y., & Petit, A. (1994). Opines as screenable for plant transformation. *Plant Molecular Biology Manual*, *C3*, 1–12.

Gamborg, O. L., Miller, R. A., & Ojima, K. (1986). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res*, *50*, 151–158.

Gaudin, V., Vrain, T., & Jouanin, L. (1994). Bacterial genes modifying hormonal balances in plants. *Plant Physiol. Biochem*, *32*, 11–29.

Jouanin, L. (1984). Restriction map of an agropine-type Ri-plasmid and its homologies with Ti-plasmids. *Plasmid*, *12*, 91–102.

Jung, K. H., Kawah, S. S., Choi, C. Y., & Liu, J. R. (1995). An interchangeable system of hairy root and cell suspension cultures of *Catharanthus roseus* for indole alkaloid production. *Plant Cell Reports*, *15*, 51–54.

Morris, R. O. (1986). Genes specifying auxin and cytokinin biosynthesis in phytopathogens. *Annu. Rev. Plant Physiol*, *37*, 509–538.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, *15*, 473–497.

Nilsson, O., & Olsson, O. (1997). Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. *Physiologia Plantarum*, *100*, 463–473.

Ondrej, M., & Protiva, J. (1987). In vitro culture of crown gall and hairy root tumors of *Atropa belladona*: differentiation and alkaloid production. *Biol. Plant*, *29*, 241–246.

Palazón, J., Altabella, T., Cusidó, R., Ribó, M., & Piñol, M. T. (1995). Growth and tropane alkaloid production in *Agrobacterium* transformed roots and derived callus of *Datura*. *Biol. Plant*, *37*(2), 161–168.

Palazón, J., Cusidó, R. M., Roig, C., & Piñol, M. T. (1997a). Effect of *rol* genes from *Agrobacterium rhizogenes* TL-DNA on nicotine production in tobacco root cultures. *Plant Physiol. Biochem*, *35*(2), 155–162.

Palazón, J., Piñol, M. T., Cusidó, R. M., Morales, C., & Bonfill, M. (1997b). Application of transformed root technology to the production of bioactive metabolites. *Recent Res. Devel. in Plant Physiol*, *1*, 125–143.

Palazón, J., Cusidó, R. M., Gonzalo, J., Bonfill, M., Morales, C., & Piñol, M. T. (1998). Relation between the amount of *rolC* gene product and indole alkaloid accumulation in *Catharanthus roseus* transformed root cultures. *J. Plant Physiol*, *153*, 712–718.

Petit, A., David, C., Dahl, G. A., Ellis, J. G., Guyon, P., Casse-Delbart, F., & Tempé, J. (1983). Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Mol. Gen. Genet*, *190*, 204–214.

Piñol, M. T., Palazón, J., & Serrano, M. (1984). Growth and nicotine content of tobacco callus cultures without organogenesis. *Plant Science Letters*, *35*, 219–223.

Piñol, M. T., Palazón, J., Cusidó, R., & Serrano, M. (1996). Effects of Ri T-DNA from *Agrobacterium rhizogenes* on growth and hyoscyamine production in *Datura stramonium* root cultures. *Bot. Acta*, *109*, 133–138.

Rhodes, M. J. C., Robins, R. J., Hamill, J. D., Parr, A. J., Hilton, M. G., & Walton, N. J. (1990). Properties of transformed root cultures. In B. V. Charlwood, & M. J. C. Rhodes, *Secondary Products from Plant Tissue Culture: Proceedings of the Phytochemical Society of Europe, Vol. 30* (pp. 201–225).

Robins, R. J., Bent, F. G., & Rhodes, M. J. C. (1991). Studies on the biosynthesis of tropane alkaloids by *Datura stramonium* L. transformed root cultures. Part 3: The relationship between morphological integrity and alkaloid biosynthesis. *Planta*, *185*, 390–395.

Robins, J. R. (1998). The application of root cultures to problems of biological chemistry. *Nat. Prod. Rep*, *15*(6), 549–570.

Saunders, J. A., & Blume, D. E. (1981). Quantitation of major tobacco alkaloids by high-performance liquid chromatography. *J. Chromatogr*, *205*, 147–154.

Toivonen, L. (1993). Utilization of hairy root cultures for production of secondary metabolites. *Biotechnol. Prog*, *9*, 12–20.

White, F. F., & Nester, E. W. (1980). Hairy root: plasmid encodes virulence in *Agrobacterium rhizogenes*. *J. Bacteriol*, *141*, 1134–1141.

White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P., & Nester, E. W. (1985). Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. *J. Bacteriol*, *164*(1), 33–44.