Changes in growth and tropane alkaloid production in long-term culture of hairy roots of *Brugmansia candida*

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ABSTRACT

Hairy roots cultures, which are the result of infection with *Agrobacterium rhizogenes*, present a number of advantages over other *in vitro* cultures. One of the most important ones is the genotypic and phenotypic stability of these cultures over the years. Our group has been working with hairy roots of *Brugmansia candida*, producer of the tropane alkaloids scopolamine and hyoscyamine, widely used in medicine as anticholinergics. Surprisingly, in our research, which spanned 5 years of culture, we encountered a pronounced increase in the production of scopolamine and a significant decrease in growth. Contradictory results have been found in the literature, where long-term stability as well as modifications in phenotypes after several subcultures were reported. Since these metabolites are involved in defense mechanisms and, in addition, *in vitro* cultures represent in themselves a stressful situation, it could be hypothesized that the whole biosynthetic pathway could be up-regulated. The limitations to this accumulation could be determined by negative feedback mechanisms or by the incapacity of the roots to tolerate its toxicity. The hairy roots obtained in our laboratory were not engineered to overexpress hyoscyamine-6-β-hydroxydase (H6H), and therefore the changes observed cannot be attributed to higher amounts of this enzyme. However, further studies, particularly at the molecular level, have to be initiated to determine if subculture of the roots affect their genetic stability. Nevertheless, from the cited data, it is clear that stability must be permanently controlled, particularly if long-term industrial processes are entailed.

INTRODUCTION

Agrobacterium tumefaciens and *A. rhizogenes* were discovered in the 1930´s, but it was only in the late 1970´s that the particular mechanism of action underlying the capacity of *A. tumefaciens* to produce crown-gall tumor was elucidated (Chilton et al., 1997). *A. rhizogenes*, while sharing many of the same characteristics as its close relative, displays some important differences. The main one is the induction of transformed (hairy) roots at the site of infection (Chilton et al., 1982). These two Gram negative bacteria have played a major role in the history of plant tissue culture and molecular biology. They allowed, for the first time, the establishment of *in vitro* cultures of cells, tissues and organs in media devoid of plant growth regulators (PGRs). At the same time, their role as vectors to transfer genes into plant cells revolutionized plant transformation (Wisniewski et al., 2002; Glaser and Matten, 2003; Christou et al., 2006).

In the present work, we will focus on the use of hairy roots for the production of plant secondary metabolites. The latter are usually synthesized in low levels but play important roles in plants (example: defense, attractants for pollinization, etc.). Secondary metabolites are particularly important for mankind because they are used in a variety of industries (Table I), especially the pharmaceutical one. The advantages of hairy root cultures are manifold. They can grow almost as fast as plant cell suspensions, but maintaining a stable differentiated phenotype. Many secondary products, among them tropane alkaloids, are not expressed in undifferentiated cell cultures efficiently because their synthesis is linked to root differentiation. Consequently, hairy root cultures can express many specific metabolic pathways, particularly secondary metabolite ones, efficiently and similarly to roots *in planta* (Hamill et al., 1986; Kamada et al., 1986; Flores et al, 1987; Pitta-Alvarez and Giulietti, 1995). In some instances, hairy roots can produce higher levels of the secondary metabolite compared to the whole plant (Payne et al.,1987). Most importantly, apparently they do not present the production instability of plant suspension cultures. Hairy roots usually have a long-term and stable production of secondary metabolites (Flores et al., 1987) and they have been employed to produce a large variety of secondary metabolites, among them tropane alkaloids.

In our laboratory, hairy roots of *Brugmansia candida* (Solanaceae) were obtained for the production of the tropane alkaloids (-)hyoscyamine and, especially, scopolamine. Both are anticholinergic agents widely used in medicine. However, scopolamine has a world demand estimated to be 10 times greater than that of (-)hyoscyamine and atropine combined (Hashimoto et al., 1993). Figure 1 shows the biosynthetic route of scopolamine. Hyoscyamine-6-β-hydroxylase (H6H) is one of the key enzymes in the route and it is involved in the hydroxylation and epoxidation of (-)hyoscyamine to render

Table I. Secondary metabolites and their uses in various industries.

Industry	Secondary metabolite	Chemical structure	Species	Uses
Pharmaceuticals	Codeine	Alkaloid	*Papaver somniferum*	Cough syrups
	Quinine	Alkaloid	*Cinchona ledgeriana*	Antimalarial
	Artemisinine	Sesquiterpenic lactone	*Artemisia annua*	Antimalarial
	Scopolamine	Alkaloid	*Datura stramonium* *Brugmansia candida*	Antispasmodic
	Vincristine	Alkaloid	*Catharanthus roseus*	Anti-leukemic
	Taxol	Diterpene	*Taxus sp.*	Anti-cancer
Agrochemical	Pyrethrin	Terpene	*Chrysanthemum cinearifolium*	Insecticide
Food	Thaumatine	Chalcone	*Cinchona ledgeriana*	Non-caloric sweetener
Cosmetics	Jasmine	Essential oil	*Jasminum sp.*	Perfume

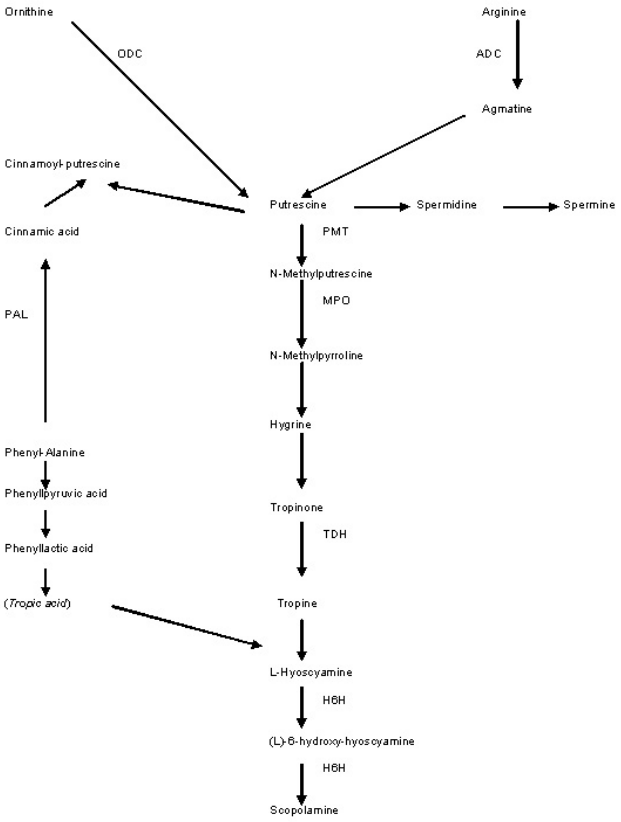

Figure 1. Biosynthesis of hyoscyamine and scopolamine: ODC, ornithine decarboxylase; ADC, arginine-decarboxylase; TDH, tropinone dehydrogenase; H6H, hyoscyamine-6-β-hydroxylase.

scopolamine. H6H is localized in the pericycle of the root and is particularly active in cultured roots, but it is absent in aerial parts of the plant (Hashimoto et al.,1991; Matzuda et al., 1991).

As was stated previously, one of the advantages of hairy roots is their production stability, as opposed to plant cell cultures that are subject to variations. However, the question remains as to the long-term stability of transformed root cultures. In the literature published so far, there are contradictory reports regarding this property of hairy roots. It is the aim of the present work to address this particular issue based on the observation of growth and tropane alkaloid production of one hairy root clone of *B. candida* during 5 years of culture.

RESULTS

Once the *B. candinda* hairy roots were obtained, one clone (clone 7) was chosen based on its growth rate and scopolamine production (data not shown). It is important to note that in every clone examined, tropane alkaloid production and growth were associated (Pitta-Alvarez, 1998). After a few subcultures, the formation of a callus derived from clone 7 was observed and, consequently, tropane alkaloid production ceased. The strategies deployed to overcome this difficulty have been described in Pitta-Alvarez and Giulietti (1995). It was observed that when the callus was subcultured in $B5_{1/2}$ medium, multiple hairy roots emerged from it. Every root was considered a different clone and, again, the one with the best growth rate and scopolamine production (Figures 2a and 2b) was chosen (clone 7X). In this case, it was evident that the change in tropane alkaloid production was due to the fact that the system had undergone a dedifferentiated state with the subsequent genetic instability derived from this situation. The use of $B5_{1/2}$ medium was used for the remaining subcultures, thus preventing dedifferentiation (Pitta-Alvarez and Giulietti, 1995). In clone 7X, alkaloid production and growth were also closely associated (Fig. 2a and 2b).

During subculture of clone 7X in $B5_{1/2}$ medium, gradual changes in tropane alkaloid production and growth were observed. After 3 years, the production of scopolamine had increased significantly, while the hyoscyamine one remained practically the same as in previous years (Figure 3a). As a result, the ratio total scopolamine/total hyoscyamine (St/Ht) increased dramatically. However, growth was affected negatively (Figure 3b). After five years of subcultures of the same clone, both scopolamine and hyoscyamine production were strongly augmented (Figure 3a). The ratio St/Ht remained practically the same as the one registered after 3 years of culture. However,

growth declined significantly and finally ceased (Figure 3b).

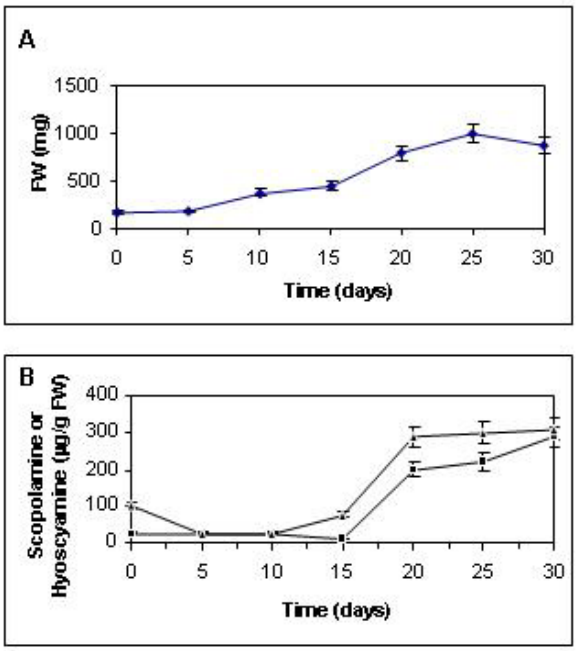

Figure 2. (a) Kinetics of growth of clone 7X immediately after re-differentiation with medium B51/2 supplemented with 15 g/l sucrose. **(b)** Kinetics of scopolamine and hyoscyamine production in clone 7X immediately after re-differentiation with medium B51/2 supplemented with 15 g/l sucrose. FW: Fresh weight. Dry weight represented in every case approximately 10% of total FW. --▲--: Scopolamine; --■--: Hyoscyamine.

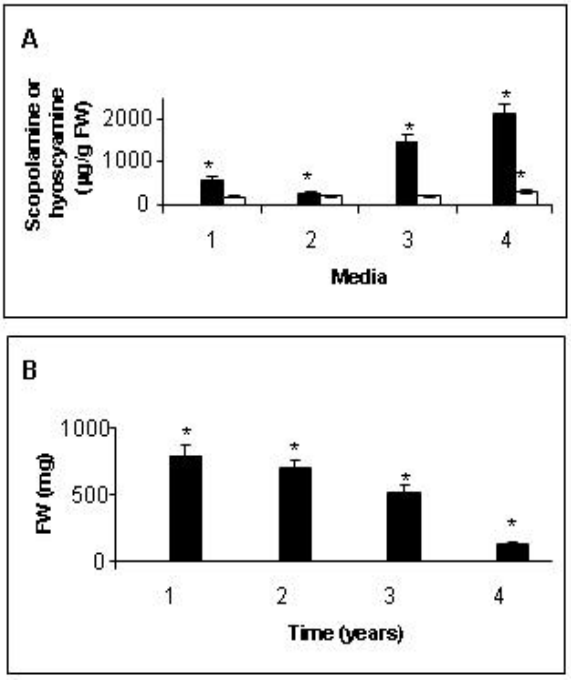

Figure 3. (a) Modifications of tropane alkaloid production over a 5 year period of subculture in two different media: 1: MS supplemented with 30 g/l sucrose employed until dedifferentiation took place; 2,3 and 4: B51/2 with 15 g/l sucrose employed (2) after re-differentiation, (3) after 3 years of subculture and (4) after 5 y. of subculture. The samples were taken after 20-d. of culture. FW: Fresh weight. Each value represents the mean of three independent determinatiosns. Data marked with an asterisk are significantly different according to Tukey´s test (p=0.05). Dry weight represented in every case approximately 10% of total FW. --■-- Scopolamine; --□—Hyoscyamine. **(b)** Modifications of growth over a five-year period of subculture in two different media: 1: MS supplemented with 30 g/l sucrose employed until dedifferentiation took place; 2,3 and 4: B51/2 with 15 g/l sucrose employed (2) after re-differentiation, (3) after 3 y. of subculture and (4) after 5 y. of subculture. The samples were taken after 15-d. of culture. FW: Fresh weight. Dry weight represented in every case approximately 10% of total FW. Each value represents the mean of three independent determinatiosns. Data marked with an asterisk are significantly different according to Tukey´s test (p=0.05).

DISCUSSION

The exact reasons for the observations described above remain unclear. Although the functions of tropane alkaloids in plants are not yet completely elucidated, they apparently are related to defense against herbivores. Since *in vitro* cultures represent in themselves a stressful situation, they could trigger in the cultures the production of these alkaloids. In addition, there is a possibility that other metabolites that appear only in *in vitro* cultures could be involved in promoting the production of tropane alkaloids through the years. For instance, cadaverine, which is absent in the plant, was detected in these hairy roots (Carrizo et al, 2001). Although this particular polyamine is possibly not responsible for the increased levels of tropane alkaloids, it suggests that other novel compounds, not found in plants, might up-regulate the enzymes involved in the biosynthesis of these alkaloids (Figure 1).

Furthermore, it has been reported that in whole plants of *B. candida*, scopolamine is transferred possibly in larger proportion than hyoscyamine (El-Dabbas and Evans, 1982) to the aerial parts. Maldonado-Mendoza et al. (1993) found that hairy roots of *Datura stramonium* produced tropane alkaloids 2 orders of magnitude higher than mother plants. They suggested that this because in mother plants both metabolites are translocated and stored in aerial parts of the plant (Payne et al.,1987). The fact that this molecule cannot be translocated in root cultures could induce a larger accumulation. The limitations to this accumulation could be determined by negative feedback mechanisms or by the incapacity of the root to tolerate its toxicity. This last speculation would explain the negative effect on growth observed as tropane alkaloid accumulation continued to rise in our hairy root system.

Yukimune et al. (1994) used repeated selection in transformed root cultures of *Duboisia myoporoides*. They found that in hairy roots the scopolamine content of the lines obtained at each selection increased with the number of selections. They also observed that the morphology of the hairy roots with improved scopolamine content differed after the repeated selection, obtainining fine root lines with extensive lateral branching. The authors suggested that the initial root tip consisted in heterogenous cells, even though it had been established from one root tip. Following

that hypothesis, they concluded that the highly productive root lines obtained from repeated selection must be the result of the removal of the heterogenous cells. Our roots could have suffered a similar phenomenon, although we did not observe morphological differences in the process. In agreement with Yukimune et al. (1994), the growth rates of clone 7X decreased after repeated selection. A similar mechanism could be at play in our hairy roots, although it is intriguing that in our case the selected phenotype was always the one with increased levels of scopolamine.

There have been numerous reports of long-term stability in alkaloid production. Maldonado-Mendoza et al. (1993) reported that, in hairy root lines of *D. stramonium*, growth patterns, biomass and tropane alkaloid production remained constant for more than 5 years. The difference in species used for these experiments could be partly responsible for the variations observed with our hairy root clone. In addition, they tested a variety of *A. rhizogenes* strains, but not LBA 9402, which is the strain we used in our experiments. At the moment we are working also with both *A. rhizogenes* strain 15834 and LBA9402 to see if the same changes in tropane alkaloids and growth are observed, or if it depends on the strain used and its interaction with the plant species. In these experiments, we are examining several clones obtained by either strain of *Agrobacterium*. Our observations thus far are in consonance to the behavior of clone 7X, with increasing concentrations of scopolamine and a gradual decrease in growth rate.

Baiza et al. (1999) observed that all the hairy roots lines of *D. stramonium* had a stable production over a period of 6 years. It has to be noted that the species used was the same as Maldonado-Mendoza (1993) and, perhaps, the hairy roots derived from them showed a higher stability than other species. They also found that there was an inverse relationship between growth and secondary metabolite production. As stated before, in our hairy roots growth and production were closely related, and this could be involved in the differences observed with hairy roots of other species.

Mano et al. (1986) found that the production of tropane alkaloids was unstable in both normal and hairy root lines established from *Scopolia japonica*. However, the hairy root cultures established for metabolite production were heterogeneous since they consisted of pools of hairy roots rather than clones. They traced the unstable metabolic activity to this initial heterogeneity. Therefore, the results obtained by this group cannot be compared to ours since our initial inoculum consisted of one root tip, thus assuring that we were starting with the same genotype.

Guivrac´h et al. (1999) raised the question as to whether the stability of transformed root phenotypes was correlated with the stability of gene expression in hairy root cultures. They considered that the viability and growth potential of the transformed roots in long-term cultures had not been fully studied. Consequently, they established hairy root clones from single root tips after the inoculation of carrot discs with a co-integrated *A. rhizogenes* comprising the wild pRi A4 T-DNA and the *gus* gene. The *gus* gene was used as a marker for the presence of TL-DNA and the *aux2* and opine synthesis genes served as indicators for the presence of TR-DNA. They then followed the evolution of the phenotype characteristics and gene expression following successive subcultures over a 2-period year. From their results, they concluded that the observed differences between clones were not correlated with the transformation events. In addition, all the clones obtained were capable of growth on hormone-free medium. Furthermore, they observed large individual variations in growth patterns between clones and, most importantly from our point of view, also inside single clones during various sub-cultures. They concluded that both phenotypes and gene expression in hairy root clones are not completely predictable. Also, the pRi T-DNA genes may be expressed unstably and gene silencing must be considered. This is closer to our observations, but in our case, the variation was always the same: gradual decrease in growth with a concomitant increase in alkaloid production. The phenotype was not subjected to any changes. There is a possibility that our particular clone was not susceptible to possible negative feedback from scopolamine. As a result, the increased amounts of scopolamine could be correlated with the up-regulation of H6H, and this, in contrast to the statements made by Guivrac´h et al. (1999) may be the consequence of transformation events.

Aird et al. (1988) suggested that hairy root cultures of several species have a stable secondary metabolite production as a consequence of their genetic stability at the chromosomal level. In this respect, Baíza et al. (1999) studied the kariotypic stability of 3 lines of hairy roots, with stable production, of *D. stramonium* versus instability of non-transformed roots of the same species. They found that the transformed cultures consisted, cytologically, exclusively of diploid cells, while non-transformed ones presented mixoploidy and aneusometry. The kariotype of the hairy root cultures were the same as that of the plant root tips, and this stability remained irrespective of the age of transformed cultures. Transformed root cultures had a stable production of hyoscyamine and scopolamine, while the normal roots showed a marked instability through time. It has been proposed that normal roots are unstable because auxins inhibit the synthesis of alkaloids through the inhibition of PMT (Wagner et al., 1986) while others consider that auxins that are applied exogenously induce chromosome alterations (Nagl, 1986; Murata, 1989). The roots obtained by our group were apparently particularly susceptible to auxins (Pitta-Alvarez and Giulietti, 1995), and this could constitute an explanation for their instability. Even though the problem of dedifferentiation was solved (Pitta-Alvarez and Giulietti, 1995) the growth pattern of the selected clone was not stable and there was a definite increase in scopolamine production. This could be explained by an apparent random event in the transformation process or to different responses to auxins.

Jouhikainene et al. (1999), who worked with hairy roots of *Hyoscyamus muticus* overexpressing the gene for H6H to

enhance the production of scopolamine, reported observing considerable variation between the clones both in morphology as well as production of hyoscyamine and scopolamine. This could have been the result of using different *A. rhizogenes* strains, but the difference was also seen within groups. Nonetheless, they also reported that scopolamine production of one of these transgenic hairy roots remained stable during 2 and a half years of cultivation.

In contrast to the results obtained by our group, Dechaux and Boitel-Conti (2005), working with hairy roots of *Datura innoxia* that overexpressed H6H from *H. niger*, observed a decrease in scopolamine levels similar to control levels after one-year of subcultures, while hyoscyamine amounts remained the same. They also studied the transcription level of the *h6h* gene and found that it did not decrease after two years of culture. As a result, they concluded that the decrease of scopolamine accumulation was not due to molecular modification of the exogenous *h6h* gene. Although growth of the hairy roots was diminished with respect to the control, they did not find a direct relation between growth ability and scopolamine production, and they concluded that, despite the toxicity of this molecule, the lower growth could be due to insufficient medium supplementation for the lines´ growth requirements. However, as the hairy roots of *D. inoxia* were not able to stably overaccumulate scopolamine, they proposed that scopolamine content may be subjected to metabolic regulation. They speculated that the high content of scopolamine accumulated in hairy roots, which have no storage structure or function, seems to be an enzymatic regulation signal. On the other hand, Palazón et al (2003), who obtained *Duboisia* lines overproducers of scopolamine, observed that adding H6H activity in this plant led to a better conversion of hyoscyamine to scopolamine. They consequently concluded that hyoscyamine may be a feedback regulation signal. In the transformed lines they obtained, the inhibition was removed by the production of scopolamine. However, they suggested that each genus seems to possess its own regulation pathway

It is important to highlight the fact that the hairy roots obtained in our laboratory were not engineered to overexpress H6H, and therefore the changes observed cannot be attributed to higher amounts of this enzyme. A distinct possibility could be that due to the stress of *in vitro* culture, the whole biosynthetic pathway could be up-regulated, in particular the bioconversion of hyoscyamine into scopolamine. In addition, this particular metabolic pathway is highly regulated. If in normal roots scopolamine was the main regulator through negative feedback mechanisms, it could be hypothesized that in hairy roots of *B. candida* this mechanism could be lost. Since this is a highly regulated pathway, there could have been a de-regulation, with scopolamine losing the ability to negative feedback.

CONCLUSIONS

Since its inception, the genetic and phenotypic stability of transformed roots with *A. rhizogenes* has been one of the pillars in their preferential use over suspension cultures. However, and as this paper demonstrates, the reports pertaining to this particular characteristic are contradictory. In our case, stability was lost over a 5 year period of subculture, and significant increases in scopolamine production as opposed to diminished growth could be observed. De-regulation of certain genes that are key in the biosynthetic pathway could be involved in the results observed. However, further studies, particularly at the molecular level and with a higher number of clones, have to be initiated to determine if subculture of the roots, and the consequent stress applied, affect their stability or if some other mechanism such as genetic instability could be playing a role. Nevertheless, from the cited data, it is clear that stability must be permanently controlled, particularly if long-term industrial processes are entailed.

MATERIALS AND METHODS

Establishment and maintenance of hairy root cultures

Transformed (hairy) root cultures were obtained by infecting explants of *B. candida* with *Agrobacterium rhizogenes* LBA 9402, employing the procedure described in Pitta-Alvarez and Giulietti (1995). The establishment of the cultures and the confirmation of their transformation were carried out according to Pitta-Alvarez and Giulietti (1995). The roots were maintained first in liquid Murashige and Skoog medium (MS) (1962) supplemented with 30 g/l sucrose. The medium used for subsequent subcultures was Gamborg (Gamborg et al., 1968) liquid medium with half-concentration of mineral salts and vitamins ($B5_{1/2}$) supplemented with 15 g/l sucrose. The roots were subcultured in the media described every 20 days and incubated at 24±2°C, in gyratory shakers at 100 rpm with a 16-h photoperiod by using cool white fluorescent lamps at a light intensity of approximately 1.8 W/m2. This procedure was followed for a period of 5 years.

Analytical methods

Fresh weight (FW) was determined by separating the root tissue from the medium by vacuum filtration. Alkaloid extraction was carried out as described by Parr et al. (1990). This consisted in the treatment of the hairy roots with 0.2% sulfuric acid during two hours. After washing the roots, they were exposed to NaOH 1N and the alkaloids were removed with $CHCl_3$. The chloroform phase was evaporated and the residue was used to determine tropane alkaloids. Hyoscyamine and scopolamine were analyzed by high-performance liquid chromatography, according to the method described by Mano et al. (1986).The determinations were carried out in a Kontron HPLC with a Kontron spectrophotometer UV 430. The column used was a Spherisorb S5 ODS2 250 X 4.6 mm. The mobile phase was constituted by 1% triethylamine:ethanol (9:1) (pH = 3.5 with formic acid). The absorption was read at a wavelength of 230 nm. Scopolamine had a retention time of 7 min., and hyoscyamine of 15 min. Dry weight (DW) was determined by drying the roots at a temperature of 100°C until constant weight.

Chemicals

Scopolamine, (-)-hyoscyamine, and all the media components were purchased from Sigma Chemical Co. (St. Louis, MO, USA)

Statistical analysis

Significance of treatments was determined by using analysis of variance. Variations among the means of the treatment were analyzed by Tukey´s procedure (1953) (p=0.05).

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