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TROPANE ALKALOID PRODUCTION IN ADVENTITIOUS ROOT CULTURES OF *SCOPOLIA CARNIOLICA* JACQ.

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Abstract: Adventitious root cultures of *Scopolia carniolica* were established from rhizogen callus. The adventitious roots were maintained in MS and in B5 liquid media, both containing 3% succrose and 1.0 mg l^{-1} IBA. The optimal medium, both for root growth and for scopolamine and hyoscyamine production, proved to be the B5 medium. Supplementing the medium of culture with 1mM putrescine caused the stimulation of both tropanic alkaloids' biosynthesis, especially scopolamine's: its level increased from 0.06 mg g^{-1} dry wt (control) to 0.15 mg g^{-1} dry wt (in the medium with putrescine). Another strong influence was exerced by incubation conditions, especially on the scopolamine/atropine ratio. Thus, at roots grown in medium with putrescine and maintained in the dark, this ratio was about 2, while at roots maintained under light (16 h photoperiod) condition, the ratio decreased to the value of 0.5, a value also registered in intact plants' roots and rhizome

Introduction

The plants from *Solanaceae* accumulate in their tissues many biologic active compounds, as tropane alkaloids. Among these, the most important are hyoscyamine (which racemises to atropine) and scopolamine, both being synthesized especially by most of the species of *Mandragora, Atropa, Hyoscyamus, Datura, Scopolia* and *Duboisia* [25]. These alkaloids antagonise acetylcholine at muscarinic receptors [9], and hence are used as muscle relaxants, particularly in eye examinations for the dilation of the pupil. Scopolamine is used as a pre-operative sedative and in commercial preparations for travel sickness (because it paralyses the nerves leading from the vestibular apparatus to the inner ear) [8].

Scopolia carniolica Jacq. is a perennial plant that exists in the mountain beech woods region, in wet and shady places. In Romania it is considered as a rare species. *S. carniolica* accumulates in its rhizome scopolamine as well as atropine [21, 30]**.** Because of abusive harvesting, especially for exportations, this species is threatened with extinction.

Since tropane alkaloid chemical synthesis is difficult and expensive, these compounds are still extracted from plants that belong to several species of the Solanaceae. Obtaining both substances through *in vitro* culture techniques is an interesting alternative, since it would guarantee a stable and uniform year-round supply, independent from seasonal variations of field-grown plants. Hyoscyamine and scopolamine are synthesised in the roots of the plant; consequently the culture of normal and transformed roots is the most appropriate *in vitro* system to produce them. On the pharmaceutical market, scopolamine is the one with the highest commercial value [26, 10]. Cell and root cultures derived from different species of *Solanaceae* have been established for the biotechnological production of these alkaloids [5, 11, 15, 16, 25]. Generally, undifferentiated cell cultures do not produce these compounds efficiently because synthesis is linked to root differentiation; therefore, root cultures are preferred [17, 25].

Several products were found to be accumulated in cultured cells at a higher level than those in native plants through optimization of cultural conditions. In this context, addition to the culture media of appropriate precursors or related compounds sometimes stimulates secondary

metabolite production. This approach is advantageous if the precursors are inexpensive. Tabata et al. [22, 23]) reported that addition of 500 mM tropic acid to the medium of the callus cultures of *Scopolia japonica* increased the amount of alkaloids by up to 14 times.

In this paper, we studied some aspects regarding biosynthesis of atropine and scopolamine in adventitious root cultures of *Scopolia carniolica* Jacq., as well as the influence of light and putrescine on the production of these tropane alkaloids.

Material and Methods

Root cultures. Adventitious root cultures of *Scopolia carniolica* were established from rhizogenic callus growth on Murashige and Skooh (MS) [13] solid medium containing 1.0 mg l^{-1} NAA or 1.0 mg l^{-1} IBA [1]. The adventitious roots were maintained in MS and in Gamborg et al. [4] (B5) liquid media, both containing 3% sucrose and 1.0 mg l^{-1} IBA. The inoculum used was about 700 mg fresh weight (40 mg dry weight) per 300-ml Erlenmeyer flask that contained 50 ml of fresh medium, after which the flask were incubated on 100 rpm rotary shaker (in the dark, as well as in a light/dark regime) in the dark and in the photoperiod regime: 16 h light (38 µmol/m^2/s) and 8 h dark, at 25±1 ºC. The root cultures were subcultured at 2-week intervals. Putrescine was added in the cultures fresh medium before autoclavation, in a concentration of 1mM.

Extraction and analysis of alkaloid. Tropane alkaloids were extracted from dry weight (80ºC, 24 h) of adventitious root. For the quantitative analysis of atropine and scopolamine, 0.1 g powder dry weight was shaken 10 min, in a Vortex-Genie2 apparatus, with 1.5 ml 1 M H_2SO_4. 0.5 ml conc. ammonia was added and then shaken for 5 min with 10 ml $(C_2H_5)_2O$. The sample was centrifuged at 4500 rpm for 5 min. The reziduum was taken with 0.2 ml phospate buffer (40 mM, pH = 2.3). 5 µl were injected in a HPLC HP Series system with Zorbax SB C18 column (3.0 mm i.d. ×100 mm). The mobile phase ((16:84 v/v) CH_3OH : KH_2PO_4 40 mM, pH = 2.3 with 85% H_3PO_4) was eluted with 1 ml/min flow rate at 45°C [2]. The alkaloids were detected using UV detector at 210 nm. The system was calibrated with standard atropine sulphate and scopolamine bromide over the concentration range 1 - 50 µg/ml.

Results and Discussion

Influence of basal medium and photoperiod regime on root growth and alkaloid production.

The gaining in weight of the *Scopolia carniolica* adventive roots, cultured in the two liquid media, MS and B5, only slightly depended on their composition of macro- and microelements. Although root cultures, especially normal ones, have a much lesser growth rate than that of the cell suspensions, the *S. carniolica* adventive roots cultures had a fairly good growth, sometimes comparable even to the growth rate of hairy root cultures induced from different solanaceae species [19, 24].

In regard to the influence of light on the root cultures' growth, the data offered by our references are few and contradictory, some of them asserting that this factor would have no effect [20]. Nevertheless, maintaining the *S. carniolica* root cultures at a photoperiod regime of 16 h light (38 µmol/m^2/s) and 8 h dark led to a growth inhibition of about 20% than the roots that were maintained in the dark, in the MS as well as in the B5 medium (Fig. 1). Another difference between the cultures incubated in the light and those incubated in the dark is that the roots that grew in the light had a green colour, thus demonstrating the chlorophyll synthesis and accumulation in their tissues (Fig. 2B, C and D). The turning green of roots that grew in the light, in vivo or in vitro, is a common process, but its intensity may vary depending on each species [3]. However, this process did not influence the morphology or the way of growing of in vitro cultured *S. carniolica* adventitious roots (Fig. 2).

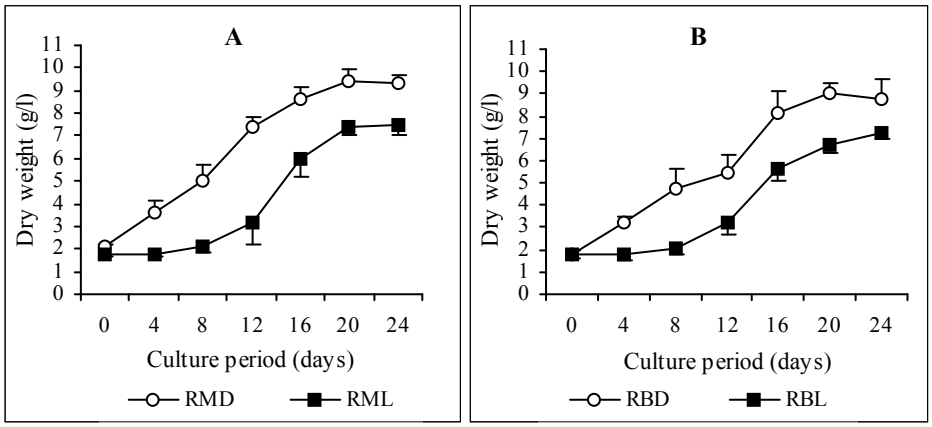

Fig. 1: The effect of basal medium composition and of light on the growth of *S. carniolica* adventitious roots. A. Roots cultured in MS medium with 1.0 mg l^{-1} IBA; RMD = root incubated under total dark condition; RML = root icubated under light (16 h photoperiod) condition. B. Roots cultured in B5 medium with 1.0 mg l^{-1} IBA; RBD = root incubated under total dark condition; RBL = root icubated under light (16 h photoperiod) condition.

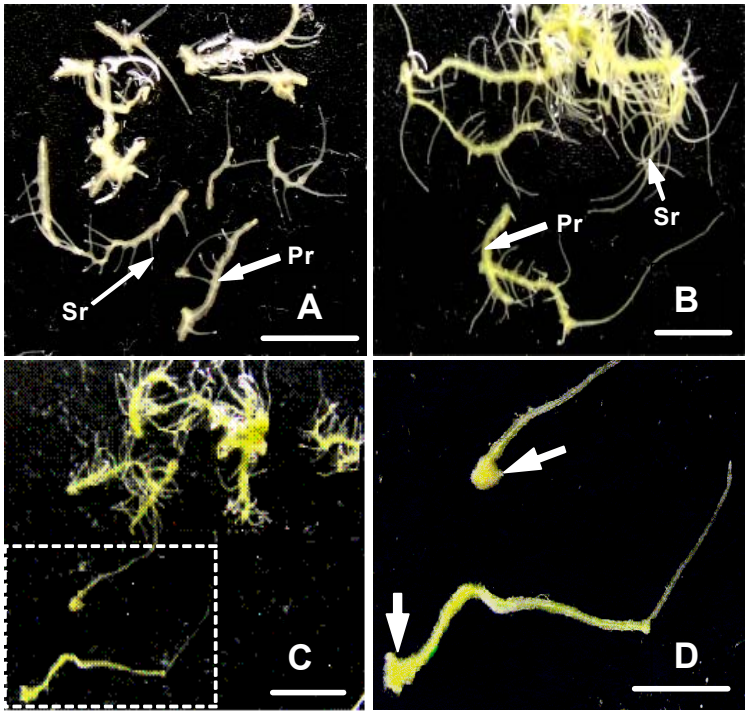

Fig. 2: Aspects concerning the growth of *S. carniolica* adventitious roots, cultured in the B5 and the MS media, both with 1.0 mg l^{-1} IBA. A and B. By the elongation and thickening of primary and secondary roots (A – 7 days after inoculation; B – 21 days after inoculation). C and D. By forming new roots from rhizogen cell nodules (marked with arrows) (C – general view and D – detail). A – root incubated under total dark condition; B,C and D – root incubated under light (16 h photoperiod) condition. Scale length = 1 cm

The stereomicroscope observations on *S. carniolica* root cultures allowed us to discover certain less known aspects. Thus, as we well know [27, 29], after inoculation, the in vitro growth of adventive or neoplastic roots, inducted from different solanaceae species, occurs by the elongation and thickening of primary and secondary (or lateral) root segments, a process that also takes place in the case of *S. carniolica* adventive roots cultures (Fig. 2A and 2B). However,

these examinations pointed out, in all variants and indifferently of the culture or light type, that, along with root segments, in the medium are also present compact cellular masses of 2-3 mm in diameter, having a nodular aspect, which form new roots. It is highly probable that these masses have a meristemoid origin and afterwards, under the influence of auxins, turn into cell masses or adventive roots generative cell nodules (Fig. 2C and 2D). It is thus obvious that *S. carniolica* adventive roots cultures gain weight by elongating, as well as by forming new roots from these nodular masses.

In a previous paper, we pointed out that, at *Scopolia carniolica*, the tropane alkaloids biosynthesis occurs, at intact plants as well as at in vitro cultured plantlets, only in the roots, and that most of these compounds accumulate in roots and rhizomes [1].

In the case of adventive roots cultured in a liquid medium, we found out that a major influence on alkaloids, respectively scopolamine and atropine production, is exercised by basal medium composition and light (Table 1). Thus, comparatively with roots grown in MS medium, those cultured in B5 medium accumulate a much larger scopolamine and atropine amount. This first experiment also showed that, after 14 culture days, the total amount of tropane alkaloids (scopolamine + hyiosciamine) accumulated in roots grown in the dark does not significantly differ from that accumulated in roots grown in a light/dark regime.

As for the level of tropanic alkaloids accumulated in *Scopolia* adventitious roots cultures, although informations are quite scarce, our results are in accordance with those obtained by other authors. Thus, Shikomura *et al.* [18] found out that in the *S. tangutica* adventitious roots culture, both scopolamine and hyosciamine accumulate after 8 weeks in an amount of about 0.05 mg g^{-1} dry wt. In the *Scopolia carniolica* root cultures obtained by us that were incubated in the dark, scopolamine accumulates, after two weeks of culture, in a 0.07 mg g^{-1} dry wt concentration, whilst atropine does the same in a 0.05 mg g^{-1} dry wt concentration.

The adventitious root of *S. carniolica* showed the highest level of scopolamine and atropine depending on the culture media. The amount of these tropane alkaloid increased remarkably when the roots were cultured in B5 medium containing 1.0 mg l^{-1} IBA and 3% sucrose. Due to this fact, the roots have been cultured further on B5 medium, in all experiments.

Table 1: The effect of culture medium composition and of light on scopolamine and atropine biosynthesis in *S. carniolica* adventitious root cultures. Analysis were performed on 14th day of culture.

Variant	Tropane alkaloid (mg g^{-1} DW)	
	Atropine	Scopolamine
RMD	0.031	0.012
RML	0.018	0.006
RBD	0.069	0.050
RBL	0.075	0.047

Roots mentained in MS medium with 1.0 mg l^{-1} IBA, under total dark condition (RMD) and under light (16 h photoperiod) condition (RML)
Roots mentained in B5 medium with 1.0 mg l^{-1} IBA, under total dark condition (RBD) and under light (16 h photoperiod) condition (RBL)

By observing the scopolamine/atropine ratio, though, it appears that light has a fairly strong influence on this ratio, given the fact that, while in the dark it approaches 1.0, in the light, the amount of atropine synthesised by the roots is almost the double of the amount of

scopolamine. Due to the fact that in this first experiment we analysed only 14 days samples, the influence of light will be further on analysed in detail.

The effect of putrescine and light on roots growth and corses of scopolamine and atropine production

One of the strategies used for increasing the yield of desired compound is the exogenous supply of an inexpensive biosynthetic precursor to culture medium. In the case of tropanic alkaloids, most of the experiments were performed using cell suspensions inducted from various solanaceae species, to the medium of which were added precursors of hyoscyamine and scopolamine, as phenylalanine, ornhitine, tropine and tropic acid [22, 25]. Of all utilized precursors, only the tropic acid had a stimulative effect upon the tropanic alkaloids biosynthesis at *Datura stramonium* and *Scopolia japonica* cultures, the level of these compounds increasing from 0.01% to 0.12%, after supplementing the media with this compound [22]. As for putrescine, one of the most important early precursors of tropanic alkaloids biogenesis way, our references' data mainly refer to the role and activity of *N*-methyltransferase putrescine enzyme (PMT), an enzyme catalysing the transformation of putrescine in *N*-methylputrescine. All experiments, especially those performed on root cultures induced from *Hyoscyamus*, *Datura* and *Atropa*, demonstrated that the PMT activity is mostly in correlation with the hyosciamine biosynthesis intensity. Thus, experiments performed with PMT inhibitors demonstrated that their addition to the roots' culture medium caused the strong inhibition of hyoscyamine biosynthesis [7, 25]. Likewise, Moyano *et al* [12] demonstrated that hairy root cultures overexpressing the PMT gene aged faster and accumulated higher amounts of tropane alkaloids than control hairy roots. Both hyoscyamine and scopolamine production were improved in hairy root cultures of *D. metel*, whereas in *H. muticus* only hyoscyamine contents were increased by *pmt* gene overexpression.

Concerning the growth of *Scopolia carniolica* adventitious roots, it has been noted that supplementing the medium with putrescine in the concentration of 1mM caused the inhibition of this process, specially in the dark conditions (Fig. 3).

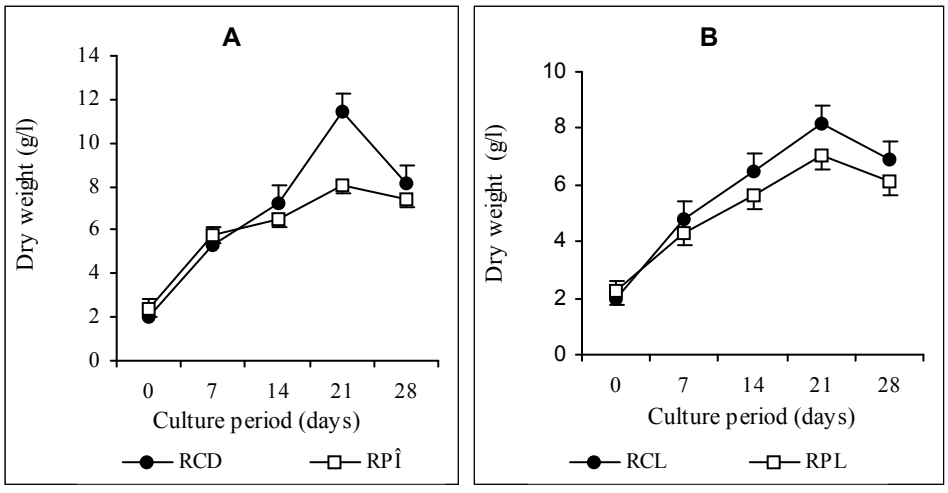

Fig. 3: The effect of putrescine (1 mM) and of light on the growth of *S. carniolica* adventitious roots cultured in B5 medium with 1.0 mg l^{-1} IBA. A. Root incubated under total dark condition. B. Root incubated under light (16 h photoperiod) condition. RC = roots grown in normal medium, control; RP = roots grown in medium with putrescine.

In the case of the two tropanic alkaloids, atropine and scopolamine, the biosynthesis of both products generally goes through the same sigmoid curve, almost parallel to that of roots' gaining dry wt. As for the intensity of scopolamine and atropine biosynthesis, putrescine had a different effect depending on the cultures incubation conditions. Thus, in the case of roots maintained in the dark, putrescine strongly stimulated scopolamine synthesis, so that, in respect to the control, in roots cultured in medium with putrescine, the scopolamine level increased approx. 2.5 times, from 0.06 mg g^{-1} dry wt reaching 0.15 mg g^{-1} dry wt, especially towards the end of the culture period. In the light, we have a reverted situation, given the fact that supplementing the medium with putrescine led to an increase of atropine's biosynthesis, its level increasing in the 14th day of culture, from 0.08 mg g^{-1} dry wt to 0.121 mg g^{-1} dry wt, a level maintained up to the end of the culture period. In exchange, the scopolamine biosynthesis was scarcely stimulated; the amount of this alkaloid gradually increasing up to a percentage of approx. 65% higher than the control at the end of the culture period (Fig. 4).

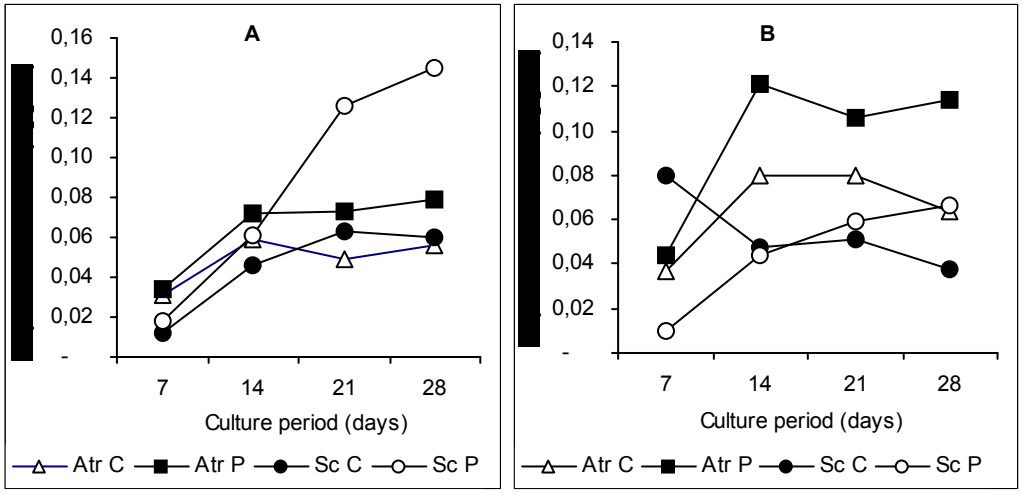

Fig. 4: The influence of putrescine (1 mM) and of light on tropane alkaloids biosynthesis in *S. carniolica* adventitious root cultures grown in B5 medium with 1 mg l^{-1} IBA. A. Root incubated under total dark condition. B. Root icubated under light (16 h photoperiod) condition. AtrC = atropine accumulated in roots cultured in normal medium, control; Atr P = atropine accumulated in roots cultured in medium with putrescine; Sc C = scopolamine accumulated in roots cultured in normal medium, control; Sc P = scopolamine accumulated in roots cultured in medium with putrescine.

If we are to observe the influence of putrescine on the dynamics of the scopolamine/atropine ratio, the two alkaloids synthesized by *S. carniolica* adventive roots, we notice that this ratio changes depending on the culture period, as well as on the light conditions. Thus, in the dark, the ratio gradually increases, beginning with the 7th day of culture and reaching at 2 in the period between the 21st and the 28th days of culture; it is a much larger ratio than that existing in intact plants' rhizome, as well as than the roots of control variant (Fig. 5). Opposedly, at the roots grown in the light, the ratio decreases beginning with the 14th day of culture and remains almost unchanged up to the 28th day, atropine accumulating in much greater amounts than scopolamine; it is a ratio resembling to the one existing in the rhizome. So that such a process is extremely hard to explain, due to the fact that we have no knowledge of similar experiments being previously performed; besides that, in the case of *Scopolia carniolica*, as we have already pointed out, the synthesis of both alkaloids occurs in roots and rhizome, where it is accumulated. Comparatively, at most of the other solanaceae species, hyoscyamine biosynthesis occurs in roots, from where it transported as hyoscyamine or a closely related metabolite to the

shoot, where it is finally converted to scopolamine in the leaf [6, 14, 28]. In the given context, we can easily explain the fact that photomixotrophic and photoautotrophic *D. stramonium* root cultures synthesize larger scopolamine amounts, depending on the photoautotrophic degree, consequently on the degree of chloroplasts development and of chlorophyll biosynthesis [3].

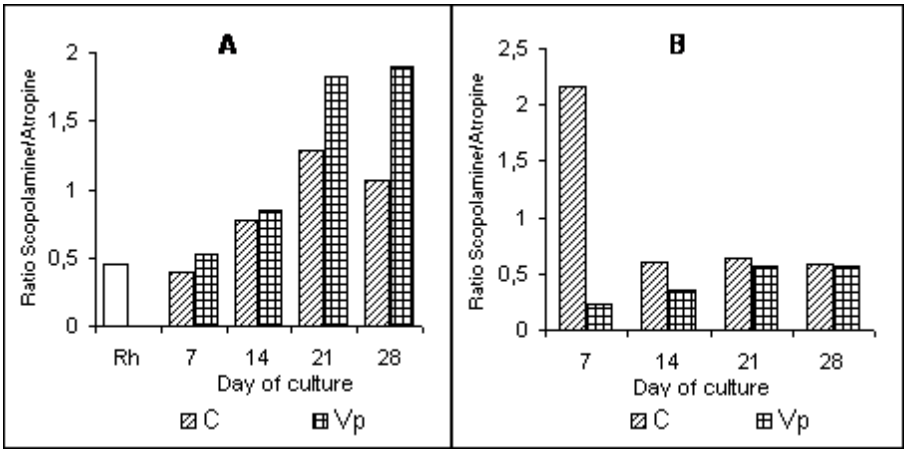

Fig. 5. The effect of putrescine (1mM) and of light on the scopolamine/atropine ratio in *S. carniolica* adventitious root cultures. A. Grown in the dark. B. Grown in the light (16 h photoperiod). R = plant rhizome; C = root mentained in the control medium; Vp = root mentained in the medium with putrescine

Conclusions

The *Scopolia carniolica* adventive roots cultures grew very well in the dark, in the B5 medium with 3% succhrose and 1.0 mg^{-1} IBA. They synthesize atropine, under normal circumstances, in amounts varying from 0.05 to 0.08 mg g^{-1} dw and 0.04 to 0.06 mg g^{-1} dw scopolamine. Light played an important part in respect to roots growth, as well as to the two tropanic alkaloids biosynthesis. Thus, the growth of incubated roots in the light was inhibited. In the case of the two alkaloids, it has been noted that, in the dark, roots tend to synthesize more scopolamine, while in the light, more atropine. Putrescine added to the roots' culture medium, in a 1 mM concentration, strongly stimulated scopolamine biosynthesis in the dark, its amount reaching, in the 28th day of culture, almost 0.15 mg g^{-1} dw, as opposed to only 0.06 mg g^{-1} dw in the case of the control; in the light, it stimulated atropine biosynthesis, making it develop from a 0.08 mg g^{-1} dw level to over 0.12 mg g^{-1} dw. Also, supplementing the medium with putrescine had a favourable effect, at roots incubated in the dark, on the scopolamine/atropine ratio, determining the clear balance in favour of scopolamine of a ratio that is exactly the opposite in the roots and rhizome of the intact plant.

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PRODUCȚIA DE ALACALOIZI TROPANICI ÎN CULTURI DE RĂDĂCINI ADVENTIVE DE *SCOPOLIA CARNIOLICA* JACQ.

(Rezumat)

Alcaloizii tropanici, hiosciamina şi scopolamina, fac parte din compuşii valoroşi din punct de vedere farmaceutic, având utilizări multiple în medicina umană. În general ei sunt extraşi şi în prezent din țesuturile plantelor intacte ce aparțin familiei *Solanaceae*. Datorită necesităților alimentare, suprafețele destinate cultivării plantelor medicinale sunt din ce în ce mai mici, iar în habitatele lor naturale, multe dintre aceste plante sunt pe cale de dispariție. În această categorie intră şi *Scopolia carniolica*, plantă care acumulează în rizomul ei atât scopolamină, cât şi hiosciamină (atropină). Una dintre alternativele cu şansă de reuşită este obținerea compuşilor faramcologic activi prin intermediul culturilor de țesuturi, organe sau celule vegetale. Culturile de rădăcini adventive de *Scopolia carniolica* au fost induse din calusuri rizogene, fiind apoi menținute în mediile lichide MS şi B5. Aceste rădăcini au crecut foarte bine la întuneric, în mediul B5 cu 3% zaharoză şi 1.0 mg^{-1} IBA. Ele sintetizează, în condiții normale, atropină în cantități cuprinse între 0,05 – 0,08 mg/g substanță uscată şi scopolamină în concentrații cuprinse între 0,04 – 0,06 mg/g substanță uscată. Lumina a jucat un rol important atât în ceea ce priveşte creşterea rădăcinilor cât şi biosinteza celor doi alcaloizi tropanici. Astfel, creşterea rădăcinilor incubate la lumină a fost inhibată. În cazul biosintezei celor doi alcaloizi, s-a constatat că la întuneric rădăcinile sintetizează mai multă scopolamină, iar la lumină mai multă atropină. Putrescina adăugată în mediul de cultură a rădăcinilor în concentrația de 1 mM a stimulat puternic, la întuneric, biosinteza scopolaminei, cantitatea acesteia ajungând în ziua a 28-a de cultură, aproape de 0,15 mg/g de substanță uscată, față de numai 0,06 mg/g de substanță uscată în cazul martorului, iar la lumină a stimulat biosinteza atropinei aceasta ajungând de la nivelul de 0,08 mg/g de substanță uscată, la peste 0,12 mg/g de substanță uscată. De asemenea suplimentarea mediului cu putrescină a avut un efect favorabil, la rădăcinile incubate la întuneric, asupra raportului dintre scopolamină şi atropină, acesta înclinând net în favoarea scopolaminei, raport care în rădăcinile şi în rizomul plantei intacte este invers.