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TROPANE ALKALOID BIOSYNTHESIS IN TISSUE CULTURES OF *SCOPOLIA CARNIOLICA* JACQ.

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Abstract: We used for the regeneration and multiplication of *Scopolia carniolica* nodal and caulinar apex explants, whereas for the calus induction, leaf, stem and root explants. These explants have been inoculated on the Murashige and Skoog (MS) medium, supplemented with various concentrations and combinations of growth regulators. The best combination of growth regulators for neoplantlets regeneration was: NAA or IBA (1.5 mg^{-1}) + BA (0.5 mg^{-1}), and for multiplication: NAA (0.1 mg^{-1}) + thidiazuron (0.5 mg^{-1}). The undifferentiated calluses have developed very well on the medium with picloram (1.5 mg^{-1}) + BA (0.2 mg^{-1}), the rhizogenesis being inducted by NAA (0.5 mg^{-1}). Chromatographic analyses indicated that only the plantlets' roots and the rhizogen calluses were able to biosynthesized just tropine and atropine. The total amount of tropanic alkaloids that accumulated in the plantlets' roots was 0.11 - 0.13% of the dry weight, while in the calluses that grew large roots (over 2 cm length), it was 0.08% of the dry weight.

Introduction

The plant kingdom supplies mankind from its beginnings with food, construction material and fuels. Plants are also an indispensable source for other products as spices, perfumes, insecticides and, last but not least, pharmaceutical compounds. In spite of the high advance of the organic chemistry of biosynthesis, plants still remain a very important source of pharmaceutical active compounds, most of them, except a few enzymes, being secondary metabolites. Because of many factors, amongst which the anthropic factor is the most important one, this reservoir of raw materials that are plants gradually decreased [19]. For all these reasons, plant tissue cultures became more important, because it is considered as an alternative method for seeds or vegetative multiplication. Nowadays, this method is highly used for either commercial propagation of many plants or in vitro preservation of rare, endemic and endangered plants [7].

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* [26]. These alkaloids antagonise acetylcholine at muscarinic receptors [13], 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 in the inner ear) [12]. Scopolamine differs from hyoscyamine only by an epoxidation of the tropane ring, a reaction that is catalysed by hyoscyamine 6β-hydroxylase [9]. Scopolamine is more valuable than hyoscyamine, and is currently commercially extracted from whole plants of the *Duboisea* genus [14].

Scopolia carniolica Jacq., perenial plant that exists in the mountain beech woods region, in wet and shady places. In our country it is considered as a rare species. *S. carniolica* accumulates in its rhizome scopolamine as well as atropine [28]. Because of abusive harvesting, especially for export, this species is threatened with extinction. Thus, although this species has been quoted in many counties, in present there have been identified merely two stations where rhyzomes are still being harvested: Remeti (Maramureş county) and Săcuieu (Cluj county) [22].

There are no studies in our country regarding the in vitro regeneration and multiplication of *S. carniolica,* and the data that could be found in foreign literature concern other species of *Solanaceae* [17,25]. In different foreign publications the synthesis of tropane alkaloids is studied especially in callus and suspension cultures [2,15,21,23,24,26] and very rarely in plants that are regenerated or multiplicated in vitro [4,11].

In this paper, we studied some aspects regarding in vitro regeneration and multiplication of *S. carniolica*, as well as undifferentiated or rhizogenic callus cultures induction. We also studied the tropane alkaloids synthesis in neoplantlets and callus cultures.

Material and methods

Tissue cultures have been induced from 15 days old plantlets of *Scopolia carniolica*, obtained by in vitro germinated seeds [5]. Explants of leaves, stems (nodes and internodes) and roots have been excised from these plantlets and inoculated on Murashige and Skoog medium (MS) [16] with 3% sucrose and 0.8% agar. For plant regeneration and multiplication, this medium was supplemented with different concentrations (0.1-1.5 mg^{-1}) and combinations of growth regulators as: 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 6-benzylaminopurine (BA), N6-[2-isopentenyl]adenine (2iP) and thidiazuron (TZ). For callus induction, MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram (P) (1.0-1.5 mg^{-1}) has been used. The pH of the media has been adjusted to 5.5 before autoclaving. The photoperiod regime was 16 h light (40 μmol/m^2/s) and 8 h dark, to 25±1 ºC. Cultures have been evaluated 6 weeks after inoculation; the multiplication rate (number of shoots/explants) and the

regeneration capacity (number of shoots with adventive roots/explant) have been followed. In the case of the callus induction experiments, the rate of callus biomass growth expressed in mg fresh weight/day/vessel has been followed. The growth rate was calculated according to the formula: $S_1 - S_0 / T_1 - T_0$, where S_1 = callus biomass at the end of the experiment; S_0 = callus biomass at the inoculation; T_1 = time at the end of the experiment; T_0 = the moment of inoculation.

Tropane alkaloids were extracted from dry weight (80ºC, 24 h) of neoplantlets and calluses. The qualitative analysis has been performed by thin layer chromatography (TLC) according to FR X [27] and Stahl [20] techniques. 0.6 g poudered plant material was shaken with H_2SO_4 0.05 M for 15 min, then filtered. 1.0 ml concentrate ammonium hydroxide was added to the filtered solution, then it was shaken twice in bolting funnel with 10 ml ether. The etheric phase was filtered through a filter containing anhydrous sodium sulphate and evaporated at drieness. The rest was dissolved in 0.5 ml methanol. Chromatographic plates: silicagel 60 G (Merck). Mobil phase: acetone:water:ammonium hydroxide conc. (90:7:3). Reference compound: atropine sulphate 0.450% (w/v) in methanol. Detection: the layer was dried at 105°C for 15 min then Dragendorff reagent was pulverized. The quantitative analysis of total alkaloids has been performed according to titrimetric technique indicated by FR X for *Belladonnae folium* [27]. 1.5 poudered plant material was thoroughly homogenized with a mixture of 0.75 ml ammonium hydroxide conc., 1.5 ml ethanol and 4.5 ml ether. After 12 h it was extracted with ether in a soxhlet apparatus for 4 h. The extract was shaken twice with H_2SO_4 0.25 M. The pH of the aqueous solution was set to 9-10 by adding ammonium hydroxide, then extracted with chlorophorm. The chlorophormic solution was dried and the rest was kept at 105°C for 1 h, then dissolved into 5 ml chlorophorm. 15 ml H_2SO_4 0.01 M were added and the remaining acid titrated with NaOH 0.02 M.

Results and discussion

Plant regeneration, multiplication and callus induction. One of the goals of this paper was to find the optimal culture medium for in vitro plant regeneration and multiplication of *Scopolia carniolica*. One of the most important factors is the influence of growth regulators. The aim of the experiments was to study the effect of cytokines and auxins on the multiplication rate and regeneration capacity. Thus, the explants of nodes and stem apexes have been inoculated on MS medium supplemented with 3% sucrose and 0.8% agar, in different hormonal variants [5]:

V_1 = NAA (1.5 mg^{-1}) + BA (0.3 mg^{-1})
V_2 = NAA (1.5 mg^{-1}) + 2iP (0.3 mg^{-1})
V_3 = 0
V_4 = NAA (0.1 mg^{-1}) + BA (0.5 mg^{-1})
V_5 = NAA (0.5 mg^{-1}) + BA (1.5 mg^{-1})
V_6 = NAA (0.1 mg^{-1}) + TZ (0.5 mg^{-1})
V_7 = NAA (1.5 mg^{-1}) + BA (0.5 mg^{-1})
V_8 = IBA (1.5 mg^{-1}) + BA (0.5 mg^{-1})
V_9 = IBA (1,5 mg^{-1}) + BA (1.5 mg^{-1})
V_{10} = IBA (0.5 mg^{-1}) + BA (0.5 mg^{-1})

A very important factor for plant regeneration, especially for in vitro plant multiplication, is the cytokinins' type and concentration, as well as their combination with auxins. Thus, we observed that in the media supplemented with a higher concentration of cytokinins than auxins (5:1), the multiplication rate was high. This happened on media, 4, 5 and especially on media 6, where the multiplication rate was 3-5 shoots/explant (Fig. 1). This medium (V_6) contained a cytokinin similar to zeatine – thidiazuron (that has a very strong cytokininic effect). Our results are in concordance with the results that Nishioka et al. [18] obtained on the tissue cultures of *Scopolia japonica.* They found that those media where the cytokinins concentration was higher than the auxins concentration stimulated the plant multiplication.

Although our results are satisfactory, we observed that on those media where the multiplication rate is high, the induced shoots are not rooted. This is the case of media 6 where the percent of shoots without the roots is 100%.

Regarding the regeneration capacity (number of rooted plants/explant) we observed that optimal media have been V_7 and V_8 (Fig. 2). One these media where the growth regulators combination have been NAA or IBA (1.5 mg^{-1}) + BA (0.5 mg^{-1}) and the cytokines:auxins ratio was 1:3, the regeneration capacity was higher than 97%. This stresses out the fact that supplementing the media with high concentration of auxins (more than 0.5 mg^{-1}) stimulated the rhizogenesis and the kept the number of plantlets with no roots extremely low (Fig. 2).

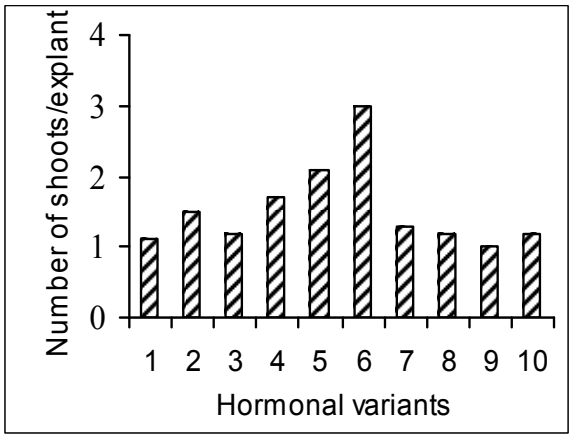

Fig. 1. The effect of the hormonal balance on the in vitro multiplication rate at *Scopolia carniolica.*

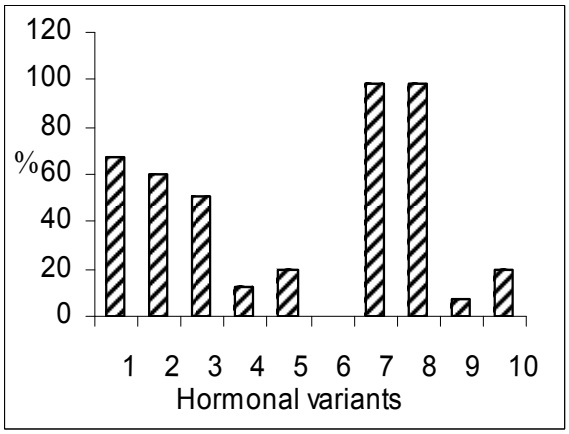

Fig. 2: The influence of the hormonal balance on the regeneration of normally conformed *Scopolia carniolica* plantlets.

For the development of callus cultures of *S. carniolica*, the calluses induced from different types of explants (root, stem, leef) have been transferred on MS

medium supplemented with 2,4-D, picloram and BA in different concentrations and combinations (Table 1).

Table 1: Growth of *Scopolia carniolica* calluses on MS medium supplemented with 2,4-D and picloram (P) in combination with BA (photoperiode regime 16/8).

Growth regulators	Explant	Fresh weight (g/flask)	Growth rate (mg fw/flask/day)	% of dry weight
2,4-D (1.0 mg^{-1}) + BA (0.1 mg^{-1})	Root	3.825	76.1	4.6
	Stem	2.712	49.2	4.7
	Leaf	3.228	61.0	5.1
2,4-D (1.5 mg^{-1}) + BA (0.2 mg^{-1})	Root	4.657	92.0	4.2
	Stem	2822	51.6	4.4
	Leaf	4.107	81.0	4.8
P (1.0 mg^{-1}) + BA (0.1 mg^{-1})	Root	3.932	76.3	4.8
	Stem	2.421	42.7	4.7
	Leaf	3.379	64.0	5.5
P (1.5 mg^{-1}) + BA (0.2 mg^{-1})	Root	4.726	93.9	4.5
	Stem	3.056	56.8	4.6
	Leaf	4.213	82.5	5.2

Our results show that those calluses that were induced from root and leaf (especially from root) developed a high quantity of cellular biomass. Thus, in this case, the growth rate expressed in fresh weight was much higher, compared to the growth rate of calluses induced from stem explants (Table1). From all 4 variants of hormonal combinations, those variants containing picloram and BA have been the best for callus development. The consistency of callus induced from roots was friable and compact in the case of the callus induced from leaf. This is also pointed out by the percentage of dry weight, which is higher in the case of foliar calluses.

For the induction of the organogenesis, respectively rhizogenesis process, peaces of callus have been transferred on media supplemented with NAA (0.5 mg^{-1}). As showed in Table 2, as well as in Table 1, the calluses induced from root and leaf have a higher growth rate. After 6 weeks, the calluses induced especially from root and leaf developed roots having cca 4 cm length. On the calluses induced from stem, the number and dimensions of induced roots have been much lower, which demonstrates the high influence that the type or origin of the explant from which the callus is originated has on the organogenesis process (Table 2).

In this experiment, we studied the influence of light on the rhisogenesis process. In this case we observed the major effect of the origin of the explant and the lower effect of the light. Although in many cases the rhizogenesis is stimulated under dark, in this case the roots grew very well under 16 h light/8 h dark regime and 40 $\mu mol/m^2/s$ light intensity.

Table 2: The effect of the explant origin and of the light on the growth rate and rhizogenesis from callus of *Scopolia carniolica* cultivated on MS medium supplemented with NAA (0.5 mg^{-1}).

Variants*	Fresh weight (g/flask)	Growth rate (mg fw/flask/day)	% of dry weight	Number of roots per callus	Length of roots (cm)
C_R D	1.567	23.7	6.3	35.4	2.9
C_R L	2.954	54.5	7.1	38.2	4.1
Cs D	1.620	23.8	6.0	11.8	0.9
Cs L	1.855	30.1	6.7	14.0	1.2
C_L D	2.065	34.7	6.1	22.5	2.1
C_L L	2.865	52.6	7.3	28.6	3.4

*C_RD and C_RL = calluses induced from roots grown in the dark and in L/D, respectively.
CsD and CsL = calluses induced from stems grown in the dark and in L/D, respectively.
C_LD and C_LL = calluses induced from leaves grown in the dark and in L/D, respectively.

Tropane alkaloid synthesis. There are only a few studies of the in situ synthesis of tropane alkaloids at *Scopolia carniolica,* and there are no studies at all about the in vitro synthesis. In our experiments some samples have been harvested in order to determin the localization of biosynthesis and of the accumulation of tropane alkaloids as: leaves and stems of whole plantlets and plantlets without roots; roots of plantlets; undifferentiated calluses; calluses with incipient stage of rhizogenesis; calluses with well developed roots (more than 2 cm length). The results of qualitative analyses (TLC) emphasized certain aspects. Thus, in the case of stems and leaves, independentely of the provenience of samples from rooted plants or from shoots with no roots, there is no tropane alkaloid synthesis (no spots on the chromatograme). The two spots have been identified only in the case of the roots extracts. Considering their Rf, color and the comparison with the control samples, they have been identified as: the first spot – tropine (R_f = 0.05), which is one of the atropine precursors; the second spot – atropine (R_f = 0.3). At the R_f > 0.9, there is another spot, probably an alkaloid unknown until now. Certainly, in vitro biosynthesis is not similar with the in situ biosynthesis that takes place in the rhizome, where, alongside atropine, there has been detected scopolamine also (as a result of the hydroxylation of hyoscyamine) [22]. These results seem to be natural, because after 6 weeks in vitro cultivated plants have only adventives roots, the rhizome being still undeveloped. Thus, probably for this reason, the tropane alkaloids synthesis stops on the hyoscyamine, respectively atropine. Of course, there could be another explanation, such as the blocking of the hyoscyamin-6β-hydroxilase (H6H) synthesis, which catalyses the transformation of hyoscyamine in scopolamine, but this process has yet to be studied.

The recent studies on the activity of some enzymes involved in tropane alkaloids biogenesis showed that their synthesis takes place especially in the roots

of many species as: *Atropa belladonna, Hyoscyamus niger, Hyoscyamus albus, Datura stramonium,* from where they could be transported in leaves, where they are stored [6]. The obtained results showed that the first, as well as the last stage of scopolamine biosynthesis of tropane alkaloids takes place in the roots' pericycle [6,10]. These data are in accordance with our results that show that the tropane alkaloids synthesis in *Scopolia carniolica* takes place only in roots. Although in 1976 Guang-Zhi and Zeng [8] observed the hyoscyamine and scopolamine accumulation in the stems of *S. acutangula* cultivated in vitro, our analysis show that the 6 weeks old plantlets of *S. carniolica* accumulate the tropane alkaloids only on the roots without, transportation to other organs.

In the case of undifferentiated calluses, independentely of their origin, on the organogenetic or embryogenetic processes that take place on them, there is no tropane alkaloid synthesis. It is known that the attempts to produce secondary metabolites in disorganised cultures (suspension, callus, protoplast, etc.) have had very limited success. And this, because of the fact that disorganised cultures are unable to accumulate many secondary compounds, and consequently have very low yields [3]. There are several reasons for the low yield of secondary metabolites in culture. Many secondary compounds are only produced in specific tissue types, and often only when the plant is subject to stress or elicitation. Since disorganised cultures lack differentiated cell types, they are often unable to synthesize tissue-specific compounds. They may also lack the ability to accumulate the secondary metabolite (i.e. in the vacuole or extracellularly) [3]. This happened in callus tissues of *S. carniolica* as well, where tropane alkaloids synthesis took place only after their transplantation on media for organogenesis, respectively rhizogenesis. On the rhizogenic calluses, depending on the intensity of the organogenetic process, tropine and atropine could be detected on the chromatograme. Thus, in the tissues with an incipient stage of rhizogenesis, only tropine could be detected, while, in the calluses with well-developed roots, both tropine and atropine could be detected on the chromatograme. There is no difference between the pattern of tropane alkaloids synthesized by plantlet roots and those roots that are induced from callus.

Our results are in accordance with some results obtained by other researchers, who observed the lack of capacity to synthesise tropane alkaloids of undifferentiated cultures of some species of *Solanaceae.* In this respect, Remeike and Koblitz (1970) quoted by Yamada and Tabata [26], showed that these authors did not detect neither scopolamine nor hyoscyamine in tissue cultures of *Datura*. In 1971 Tabata et al. [23] found that in callus cultures of *Datura* and *Scopolia* the amount of tropane alkaloids is very low and it became even lower during the successive subcultivations. Several authors [1,11,24] pointed out that in the case of cell and callus cultures of *Scopolia, Datura,* and *Hyoscyamus*, there is a direct relationship between morphological and chemical differentiation confirmed by

tropane alkaloids synthesis, which is installed progressively, together with the organogenic or rhizogenic process expression.

Quantitative analysis showed that in vitro regenerated roots of *Scopolia carniolica* plantlets accumulate 0.11 – 0.13% of the dry weight of total tropane alkaloids. There are no significant differences between the two hormonal variants (NAA + BA) and (IBA + BA). In rhizogenic calluses the amount of tropane alkaloids depends on the intensity of the rhysogenic process. Thus, in the case of calluses with incipient rhizogenesis, the level of these compounds was 0.04% of the dry weight, and in those calluses with 4 cm long roots the amount was 0.08% of the dry weight.

Conclusions

For the regeneration of *Scopolia carniolica* plantlets, the best combination in culture media was NAA or IBA (1.5 mg^{-1}) + BA (0.5 mg^{-1}), and for plant multiplication: NAA (0.1 mg^{-1}) + TZ (0.5 mg^{-1}). Undifferentiated calluses have been developed very well on MS medium supplemented with picloram (1.5 mg^{-1}) + BA (0.2 mg^{-1}), the rhizogenesis being induced by NAA (0.5 mg^{-1}). The tropane alkaloids synthesis (tropine and atropine) in 60 days old plants of *Scopolia carniolica* took place only in roots and their accumulation as well takes place in this organ, in an amount of 0.11 – 0.13% of the dry weight. Also, rhizogen calluses synthesize and accumulate different quantities of tropane alkaloids, depending on the organogenic process intensity (0.04 to 0.08% of the dry weight) and there is no difference between the pattern of tropane alkaloids synthesized by plantlets roots and by the roots induced from callus.

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BIOSINTEZA ALCALOIZILOR TROPANICI ÎN CULTURI DE ȚESUTURI DE *SCOPOLIA CARNIOLICA* JACQ.

(Rezumat)

Pentru regenerarea şi multiplicarea speciei *Scopolia carniolica* s-au utilizat explante de noduri şi apexuri caulinare, prelevate de la plante dezvoltate din semințe germinat aseptic, iar pentru inducerea calusurilor - explante de frunză, tulpină şi rădăcină. Acestea au fost inoculate pe mediul Murashige şi Skoog (MS) suplimentat cu diferite concentrații şi combinații de regulatori de creştere. Pentru regenerarea de neoplantule, cea mai bună combinație s-a dovedit a fi ANA sau AIB (1,5 mg^{-1}) + BA (0,5 mg^{-1}), iar pentru multiplicare: ANA (0,1 mg^{-1}) + TZ (thidiazuron) (0,5 mg^{-1}). Calusurile neorganogene s-au dezvoltat foarte bine pe mediul MS cu picloram (1,5 mg^{-1}) + BA (0,2 mg^{-1}), organogeneza, respectiv rizogeneza fiind indusă pe mediul MS cu ANA (0,5 mg^{-1}). Analizele cromatografice au indicat faptul că numai rădăcinile neoplantulelor şi calusurile rizogene posedă capacitatea de a biosintetiza şi acumula alcaloizii tropanici şi anume tropină şi atropină. Cantitatea totală de alcaloizi tropanici acumulată în rădăcinile plantulelor a fost de 0,11 – 0,13% din substanța uscată, iar în calusurile cu rădăcini bine dezvoltate (peste 2 cm în lungime) de 0,08%. Între tiparul alcaloizilor sintetizați de rădăcinile plantulelor şi cel al calusurilor rizogene nu există nici o diferență.