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Communication

In vitro plant tissue cultures accumulate polyisoprenoid alcohols★☆

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***In vitro* cultivated plant cells and tissues were found to synthesize polyisoprenoids. *Taxus baccata* suspension cell cultures accumulated polyisoprenoids of the same pattern as the parental tissue; methyl jasmonate or chitosan treatment almost doubled their content. All the root cultures studied accumulated dolichols as predominant polyisoprenoids. Roots of *Ocimum sanctum* grown *in vitro* accumulated approx. 2.5-fold higher amount of dolichols than the roots of soil-grown plants. Dolichols dominated over polyprenols in all *Triticum* sp. tissues studied.**

Keywords: dolichols, polyprenols, isoprenoids, HPLC

INTRODUCTION

Polyisoprenoid alcohols (dolichols and polyprenols) are linear five-carbon unit polymers present in almost all living cells. Dolichols (α-saturated isoprenoid alcohols) are found mainly in animal tissues (e.g. in rat and human organs) and yeast cells (Hemming, 1985), however, they have also been detected as a predominant form in plant roots (Tateyama *et al*., 1999; Skorupinska-Tudek *et al*., 2003). Polyprenols (α-unsaturated isoprenoid alcohols) are characteristic for bacterial cells and plant photosynthetic tissues. They have also been identified in wood, seeds and flowers. Analysis of the subcellular distribution of these compounds in leaves showed that polyprenols were mostly located in chloroplasts (Wellburn & Hemming, 1967; Hemming, 1983; Swiezewska *et al*., 1993; Kurisaki *et al*., 1997) while small amounts of dolichols were detected in microsomes (Sakaihara *et al*., 2000). Polyisoprenoid alcohols always constitute 'families' of prenologues differing in the number of isoprene residues. The 'family pattern' is considered as a species-specific feature and can be applied as a chemotaxonomic marker (Swiezewska *et al*., 1994). Dolichol families isolated from animal or yeast cells as well as from plant roots consist of six to eight compounds irrespective of the organism (e.g. Dol-16, -18 and -19 are predominant in yeast, rat and human, respectively). In contrast to dolichols, polyprenols represent a large diversity in chain length from Pren-6 to Pren-130. The content of all isoprenoid alcohols increases during the life-span of the organism. Seasonal fluctuations of polyprenol levels have also been described. The occurrence, structure, biosynthesis and function of polyisoprenoid alcohols have been summarized in recently published

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☆Supplementary information available at: www.actabp.pl

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Abbreviations: HPLC/ESI-MS, high-performance liquid chromatography/electrospray ionization mass spectrometry; Pren-n/Dol-n, prenol/dolichol composed of n isoprenoid units.

reviews (Rezanka & Vortruba, 2001; Swiezewska & Danikiewicz, 2005).

In cells both dolichols and polyprenols are found in the form of free alcohols and esters with carboxylic acids. A fraction of polyisoprenoid phosphate has also been detected. The biological role of phosphorylated polyisoprenoid alcohols: cofactors in the biosynthesis of glycoproteins, glycosylphosphoinositol (GPI) anchor and bacterial peptidoglycans is well characterized. They are also postulated to serve as a donor of isoprenoid groups during protein prenylation. In contrast, the role of free polyisoprenoid alcohols and carboxylic esters is uncertain. Biophysical studies on model membranes have shown that they increase their fluidity and permeability and also enhance membrane fusion. These compounds are also postulated to be involved in the transport of endoplasmic reticulum and vacuolar proteins.

In vitro cultures of plant tissues are widely studied as a source of pharmacologically important compounds (Rao & Ravishankar, 2002).

The aim of this study was to investigate the potential capacity of *in vitro* cultures from *Taxus baccata* and other plant species to biosynthesize polyisoprenoids.

MATERIALS AND METHODS

Plant material and chemicals. *In vitro* root cultures of plants were cultivated in darkness as follows. Transformed roots of *Withania somnifera* (local name Ashwagandha, *Solanaceae*) obtained using *Agrobacterium rhizogenes* strain ATCC 15834 were grown for 6 months in liquid B5 medium (Gamborg *et al*., 1968). Transformed roots of *Platycodon grandiflorum* (Ballon Flower, *Campanulaceae*) obtained using *A. rhizogenes* strain ATCC 15834, were grown for 5 months in liquid WPM medium (Llyod & McCown, 1980). Hairy roots of *Ocimum sanctum* (Holly Basil, Sacred Basil, *Lamiaceae*) initiated by inoculation of leaves with *A. rhizogenes* strain LBA 9402 were grown for 45 days in liquid WPM medium. *Taxus x media* var. *Hicksii* Rehd (*Taxaceae*) hairy roots were cultivated for 55 days in modified solid DCR medium (Furmanowa & Syklowska-Baranek, 2000).

Suspension culture of *T. baccata* (*Taxaceae*) was established from a callus obtained from stem of a female tree (Botanical Garden of Warsaw University) and was further cultivated in liquid WR II medium (Furmanowa *et al*., 1995) illuminated with white light at 5×10^{-5} E s^{-1} m^{-2} on a gyratory shaker at 110 rpm. Approximately 6.0 g (fresh weight) of 30 day-old cells was used to inoculate 100 ml of fresh medium. One week after inoculation elicitors, methyl jasmonate (100 μM final concentration) or chitosan (1.8 mg/l final concentration) were added to the flasks, and the cells were harvested 7 days later.

Seeds of *Triticum* sp. (*Poaceae*) were bought in an open-air local market. Wheat seedlings were obtained by germination of seeds in darkness for 14 days in vermiculite at room temperature. Cotyledons and roots were collected separately.

Branches with needles (one and two year-old) of a female tree of *T. baccata* and organs of *O. sanctum* plants (leaves, stems and roots) were from the Botanical Garden of Warsaw University and the field of the Medical University of Warsaw, respectively.

All dolichol and polyprenol standards were from the Collection of Polyprenols (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warszawa). Chromatographic materials were from Merck, HPLC solvents from Baker B.V. (Deventer, Holland). All the other chemicals of analytical grade were purchased from Sigma.

Isolation of polyisoprenoid alcohols. Organs of *W. somnifera*, *P. grandiflorum*, *T. media*, *O. sanctum*, *Triticum* sp. were dried in the open air, pulped in a ceramic mortar and pestle and extracted with acetone/hexane (1 : 1, v/v) for 48 h at 40°C. *T. baccata* cells grown in suspension culture were spun down, lyophilized and subsequently extracted as described above. Branches and needles of *T. baccata* were homogenized separately with Ultra Turrax homogenizer (Ultra Turrax, Germany) in a mixture of acetone/hexane (1 : 1, v/v) and extracted as above. Polyisoprenoid content, after HPLC estimation was further recalculated per dry weight of the tissue.

After filtration and evaporation of the solvents the residue was treated with alkali for 1 h at 90°C (Skorupinska-Tudek *et al*., 2003). The mixture was fractionated into two phases with addition of diethyl ether and water. The upper phase containing lipids was collected, evaporated, dissolved in hexane and purified on Silica Gel 60 (230–400 mesh, Merck, Darmstadt, Germany) column equilibrated with hexane and eluted with hexane containing increasing concentrations of diethyl ether (0–15%). Fractions were analyzed by thin-layer chromatography (TLC) on Silica Gel plates in solvent A (toluene/ethyl acetate, 9 : 1, v/v) and on RP-18 plates in solvent B (acetone). Spots of lipids were visualized with iodine vapors. Fractions with detectable amounts of polyisoprenoid alcohols were subsequently subjected to HPLC analysis.

HPLC analysis of polyisoprenoid lipids. Analysis of lipids was performed using a Waters dual pump apparatus (Waters Ass., USA) and a Hypersil ODS column (4.6 × 60 mm, 3 μm; Knauer, Germany) with the solvent system: A — methanol /water, 9 : 1 (v/v); B —methanol/propan-2-ol/hexane, 2 : 1 : 1 (by vol.) and a flow rate of 1.5 ml/min controlled by a Waters gradient programmer. The UV

detector (Waters 484) was set at 210 nm as described earlier (Skorupinska-Tudek *et al*., 2003). The amount of dolichols and polyprenols was estimated by comparison with an external standard of Dol-23. The chain length and identity of lipids was confirmed by applying qualitative standards (Pren-16, Dol-16), mixture of polyprenols (Pren-11, to Pren-23) and a mixture of dolichols (Dol-17, -18, -19, -20, -21). All the estimations were performed in duplicate.

RESULTS AND DISCUSSION

Polyisoprenoid alcohols from *in vitro* cultures

For many years plant photosynthetic tissues were considered the best available source of polyisoprenoids as documented during 40 years of studies carried on at the Department of Lipid Biochemistry (IBB PAS, Warszawa, Poland). More recently composition of other tissues such as seeds and roots was also investigated. This observation raised the question whether *in vitro* cultivated plant tissues might be the source of long chain polyisoprenoid alcohols.

T. baccata cells grown in suspension were found to synthesize a two-family mixture of polyprenols (ranging from Pren-15 to Pren-25, with Pren-17 and Pren-22 dominating, respectively) (Supplementary Fig. 1e). In younger, two week-old culture cells the mixture of polyprenols was accompanied by traces of dolichols (not shown). Interestingly, the pattern of polyprenols was identical as that found previously in *T. baccata* needles (Swiezewska & Chojnacki, 1988). The content of polyisoprenoids was approx. 4-fold increased for 4-week-old cells (Table 1). In an attempt to increase the accumulation of polyisoprenoids *T. baccata* cells were treated with methyl jasmonate or chitosan. Indeed, polyprenol content reached approx. 190% and 170% of the control for jasmonate and chitosan treatment, respectively. Additionally, older and chitosan-treated cells were found to produce slightly higher amounts of longer polyprenols constituting the second family in the mixture. Age-dependent stimulation of production of longer polyisoprenoid alcohols was reported earlier for the yeast *Saccharomyces cerevisiae* (Szkopinska *et al*., 2002).

In all root cultures studied the amount of dolichols highly exceeded polyprenols (see Supplementary Fig. 1 and Table 1). In *T. media* and *O. sanctum* roots (Supplementary Fig. 1c and d) two families of dolichols were observed. In *T. media* the polyisoprenoid mixture was composed of dolichols ranging from Dol-14 to Dol-25, with Dol-17 and -22 dominating, respectively. A mixture of somewhat longer dolichols was detected in the roots of *O. sanctum*, namely Dol-15 to Dol-31, with Dol-18 and -26 dominating, respectively. In contrast, a single family of dolichols was extracted from *W. somnifera* (Dol-14 to -21, with Dol-17 dominating) (Supplementary Fig. 1a) while the one isolated from *P. grandiflorum* was slightly broader, from Dol-14 to Dol-28, with Dol-16 dominating (Supplementary Fig. 1b). In all the root samples small amounts of polyprenols of the respective chain-length were observed which upon HPLC analysis formed tiny, not well-separated pre-peaks accompanying a much higher signal of dolichols. The occurrence of minute amounts of polyprenols accompanying dolichols should be considered as typical feature of roots as recently documented by application of HPLC/ESI-MS for the roots of *Coluria geoides* (Skorupinska-Tudek *et al*., 2003).

Total polyisoprenoid content was lowest in the roots of *O. sanctum* (10.6 μg/g d.w.), and highest in *T. media* (21.8 μg/g d.w.) (Table 1). However one should keep in mind that accumulation of polyisoprenoids increases during the senescence of the tissue and might differ for different species.

Table 1. The content of polyisoprenoid lipids in various *in vitro* cultures.

Each experiment was performed in duplicate.

In vitro cultures	Content of isoprenoid alcohols (μg/g of d. w.)			Dominating prenologues
	Experiment #1	#2	Mean	
T. baccata suspension culture				
2 week-old	18	28	23	Pren-17, Pren-22
4 week-old	67	111	89	Pren-17, Pren-22
2 week-old + jasmonate	30	56	43	Pren-17, Pren-22
2 week-old + chitosan	26	52	39	Pren-17, Pren-22
Hairy roots				
T. media	15	29	22	Dol-17, Dol-22
O. sanctum	6	15	11	Dol-18, Dol-26
W. somnifera	10	20	15	Dol-17
P. grandiflorum	14	26	20	Dol-16

Table 2. Pattern and content of polyisoprenoid lipids in plant organs.

Numbers in bold indicate the dominating prenologues; nd – not detected.

Plant	Organ	Total isoprenoid alcohols (μg/g of d.w.)			Polyprenols (number of isoprene units)
		Experiment #1	#2	Mean	Dolichols (number of isoprene units)
Triticum sp.	seeds	4	14	9	13 14 **15** 16 17 12 13 14 **15** 16 17 18
	cotyledons	8	18	13	13 14 **15** 16 17 13 14 **15** 16 17
	roots	56	112	84	12 13 14 **15** 16 17 18 12 13 14 **15** 16 17 18
O. sanctum	leaves	77	133	100	10 11 12 13 14 15 16 17 **18** 19 20 21 22 23 24 **25** 26 27 ...60 14 15 16 17 **18** 19 20 21 22
	stems	3	9	6	traces 15 16 17 **18** 19 20 21 22 23 24 25 **26** 27 28
	roots	2	6	4	traces 15 16 17 **18** 19 20 21 22 23 24 25 **26** 27 28 29
T. baccata	1 y.o. needles	482	724	603	15 16 17 **18** 19 20 21 **22** 23 24 25 26 27 nd
	2 y.o. needles	4200	5880	5040	15 16 17 **18** 19 20 21 **22** 23 24 25 26 27 .... 30 nd
	1 y.o. branches	499	831	665	15 16 17 **18** 19 20 21 **22** 23 24 25 26 nd
	2 y.o. branches	1218	1828	1523	15 16 17 **18** 19 20 21 **22** 23 24 25 26 nd

Polyisoprenoid lipids in plant organs

Accumulation of polyisoprenoids in tissues of *T. baccata* and *O. sanctum* grown in the soil was further analyzed (Supplementary Fig. 2 and Table 2). Branches and needles of *T. baccata* accumulated a mixture of polyprenols with a pattern identical to four-week-old suspension culture (Supplementary Fig. 2e and Table 2), again the same as found earlier for the needles (Swiezewska & Chojnacki, 1988). As expected, in needles and branches the total content of polyprenols increased with age, however, the former one (Table 2) was lower than reported earlier. This was probably caused by different climate and environmental conditions in which the yews were grown. According to the literature photosynthetic tissues exposed to direct sunlight accumulate higher amounts of polyprenols (Bajda *et al*., 2005).

Concurrently this is the first evidence of accumulation of polyprenols in the branches of *T. baccata*. Neither needles nor branches accumulated dolichols. Upon quantitative analysis of HPLC spectra the proportion of longer polyprenols was found to be to some extent increased in older tissue, similarly to the observation described above for *Taxus* cells grown in suspension culture.

An identical dolichol pattern was found for both types of basil roots (Supplementary Figs. 1d and 2d). Unexpectedly, 2.5-fold lower dolichol content was found in roots grown in the soil in comparison to root culture. Leaves of *O. sanctum* accumulated a two-family mixture of polyprenols (Pren-10 to Pren-60, with Pren-18 and -25 dominating, respectively) (Supplementary Fig. 2c). HPLC/ESI-MS analysis confirmed their structure and additionally proved that polyprenols were accompanied by traces (approx. 10% of polyprenol content) of dolichols (Dol-14 to Dol-22, Dol-18 dominating) (Table 2). Polyisoprenoid composition of basil stems was identical to that found for its roots (Table 2).

As expected, in photosynthetic tissues of both angiosperm and gymnosperm plants the content of polyisoprenoid lipids was much higher than in other tissues of the same plant, with one exception of the roots of *Triticum* sp. (Table 2), though in the latter case etiolated seedlings were analyzed. For all the tissues of *Triticum* sp. (seeds, cotyledons, roots) an identical polyisoprenoid pattern comprising a mix-

ture of polyprenols and dolichols (Pren/Dol-12 to Pren/Dol-18, Pren/Dol-15 dominating) was observed with comparable ratio of polyprenols and dolichols (prenol/dolichol approx. 1 : 3) (Supplementary Fig. 2a and b). A similar atypical composition of the polyisoprenoid mixture isolated from photosynthetic tissue has been observed earlier for some plant species, i.e., leaves of *Capparis coriacea* (Jankowski & Chojnacki, 1991), leaves of the fern (Wojtas *et al*., 2005), shoots of *Hevea brasiliensis* (Tateyama *et al*., 1999) and seeds of some dicotyledonous plants (Ravi *et al*., 1983), however, contradictory results indicating polyprenols as dominating prenologues in seeds of several gymnosperm and angiosperm plants were presented recently (Skorupinska-Tudek *et al*., 2003; Chouda & Jankowski, 2005). Typically, for leaves polyprenols are significantly dominating over dolichols reaching the ratio 800 : 1 in old leaves of *Gingko biloba* (Tateyama *et al*., 1999).

CONCLUSIONS

In vitro root cultures might be a potential source of polyisoprenoid alcohols and this seems particularly important for short- and long-chain dolichols with limited abundance in the animal kingdom.

It is still not clear why in some plant tissues polyprenols are the predominant compounds whereas dolichols dominate in the others. It could be the result of change in balance of particular compounds as end-products of biosynthesis. Observed developmental changes of the dolichol/polyprenol ratio (increased dolichol content in young tissues) might be possibly explained by the higher requirement for dolichyl phosphate, a cofactor of protein glycosylation, in the rapidly dividing cells.

Worth noting is the similarity of polyisoprenoid composition of the *T. baccata* suspension cell culture and the parental material. There are numerous examples of secondary metabolites which are almost not produced in suspension cell cultures (Bourgaud *et al*., 2001; Pasquali *et al*., 2006). This might be an indication of the significant yet still unidentified role of polyisoprenoids in primary cellular metabolism.

Additionally, this observation might lead to a really interesting experimental design. According to the literature polyprenyl diphosphates which are the end products of *cis*-prenyltransferase, an enzyme responsible for the synthesis of the polyisoprenoid backbone (Kharel & Koyama, 2003), are further converted towards dolichols by a still unresolved mechanism. Induction of organogenesis of cells grown in suspension (producing polyprenols) leading to the formation of roots (synthesizing dolichols) could make possible the study of regulation of the terminal steps of dolichol biosynthesis.

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REFERENCES

Bajda A, Chojnacki T, Hertel J, Swiezewska E, Wojcik J, Kaczkowska A, Marczewski A, Bojarczuk T, Karolewski P, Oleksyn J (2005) *Acta Biochim Polon* **51:** 233–241.

Bourgaud F, Gravot A, Milesi S, Gontier E (2001) *Plant Science* **161**: 839–851.

Chouda M, Jankowski W (2005) *Acta Biochim Polon* **52**: 243–253.

Furmanowa M, Syklowska-Baranek K (2000) *Biotechnol Lett* **22**: 683–686.

Furmanowa M, Glowniak K, Zobel A, Guzewska J, Zagorka G, Rapczewska L, Jozefczyk A (1995) Taxol in *Taxus baccata* L. var. Elegantissima organs and in tissue culture. *Ninth Forum for Applied Biotechnology. 27–29 September Gent (Belgium) Proceedings Part*: 2115–2118.

Gamborg OL, Miller RA, Ojima K (1968) *Exp Cell Res* **50**: 151–158.

Hemming FW (1983) *Biochem Soc Trans* **11**: 497–504.

Hemming FW (1985) In *Glycolipids*. Wiegandt H eds, pp 261–305. Elsevier Science Publishers B. V., Amsterdam.

Jankowski WJ, Chojnacki T (1991) *Acta Biochim Polon* **38:** 265–276.

Kharel Y, Koyama T (2003) *Nat Prod Rep* **20**: 111–118.

Kurisaki A, Sagami H, Ogura K (1997) *Phytochemistry* **44**: 45–50.

Lloyd GB, McCown BH (1980) *Proc Int Plant Propag Soc* **30**: 421–427.

Pasquali G, Porto DD, Fett-Neto AG (2006) *J Biosci Bioeng* **101**: 287–296.

Rao SR, Ravishankar GA (2002) *Biotechnol Adv* **20**: 101–153.

Ravi K, Rip JW, Carrol KK (1983) *Biochem J* **213**: 513–518.

Rezanka T, Votruba J (2001) *J Chromatogr A* **936**: 95–110.

Sakaihara T, Honda A, Tateyama S, Sagami H (2000) *J Biochem* (Tokyo) **128**: 1073–1078.

Skorupinska-Tudek K, Bienkowski T, Olszowska O, Furmanowa M, Chojnacki T, Danikiewicz W, Swiezewska E (2003) *Lipids* **38:** 981–990.

Swiezewska E, Chojnacki T (1988) *Acta Biochim Polon* **35:** 131–147.

Swiezewska E, Dallner G, Andersson B, Ernster L (1993) *J Biol Chem* **268**: 1494–1499.

Swiezewska E, Sasak W, Mańkowski T, Jankowski WJ, Vogtman T, Krajewska I, Hertel J, Skoczylas E, Chojnaki T (1994) *Acta Biochim Polon* **41**: 221–260.

Swiezewska E, Danikiewicz W (2005) *Progr Lipid Res* **44**: 235–258.

Szkopinska A, Swiezewska E, Rytka J (2002) *Acta Biochim Polon* **49**: 781–787.

Tateyama S, Wititsuwannakul R, Wititsuwannakul D, Sagami H, Ogura K (1999) *Phytochemistry* **51**: 11–15.
Wellburn AR, Hemming FW (1967) *Biochem J* **104**: 173–177.
Wojtas M, Bienkowski T, Zelman-Femiak M, Tateyama S, Sagami H, Chojnacki T, Danikiewicz W, Swiezewska E (2005) *Acta Biochim Polon* **52**: 255–259.