[Chem. Pharm. Bull. 33(12)5445—5448(1985)]

Alkaloid Composition and Atropine Esterase Activity in Callus and Differentiated Tissues of *Duboisia myoporoides* R. BR.

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(Received May 9, 1985)

Alkaloid composition and atropine esterase activity in callus and differentiated tissues from callus and from the original plant of *Duboisia myoporoides* were determined. Callus and differentiated shoots from callus contained no tropane alkaloids, though they contained very small amounts of anabasine and nicotine. In contrast, differentiated roots from callus contained all the kinds of alkaloids found in the original plant, *i.e.*, atropine, scopolamine, anabasine, nornicotine and nicotine. Neither callus nor differentiated leaves showed atropine esterase activity, but differentiated roots did show atropine esterase activity. These results indicate that the root plays an important role in both tropane alkaloid biosynthesis and degradation, whereas the leaf does not.

Keywords——*Duboisia myoporoides*; callus; differentiated tissue; alkaloid; atropine esterase

Scopolamine and atropine are important anesthetic and antispasmodic drugs, the production of which depends on plant sources. For such drug production, it is important to know about alkaloid degradation as well as biosynthesis in the plants. Atropine esterase is an enzyme involved in the decomposition of atropine to tropine and tropic acid. So far, there are a few reports on the atropine esterase activity of *Datura*,[1-4] but no papers have been published on the enzyme activity in the cultured tissues of plants such as *Datura*, *Atropa*, *Scopolia*, *Hyoscyamus* and *Duboisia*.

In the present study, we investigated the atropine esterase activity, as well as alkaloid composition, in both cultured tissues and the original plant of *Duboisia myoporoides*. The results show that the root has the capability for both tropane alkaloid biosynthesis and degradation, but the leaf is the organ of alkaloid accumulation, in which no degradation is observed.

Materials and Methods

Plant Materials and Culture Methods——*Duboisia myoporoides* R. BR. was obtained from the Medicinal Plant Garden of Nagasaki University. Leaves, stems and roots of the original plant were harvested from a mature tree of 5 m in height. Callus cultures were derived from a stem of the tree on the Murashige-Skoog basal agar medium[5] (BM) supplemented with 2,4-dichlorophenoxyacetic acid (1 mg/l) and kinetin (0.01 mg/l) in the dark at 26°C. Callus was subcultured every month on the same medium under the same circumstances. Differentiated roots were induced when the callus pieces were placed on BM containing 3-indolebutyric acid (5 mg/l) for 2—3 months in the dark. Differentiated shoots were obtained from callus pieces which were cultured on BM containing 6-benzylaminopurine (5 mg/l) for 2—3 months under continuous light.

Extraction and Qualitative Analysis of Alkaloids——Original plant materials and cultured tissues were lyophilized and then powdered. One gram of a sample was extracted overnight with 20 ml of 80% MeOH three times. The combined extract was evaporated to dryness under reduced pressure at below 50°C, and the residue was dissolved in 20 ml of distilled water followed by filtration. The filtrate was extracted with 10 ml of $CHCl_3$ three times. The aqueous layer was adjusted to pH 9.0 with 0.1 N NaOH and extracted with 10 ml of $CHCl_3$ three times. The combined $CHCl_3$ layer was used for analysis after being dried with Na_2SO_4. Identification of the alkaloids was reported previously.[6] A sample was subjected to thin-layer chromatography (TLC) in comparison with authentic alkaloids.

The alkaloids were separated on a Silica gel GF_{254} plate with $CHCl_3$–EtOH–28% NH_4OH (85 : 14 : 1) and with 70% EtOH–28% NH_4OH (99 : 1). The tropane and pyridine alkaloids were detected by spraying with Dragendorff's reagent. For detection of pyridine alkaloids, BrCN vapor[7] was also used.

Quantitative Estimation of Alkaloids by Dual-Wavelength Densitometry——The alkaloid extract corresponding to 0.01—0.05 g (dry weight) of the original plant or to 0.1—1.0 g (dry weight) of the cultured tissues was prepared for TLC. The alkaloids were separated on the plates (Silica gel GF_{254}, Merck, 20 cm × 20 cm) using the solvent mixture of $CHCl_3$–EtOH–28% NH_4OH (85 : 14 : 1). Pyridine alkaloids and tropane alkaloids were visualized by using BrCN vapor and Dragendorff's reagent, respectively. The alkaloids were determined by dual-wavelength densitometry (Shimadzu CS-910). The wavelengths of samples ($=\lambda_S$) and references ($=\lambda_R$) used were as follows: atropine and scopolamine, 505 nm (λ_S) and 560 nm (λ_R); anabasine, 535 nm (λ_S) and 660 nm (λ_R); nornicotine and nicotine, 465 nm (λ_S) and 560 nm (λ_R).

Assay of Atropine Esterase Activity——Atropine esterase activity of the original plant and cultured tissues was determined by a radiochemical method using [carbonyl-^{14}C]atropine sulfate-monohydrate as a substrate, according to the procedure described in the previous paper.[4] Fresh plant material or cultured tissues (200—500 mg) were homogenized in a chilled mortar with 0.2 M phosphate buffer (pH 7.0, 1—3 ml). After centrifugation at 14000g at 4°C for 20 min, the supernatant was dialyzed for 3 h against the extraction buffer at 4°C. A portion of the dialyzed extract (1.0 ml) was mixed with 0.1 ml of the substrate solution containing 0.072 μmol [carbonyl-^{14}C]atropine sulfate-monohydrate (50 μg, 0.04 μCi). The mixture was incubated for 20 h at 26°C with a drop of toluene as a preservative in a covered test tube, then the enzyme reaction was terminated by addition of 10% HCl (0.2 ml). A mixture of atropine (0.25 mg) and tropic acid (0.25 mg) in water (0.5 ml) was added as a carrier. The whole was extracted with ether (7 ml) 3 times, dried over Na_2SO_4 and concentrated to dryness. The residue was dissolved in MeOH (1.0 ml) and mixed with 10 ml of toluene scintillator. The radioactivity was measured with a liquid scintillation counter (Aloka LSC-703). The radioactivity of the blank, obtained by the same method as described above using previously boiled enzyme, was subtracted from the total radioactivity of the sample. The enzyme activity was expressed as dpm per mg of protein. One thousand dpm correspond to 0.025 μg of tropic acid. Protein was determined according to the method of Bradford, using bovine serum albumin as the standard.[8]

Chemicals——Authentic samples of atropine, scopolamine, anabasine, nornicotine and nicotine were purchased commercially. [Carbonyl-^{14}C]atropine sulfate-monohydrate was synthesized according to the known methods,[9,10] and its specific activity was 593 μCi/mmol.

Results and Discussion

Alkaloid content of callus, and differentiated shoots and roots from callus of *Duboisia myoporides* were compared with that of the original plant (Table I). Atropine, scopolamine, anabasine, nornicotine and nicotine were found in the leaves, stems and roots of the original plant. No tropane alkaloids were detected in callus or differentiated shoots, though very small amounts of anabasine and nicotine were detected. In contrast, differentiated roots from callus produced all five kinds of alkaloids found in the original plant.

TABLE I. Alkaloid Content in Callus, Differentiated Tissues from Callus and the Original Plant of *Duboisia myoporoides*

Material	Alkaloid content (mg/g dry weight)				
	Atropine	Scopolamine	Anabasine	Nornicotine	Nicotine
Original plant					
Leaves	1.00±0.11	1.75±0.16	11.90±0.81	2.77±0.21	0.18±0.04
Stems	2.03±0.13	0.35±0.03	2.36±0.90	0.90±0.11	0.08±0.02
Roots	0.14+0.03	0.06+0.02	0.43+0.04	0.16+0.04	0.17+0.03
Callus	n.d.	n.d.	0.0010±0.0004	n.d.	0.0007±0.0004
Differentiated tissues					
Shoots					
Leaves	n.d.	n.d.	0.0005±0.0002	n.d.	0.0008±0.0004
Stems	n.d.	n.d.	0.0004±0.0002	n.d.	0.0005±0.0002
Roots	0.052±0.011	0.040±0.009	0.008±0.003	0.034±0.008	0.097±0.014

Average of three determinations (mean±S.E.); n.d., not detected (<0.0001).

TABLE II. Atropine Esterase Activity in Callus, Differentiated Tissues from Callus and the Original Plant of *Duboisia myoporoides*

Material	Sample (dpm/mg protein)	Blank (dpm)	Atropine esterase activity (dpm/mg protein)
Original plant			
Leaves	461 ± 10	450 ± 10	11 ± 14
Stems	537 ± 11	431 ± 10	106 ± 15
Roots	14005 ± 119	436 ± 10	13569 ± 119
Callus	443 ± 10	440 ± 10	3 ± 14
Differentiated tissues			
Shoots			
Leaves	437 ± 10	429 ± 10	8 ± 14
Stems	704 ± 12	422 ± 10	282 ± 16
Roots	950 ± 21	441 ± 10	509 ± 23

Atropine esterase activities in callus and differentiated tissues were also assayed and compared with that of the original plant (Table II). No atropine esterase activity was found in callus or leaves of differentiated shoots or the original plant. On the other hand, both differentiated roots from callus and from the original plant exhibited atropine esterase activity, though the roots of the original plant had much higher activity than differentiated roots from callus. There were also low atropine esterase activities in stems of the original plant and of differentiated shoots.

The original plant of *Duboisia myoporoides* contained not only tropane alkaloids but also pyridine alkaloids, as previously reported.[6,11] Alkaloid production by cultured tissues of *Duboisia* has been studied so far. Sipply and Friedrich detected atropine and valtropine in callus from leaves of *Duboisia myoporoides*,[12] while Kagei *et al.* detected valtropine and nicotine in cultured cells of *Duboisia leichhardtti*.[13] On the other hand, Griffin reported that neither callus nor differentiated shoots from callus of the hybrid of *Duboisia myoporoides* and *Duboisia leichhardtii* contained the normal *Duboisia* alkaloids.[14] The same results were obtained with *Duboisia leichhardtii* cultured tissues by Yamada and Endo.[15] We did not detect any tropane alkaloids, either, but found very small amounts of anabasine and nicotine in callus and differentiated shoots from callus of *Duboisia myoporoides*. The production of anabasine in callus and differentiated shoots of *Duboisia* has not been reported previously. Tropane alkaloids were found only in differentiated roots of *Duboisia myoporoides*, as in the cases of *Datura*, *Scopolia* and *Atropa*.[16-18] Clearly roots have the ability to biosynthesize both tropane and pyridine alkaloids.

Atropine esterase activity in roots of the original plant of *Duboisia myoporoides* was much higher than that in the other parts, as also found in *Datura*.[1-4] Stems of the original plant and differentiated shoots showed low activities, and leaves had no activity. Differentiated roots from callus showed atropine esterase activity, but the activity was very much lower than that of the roots of the original plant. This may be caused by differences of growth stages, cultural conditions and morphological states.

Differentiated roots from callus contained tropane alkaloids and displayed atropine esterase activity. Hydrolysis and synthesis of atropine are not reversible reactions, because the *in vitro* synthesis occurred only in the presence of the essential cofactors, adenosine triphosphate (ATP) and coenzyme A.[19] Since differentiated roots without aerial parts from callus contained tropane alkaloids, the alkaloids were synthesized in the roots. Grafting experiments with *Lycopersicum esculentum/Duboisia myoporoides*[20] and *Datura stramonium/Nicotiana tabacum*[21] support the view that the alkaloids are synthesized in the

roots and transported from the roots to the aerial parts. Thus the root is the most important organ for both tropane alkaloid biosynthesis and degradation. Differentiated leaves from both callus and the original plant showed no atropine esterase activity. Differentiated rootless leaves from callus contained no tropane alkaloids, but the leaves of the original plant showed high contents of the alkaloids. These results led us to the conclusion that in the original plant of *Duboisia myoporoides* atropine is synthesized in roots, then transferred to and accumulated in the leaves, where atropine is not hydrolyzed. Decomposition of atropine happens mainly in the roots. However, it is not clear whether atropine which is synthesized in the roots is hydrolyzed directly there or whether atropine which is accumulated in the leaves is transported back to the roots and then hydrolyzed there.

Acknowledgement We thank Miss Ooba for her technical assistance.

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