# Evidence for a wide-spread occurrence of the genes of quinolizidine alkaloid biosynthesis

## Induction of alkaloid accumulation in cell suspension cultures of alkaloid-'free' species

M. Wink and L. Witte*

*Institut für Pharmazeutische Biologie der Technischen Universität Braunschweig, D-3300 Braunschweig* and **Gesellschaft für Biotechnologische Forschung, D-3300 Braunschweig, FRG*

Received 13 June 1983

A short-time increase of quinolizidine alkaloid accumulation can be induced in cell suspension cultures of *Lupinus polyphyllus* by application of foreign alkaloids such as papaverine, coniine, and other compounds like cAMP, polyamines, or even by transfer of the cells into fresh, autoclaved cell culture medium. This induction can be inhibited by cycloheximide. Using the same method of induction we were able to show quinolizidine alkaloid accumulation in cell cultures of 6 species (*Conium maculatum, Daucus carota, Atropa belladonna, Chenopodium rubrum, Spinacia oleracea, Symphytum officinale*), which are supposed to produce other alkaloids or no alkaloids at all. This indicates that the genes of quinolizidine alkaloid biosynthesis are obviously not restricted to the Fabaceae family but are widely distributed in higher plants.

*Quinolizidine alkaloids* *Induction* *Cell suspension culture* *Alkaloid-'free' species* *Gene, of alkaloid biosynthesis*

## 1. INTRODUCTION

Plant natural products, such as flavonoids and lignins, are present in most species, whereas other secondary compounds (e.g., alkaloids) are thought to be present in a limited number of species, genera, or families. Quinolizidine alkaloids are widely distributed in the Fabaceae and are assumed to be specific for this plant family [1]. Stimulated by experiments [2,3], which implied a wider distribution of lupin alkaloids, we studied if quinolizidine alkaloid biosynthesis takes place also in families other than the Fabaceae. As an experimental approach, we tried modulating the alkaloid metabolism of plant cell suspension cultures of alkaloid-producing species and of alkaloid-'free' species.

## 2. MATERIALS AND METHODS

Cell suspensions of *Lupinus polyphyllus, L. luteus, Conium maculatum, Daucus carota, Atropa belladonna, Chenopodium rubrum, Spinacea oleracea*, and *Symphytum officinale* were cultured at 25°C, and 16 h of daily illumination (3000 lux) as in [3–6].

The activity of oxosparteine synthase [7,8] was assayed according to [9].

### 2.1. *Alkaloid extraction*

Cells were homogenized in 0.5 M HCl and left standing at room temperature for 30 min. After filtering the homogenate through nylon gauze (100 $\mu$m mesh) the filtrate was alkalinized with 25% ammonium hydroxide and applied onto a standard



*Published by Elsevier Science Publishers B.V.*
00145793/83/$3.00 

Extrelut column (Merck, Darmstadt). The alkaloids were eluted wiht methylene chloride and the solvent evaporated in vacuo.

### 2.2. *Capillary gas–liquid chromatography (GLC)*

Alkaloid extracts were separated by high resolution GLC on fused silica capillary columns (15 cm × 0.25 mm) coated with DB 1 (J and W Scientific). A Perkin Elmer gas chromatograph (Sigma 1 b), equipped with flame ionization and nitrogen specific detectors was employed [4–6]. Owing to the high resolution and reproducibility of the columns, the alkaloids could be easily identified by their specific retention indexes [4–6,10].

### 2.3. *GLC–mass spectrometry (MS)*

To confirm the GLC identifications we analyzed all alkaloids by capillary GLC/MS employing a Kratos MS 30 combined with the data system DS 50 as in [4–6,10].

## 3. RESULTS AND DISCUSSION

### 3.1. *Induction of alkaloid accumulation in cell suspension cultures of alkaloid-producing species*

Photoheterotrophic, chloroplast-containing cell suspension cultures to *Lupinus polyphyllus, Cytisus scoparius* and other Fabaceae accumulate quinolizidine alkaloids such as lupanine at a concentration usually 1–3 orders of magnitude lower than the alkaloid level of the differentiated plant [4,5], similarly to the situation in many other cell culture systems [11–13]. However, the activity of the enzymes of lupanine biosynthesis, lysine decarboxylase [3,14] and oxosparteine synthase [7,8], which are localized in the chloroplast [9], is similar to, or lower only by one order magnitude than, the respective enzyme activities in the plant [15]. We concluded that lupin alkaloids are actually formed by the cells, but are not accumulated to a marked degree, since they are rapidly degraded which is also an important feature of lupin plants and of other plant species [16,17]. Thus, the alkaloid turnover seemed to us an interesting possibility for the manipulation of alkaloid metabolism.

Compounds which potentially interact with DNA (e.g., alkaloids and polyamines) may affect gene expression in many biological systems [18–20]. In plants (e.g., in *Pisum sativum*) these compounds and other elicitors induce phenylalanine ammonia-lyase (PAL) and pisatin synthesis [20–22].

We have added foreign alkaloids, polyamines and other potential bioregulators to cell suspension cultures of *Lupinus polyphyllus* (table 1). High resolution GLC of quinolizidine alkaloids from the cells and the cell culture medium showed a significant increase in quinolizidine alkaloids as early as 2 h after the application of the foreign compounds. During the first 6 h, the rate of alkaloid accumulation was about 10–30 nmol lupanine $h^{-1}$.g fresh $wt^{-1}$. This value is in the same or one order of magnitude lower than the respective value obtained from the intact leaf [15]. The main alkaloid of the induced cells was lupanine, which was accompanied by minor alkaloids such as sparteine, 17-oxosparteine, 17-oxolypanine, 4-hydroxylupanine, 13-hydroxylupanine, tetrahydrorhombifoline, and 13-tigloyloxylupanine. The minor alkaloids are present in the leaves of *Lupinus polyphyllus* [5,10] but are usually not detectable in non-induced cell cultures.

Polyamines induce alkaloid accumulation (table 1), spermidine being the most active compound. Of the polyamines tested, cadaverine is a substrate of oxosparteine synthase, the key enzyme of lupanine biosynthesis [7,8]. Therefore, we cannot distinguish between an inducer or precursor activity of cadaverine. Also, cyclic AMP was an active in-

Table 1

Induction of quinolizidine alkaloid accumulation in cell suspension cultures of *Lupinus polyphyllus*

| Conditions | Alkaloid content | | |
|---|---|---|---|
| | $\bar{x}$ + SE | | *n* |
| Non-induced controls | 100% | | 70 |
| Culture medium-induced cells | 368 ± | 119% | 13 |
| Coniine-induced cells (3 mM) | 4679 ± | 1231% | 14 |
| Papaverine-induced cells (1 mM) | 7554 ± | 2847% | 7 |
| Cadaverine-induced cells (3 mM) | 1278 ± | 96% | 4 |
| Spermidine-induced cells (0.5 mM) | 15367 ± | 10563% | 4 |
| Cyclic AMP-induced cells (0.2 mM) | 17818 ± | 10481% | 3 |

Filter-sterilized inducers were added to cell cultures directly after transfer of the cells into fresh medium in [30]. The cells were harvested after 24 h and the alkaloids in the cells and in the medium were determined by capillary GLC according to [30,31].

ducer. Recent evidence suggests that cAMP might be a powerful plant bioregulator [23]. Besides a role as a second messenger it is considered to be mainly involved in the regulation of gene expression, similarly to the situation in bacteria.

The cell culture medium itself had some inducing activity (table 1). The amount of free alkaloid was 0.1 nmol/g fresh wt in cells cultured in a filter-sterilized medium for 24 h, it was 0.25 nmol/g fresh wt in the normal medium autoclaved for 20 min, but increased to 0.35 nmol/g fresh wt in a medium autoclaved for 60 min. Since the medium contains sucrose and ammonium salts, it is possible that inducing compounds are formed by Maillard reactions during autoclaving. Autoclaved medium also induced PAL and indole alkaloid biosynthesis [24,25].

The application of cycloheximide (6 μg/ml) together with inducers inhibited the induction of alkaloid accumulation by 67 ± 7%. However, the activity of oxosparteine synthase was not influenced by induction or the addition of cycloheximide (table 2). We do not yet understand the complicated metabolic and regulatory interactions which take place upon alkaloid induction. Since the activity of the key enzyme of lupin alkaloid synthesis was not affected by the inducers or cycloheximide, we think that the rate of alkaloid degradation and not that of alkaloid biosynthesis is changed. This assumption is consistent with the hypothesis that modulation of inactivating systems is an important form of regulation in eukaryotic cells [26].

### 3.2. *Induction of quinolizidine alkaloid accumulation in cell cultures of alkaloid-'free' species*

In the second part of our study we tried to induce and detect quinolizidine alkaloid biosynthesis in chloroplast-containing cell suspension cultures of 3 alkaloid-'free' species, including spinach and carrot, and of 3 species which accumulate biogenetically different alkaloids. A prerequisite for this study was the availability of a separating system of high resolution, such as capillary GLC with a sensitive nitrogen-specific detector, such as capillary GLC with a sensitive nitrogen-specific detector, to record pM and even fM levels of an

Table 2

Induction of quinolizidine accumulation in cell suspension cultures of alkaloid-producing and alkaloid-'free' species. Experimental conditions as in table 1. To calculate the degree of induction, the alkaloid content of non-induced cells, determined in cells after 10–14 days of culture, was set 100%

| Species | Family | Alkaloid content | | | | | Oxosparteine synthase | |
|---|---|---|---|---|---|---|---|---|
| | | Leaf (μg/g fresh wt) | NIC (μg/g fresh wt) | MIC % $\bar{x}(n)$ | AIC % $\bar{x}(n)$ | PIC % $\bar{x}(n)$ | NIC (pmol.h$^{-1}$.g fresh wt$^{-1}$) | IC |
| *Lupinus polyphyllus* | Fabaceae | 200–4000 | <0.001–1.0 | 368 (13) | 5332 (21) | 7476 (8) | 15.8 | 13.2 |
| *Lupinus luteus* | Fabaceae | 200–2000 | <0.001–0.1 | 310 (2) | 1266 (3) | 940 (1) | 9.7 | 8.3 |
| *Conium maculatum* | Umbelliferae | + | <0.001 | 1170 (2) | 660 (1) | 550 (2) | 8.9 | 7.8 |
| *Daucus carota* | Umbelliferae | | <0.001 | n.d. | 2600 (1) | 670 (1) | 10.7 | 10.5 |
| *Atropa belladonna* | Solanaceae | + | <0.001 | 250 (1) | 7725 (2) | 750 (1) | 10.9 | 12.3 |
| *Chenopodium rubrum* | Chenopodiaceae | | <0.001 | 1100 (1) | 750 (1) | n.d. | 10.4 | 10.8 |
| *Spinacia oleracea* | Chenopodiaceae | | <0.001 | 464 (3) | 563 (2) | 3840 (1) | 15.5 | 15.4 |
| *Symphytum officinale* | Boraginaceae | + | <0.001 | 440 (1) | n.d. | 850 (1) | 10.2 | 13.4 |

+ = biogenetically different alkaloids present in the plants; NIC = non-induced cells; IC = induced cells; AIC = alkaloid-induce cells; MIC = medium-induced cells; PIC = polyamine-induced cells; n.d. = not determined.

alkaloid. Capillary GLC/MS has already been employed as a powerful method for the identification of lupin alkaloids in complex mixtures [5,6,10].

To our surprise, these cell suspension cultures contained nM levels of lupanine. We could also detect oxosparteine synthase activity which was in the same range as in lupin cell cultures (table 2). Upon application of the compounds which induce alkaloid accumulation in lupins (table 1) we could observe a significant increase in quinolizidine alkaloids 2–72 h after the treatment (table 2). These alkaloids could be unambiguously identified by GLC/MS and authentic reference compounds [5,6,10] as lupanine, the major alkaloid, along with sparteine, tetrahydrorhombifoline, and 17-oxosparteine as minor alkaloids. The activity of oxosparteine synthease was not significantly influenced by the inducers. In analogy to the lupin cell cultures, we conclude that quinolizidine alkaloids are actually produced by these cells but they are accumulated at a very low level only, probably due to an alkaloid-degrading system [16]. In the induction experiments we also observed an increase in the amount of hyoscyamine in *Atropa belladonna* cultures and of other compounds which we did not identify.

## 4. CONCLUSIONS

We conclude that the genes of quinolizidine alkaloid biosynthesis are not restricted to the Fabaceae family but are distributed widely in the plant kingdom. Sporadic occurrence of quinolizidine alkaloids has already been recorded for a few non-fabaceous species in [1]. This would explain why lysine decarboxylase, the first enzyme of lupanine synthesis, is present in 46 species of the 17 families studied [3], and why cell cultures of *Conium* and *Symphytum* are able to perform specific steps of lupanine biosynthesis [2].

Also, other evidence indicates that the genetic information for secondary metabolism may have a much wider distribution than generally anticipated: cell cultures are able to transform exogenous compounds in a way which is thought to be specific for a few species only [27,28]. Some specific enzymes of secondary metabolism are obviously present in a number of higher plants, whether they actually accumulate a compound or not [29–31]. Employing sensitive assay methods, morphine was detected, which is thought to be a specific alkaloid of Papaveraceae in lettuce and hay [32]. A study of the residues of sugar refining revealed the presence of acetidine carboxylic acid which was assumed to be present in *Convallaria* plants only [33]. Some structurally complicated secondary products such as ergot alkaloids are present in fungi [34] (*Claviceps, Aspergillus, Penicillium*, and *Rhizopus*) and in some higher plants (Convolvulaceae). The betalaines are found in the mushroom, *Amanita muscaria*, and in higher plants, the Centrospermae [35]. One might conclude that the pathways leading to these compounds evolved independently during evolution but in view of these findings it is tempting to assume that the genetic information for secondary metabolism has a wider distribution, or is even universally present, in the plant kingdom. The specific information seems to be expressed in a few species only and the compounds accumulate to a significant, easily detectable degree. In these plants some of the secondary compounds seem to have been selected during evolution because they are compounds active in plant–plant or plant–herbivore interactions [17,36–39]. But even there, plants can be selected which do not accumulate high levels of a natural product; e.g., in the case of lupins, the 'sweet' varieties. In the 'sweet' lupins and in other plants the genes may be represented or are expressed to a small extent [3]. Furthermore, the products may be degraded as rapidly as they are produced. Their formation, therefore, escapes the attention of phytochemists and chemotaxonomists, although the consequences might be important for the manipulation of plants; e.g., genetic engineering, plant breeding or the production of secondary compounds.

## ACKNOWLEDGEMENTS

This work was supported by grants of the Land Niedersachsen and the Deutsche Forschungsgemeinschaft (to Professor Dr T. Hartmann). Excellent technical assistance of C. Theuring is gratefully acknowledged. We would like to thank Professor Dr T. Hartmann, B.C. Homeyer for reading of the manuscript and Dr M. Roberts (university of London) for helpful comments.

## REFERENCES


[1] Schütte, H.R. (1969) in: Biosynthese der Alkaloide (Mothes, K. and Schütte, H.R. eds) pp. 324–343, VEB-Berlin.
[2] Wink, M., Hartmann, T. and Witte, L. (1980) Planta Med. 40, 31–39.
[3] Schoofs, G., Teichmann, S., Hartmann, T. and Wink, M. (1983) Phytochemistry 22, 65–69.
[4] Wink, M. and Hartmann, T. (1980) Planta Med. 40, 149–155.
[5] Wink, M., Witte, L., Hartmann, T., Theuring, C. and Voss, V. (1983) Planta Med. in press.
[6] Wink, M., Witte, L., Schiebel, H.M. and Hartmann, T. (1980) Planta Med. 38, 238–245.
[7] Wink, M. and Hartmann, T. (1979) FEBS Lett. 101, 343–346.
[8] Wink, M., Hartmann, T. and Schiebel, H.M. (1979) Z. Naturforsch. 34c, 704–708.
[9] Wink, M. and Hartmann, T. (1982) Plant Physiol. 70, 74–77.
[10] Wink, M., Schiebel, H.M., Witte, L. and Hartmann, T. (1982) Planta Med. 44, 15–20.
[11] Barz, W. and Ellis, B.E. (1981) in: Natural products as medicinal agents (Beal, J. and Reinhard, E. eds) pp. 471–507, Hippokrates Verlag, Stuttgart).
[12] Böhm, H. (1982) in: Plant tissue culture 1982 (Fujiwara, A. ed) Proc. 5th Intl. Cong. Plant Tissue and Cell Culture pp. 325–328.
[13] Zenk, M. (1978) in: Frontiers of plant tissue culture (Thorpe, T.A. ed) pp. 1–14, Int. Ass. Plant Tissue Culture, Calgary.
[14] Hartmann, T., Schoofs, G. and Wink, M. (1980) FEBS Lett. 115, 35–38.
[15] Wink, M. and Hartmann, T. (1982) in: Plant Tissue Culture 1982 (Fujiwara, A. ed) Proc. 5th Intl. Cong. Plant Tissue and Cell Culture, pp. 333–334.
[16] Wink, M. and Hartmann, T. (1982) Z. Naturforsch. 37c, 369–375.
[17] Robinson, T. (1974) Science 184, 430–435.
[18] Bagni, N., Adamo, P. and Serafini-Fracassini, D. (1981) Plant Physiol. 68, 727–730.
[19] Beljanski, M., LeGoff, L. Beljanski, M. (1982) Exp. Cell Biol. 50, 271–280.
[20] Hadwiger, L.A. and Schwochau, M.E. (1971) Plant Physiol. 47, 346–351.
[21] Loschke, D.C., Hadwiger, L.A., Schröder, J. and Hahlbrock, K. (1981) Plant Physiol. 68, 680–685.
[22] Hahlbrock, K., Lamb, C.J., Purwin, C., Ebel, J. Fautz, E. and Schäfer, E. (1981) Plant Physiol. 67, 768–773.
[23] Brown, E.G. and Newton, R.P. (1981) Phytochemistry 20, 2453–2463.
[24] Hahlbrock, K. and Schröder, J. (1975) Arch. Biochim. Biophys. 171, 500–506.
[25] Knobloch, K.-H., Hansen, B. and Berlin, J. (1981) Z. Naturforsch. 36c, 40–43.
[26] Marcus, A. (1971) Annu. Rev. Plant Physiol. 22, 313–336.
[27] Veliky, I.A. and Barber, K.M. (1975) Lloydia 38, 125–130.
[28] Grützmann, K.D. and Schröter, H.-B. (1966) Abh. Dtsch. Akad. Wiss. Berlin, Kl. Chemie 3, 347.
[29] Wink, C. and Hartmann, T. (1981) Z. Naturforsch. 36c, 625–632.
[30] Hartmann, T., Ihlert, H.-I. and Steiner, M. (1972) Z. Pflanzenphysiol. 68, 11–18.
[31] Miller, J.M. and Conn, E.E. (1980) Plant Physiol. 65, 1199–1202.
[32] Hazum, E., Sabatka, J.J., Chang, K.-J., Brent, D.A., Findlay, J.W.A. and Cuatrecasas, P. (1981) Science 213, 1010–1012.
[33] Fowden, L. (1972) Phytochemistry 11, 2271–2276.
[34] Gröger, D. (1975) Planta Med. 28, 269–288.
[35] Mothes, K. (1976) Rec. Adv. Phytochem. 10, 385–405.
[36] Rice, E.L. (1979) Bot. Rev. 45, 15–109.
[37] Rosenthal, G.A. and Janzen, D.H. (1979) Herbivores: Their interaction with secondary plant metabolites, Academic Press, New York.
[38] Wink, M., Hartmann, T., Witte, L. Rheinheimer, J. (1982) Z. Naturforsch. 37c, 1081–1086.
[39] Wink, M. (1983) Planta, in press.