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THE EFFECT OF LIGHT ON THE PRODUCTION OF *HEIMIA* ALKALOIDS

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Key Word Index—*Heimia salicifolia*; Lythraceae; shoot cultures; alkaloids; effect of light.

Abstract—Alkaloid extracts obtained from light- and dark-grown shoot cultures of *Heimia salicifolia* were partially fractionated and semi-quantitatively analysed for phenyl- and biphenylquinolizidine alkaloids. The concentrations of the two structural types of alkaloids in extracts of shoots grown with illumination increase over a 4 week culture period. In extracts obtained from shoots grown in darkness for 4 weeks, however, we observed substantial decreases in the concentrations of both the phenyl- and biphenylquinolizidine alkaloids. We observed that growth in the dark for periods of up to 9 weeks resulted in a further reduction in the levels of biphenyl alkaloids relative to those of the phenylquinolizidinols.

INTRODUCTION

It has recently been reported [1] that shoot cultures of *Heimia salicifolia* Link and Otto, which we presently maintain, produce a number of the biphenylquinolizidine lactones found in the field-grown plant, as well as two phenylquinolizidinols previously found only in young seedlings. According to the proposed biogenic pathway for the *Heimia* alkaloids [2, 3], the first committed steps towards these secondary metabolites are the decarboxylation of lysine, yielding 1,5-diaminopentane (cadaverine), and the elimination of ammonia from phenylalanine yielding *trans*-cinnamic acid.

Studies of the biosynthesis of quinolizidine alkaloids in *Lupinus polyphyllus* have demonstrated that the biosynthesis of lysine [4] and lysine decarboxylase activity [5] are both localized in the chloroplasts. Moreover, the stimulatory effect of light on the activity of phenylalanine ammonia-lyase and other enzymes of the general phenylpropanoid pathway have been well-documented [6]. Since these facts imply a relationship between illumination and alkaloid formation, we have investigated the possible existence of such a correlation in *H. salicifolia*.

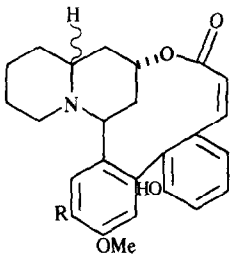
The phenyl- and biphenylquinolizidines present in cultured shoots grown both with and without illumination were analysed by TLC at various times during the culture period. The two structural types of alkaloids were partially resolved by differential extraction through pH adjustments [1] and the use of organic solvents of different selectivities. Chlorophyll was also determined in the shoots as a measure of chloroplast development. We report on the positive relationship between illumination or chloroplast development and alkaloid formation in *H. salicifolia*.

RESULTS

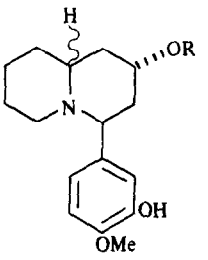
After extraction and partial fractionation of the alkaloids from fresh tissue (see Experimental) we found that the first chloroform extract, obtained at pH 1, contained the less polar biphenyl lactones **1**–**3** and ester alkaloids **4** and **5**. Small amounts of at least two unidentified alkaloids were also present in this extract. The second organic extract, which was obtained at pH 8.5, contained mostly the more polar phenylquinolizidinols **6** and **7**; a considerable amount of lyfoline (**2**) was also present. Since lyfoline partitioned into both organic extracts, we were unable to quantitate this alkaloid by analysis of a single fraction. Since vertine (**1**) is by far the major alkaloid produced by the shoot cultures [1], we chose to quantitate this alkaloid as the representative biphenyl lactone. We chose arbitrarily to study demethyllasubine I (**6**) as the representative phenylquinolizidinol since it and demethyllasubine II (**7**) are present in approximately equal concentrations. Ester alkaloids are very minor components of the total alkaloid content of the tissues; changes in their concentrations would have been difficult to measure using our method.

Samples of both light- and dark-grown shoot cultures were taken 24 hr after subculture and approximately weekly thereafter, for 4 weeks. Simultaneous TLC analysis of the organic extracts obtained from light-grown shoots revealed a progressive and marked increase in the concentrations of the three major biphenyl lactones [vertine (**1**), lyfoline (**2**), and lythrine (**3**)] up to *ca* 2 weeks after subculture. Thereafter the concentrations of these alkaloids appeared to remain relatively constant. The increase with time in the concentrations of the alcohols **6** and **7** was less dramatic, but still quite obvious from the increasing intensities revealed on spraying the chromatograms. Alkaloids **1** and **6** were then analysed semi-quantitatively in each of the extracts in an attempt to measure the changes in their concentrations during the culture period. These results are shown in Figs 1 and 2.

Vertine (**1**) was found to triple in concentration from 7 to 21 μg/g fr. wt between days 1 and 16 of culture. After this time, there was a slight decrease in its concentrations to 17 μg/g fr. wt on day 28. The concentrations of alkaloid **6** remained essentially unchanged over the entire 28-day period, at *ca* 4–6 μg/g fr. wt. The chlorophyll content of the shoots harvested 8 and 16 days after subculture were 8% and 21% higher than that of shoots harvested on the first day after subculture. By day 28, chlorophyll had returned to the level measured on day 1. Thus, while the



	Alkaloid	Stereochemistry at ring juncture	R
1	vertine	*cis*	OMe
2	lyfoline	*trans*	OH
3	lythrine	*trans*	OMe



4	*cis*	*p* - hydroxycinnamoyl
5	*trans*	*p* - hydroxycinnamoyl
6	*cis*	H
7	*trans*	H

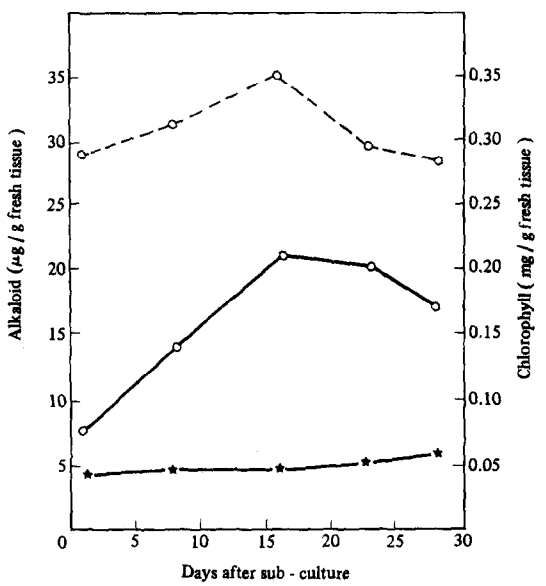


Fig. 1. Changes in alkaloid and chlorophyll concentrations in light-grown shoots with time after subculture. (✱——✱) Alkaloid **6** (demethyllasubline I); (⊙——⊙) alkaloid **1** (vertine); (⊙- - - -⊙) chlorophyll.

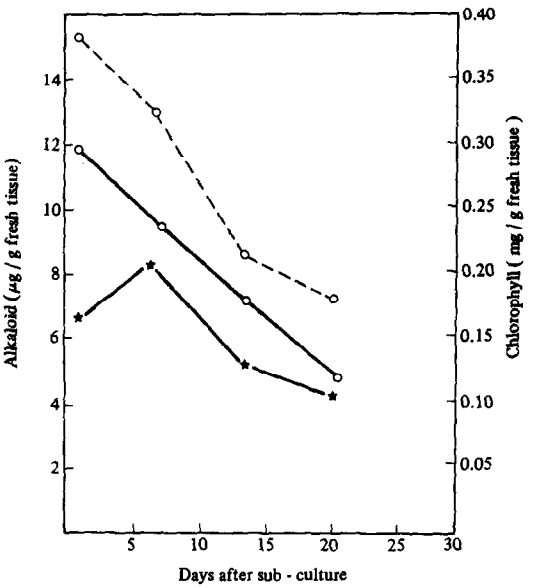


Fig. 2. Changes in alkaloid and chlorophyll concentration in dark-grown shoots with time after subculture. (✱ —— ✱) Alkaloid **6** (demethyllasubline I); (⊙ —— ⊙) alkaloid **1**; (⊙- - - -⊙) chlorophyll.

biosynthesis of the phenylquinolizidinols appeared to occur at a rate which closely paralleled the increase in fr. wt of the tissues, the rate of accumulation of the biphenyl lactones apparently increased rapidly in the first 16 days after subculture and then declined. In addition, there appeared to be a strong correlation between chloroplast development and alkaloid accumulation at least in the first 16 days after subculture. The apparent correlation was better with vertine (99%) than with **6** (79%).

It should be noted that the levels of alkaloids reported here are not directly comparable to those reported previously [1], as the present results are based on the fr. wt of extracted tissue rather than dry wt. The extraction procedures and periods of time between last subculture and harvest also differ significantly.

Simultaneous TLC analysis of alkaloid extracts obtained from shoots grown in darkness revealed a pattern of alkaloid accumulation essentially opposite to that found in light-grown shoots. That is, the concentrations of both phenyl- and biphenylquinolizidines decreased over

the course of the dark-grown period. The concentration of vertine (**1**) fell steadily from 12 μg/g fr. wt on day 1 after subculture to 5 μg/g fr. wt on day 21, representing a decrease of 60% overall. The concentration of **6** showed an initial increase of 27%, followed by a substantial decrease after 2 and 3 weeks in darkness. The overall change for **6** between days 1 and 21 is a decrease of 34%. The chlorophyll content of these tissues fell from 0.379 to 0.174 mg/g fr. wt, a 55% decrease over the same period. The correlation between alkaloid accumulation and chloroplast development is again very strong, at 98% for vertine (**1**), and 78% for **6**. Again, the level of **1** seems more closely to reflect the changes in the chlorophyll content of the shoots than does the level of the phenylquinolizidinol **6**.

Shoots which were kept in darkness for periods of up to 9 weeks were analysed only qualitatively for alkaloids. After 6 weeks of darkness, the spectrum of alkaloids was still unchanged, however, a more pronounced decrease in the levels of biphenyl lactones relative to the phenylquinolizidinols **6** and **7** was noted. After 9 weeks, the biphenyl alkaloids were almost undetectable, while **6** and **7** were still clearly present. These findings support the trend observed in the first 3 weeks of darkness, i.e. a more dramatic (60%) decrease in the level of vertine (**1**) compared with the decrease of 34% in the level of **6**.

DISCUSSION

The decreased accumulation of alkaloids in dark-grown shoots was not unexpected since many of the enzymes thought to be involved in early steps of the biosynthetic pathway are known to be more active in the light. Phenylalanine ammonia-lyase and cinnamate-4-hydroxylase are well-known examples [6, 7]. In addition, the existence of a positive correlation between chlorophyll content and lupine alkaloid formation in *Lupinus polyphyllus* [8] suggested that the lysine branch of the biosynthesis of the *Heimia* alkaloids might also be affected by light. The results of this study provide evidence for a similar correlation between greening and the production of the *Heimia* alkaloids. While the accuracy of the TLC method used in our determinations was limited by the accuracy of the determination of fr. wt, by the sensitivity of the phenol spray and the obvious difficulties involved in the visual evaluations of the intensities of the coloured areas of the chromatograms, as described in the Experimental, the method clearly demonstrates that the alkaloid content of the tissues undergoes dynamic changes with time after subculture. Moreover, the course of these changes is altered significantly by the absence of light.

The relationship between chlorophyll and alkaloid contents of the shoot cultures analysed implies that at least part of the biosynthetic pathway may take place in the chloroplasts. Localization of the pertinent enzyme activities has identified chloroplasts as the site of biosynthesis of the tetracyclic quinolizidine alkaloids of the lupine type [5]. Enzymatic studies with *H. salicifolia* which have been initiated will undoubtedly enhance our knowledge concerning the subcellular localization of the pathway. They will also address questions pertaining to the regulation of the expression of the pathway.

The observation that the concentration of phenylquinolizidinols fell less than that of the biphenyl lactones in those tissues grown in darkness might suggest that the production of biphenyl alkaloids from their presumed phenylquinolizidine precursors [1–3] involves one or more light-dependent reactions. One obvious possibility is the phenol-oxidative coupling reaction. Another might well be the isomerization of the *trans*-cinnamoyl double bond to the *cis* conformation that must take place prior to coupling of the phenyl systems; that this transformation requires light *in vivo* has been well documented [9] in the studies of coumarin biosynthesis, and the photochemical interconversion has long been established [10]. It is always possible that the greater reduction in biphenyl vs phenyl alkaloids may, at least in part, reflect a faster rate of degradation of the former alkaloids.

EXPERIMENTAL

Plant material. Both light- and dark-grown shoot cultures were developed from seeds as previously described [1] and were maintained on medium G described therein. Light-grown shoots were subject to a 16 hr daylight cycle of 500 lux, 40 W. To obtain 'dark-grown' shoots, cultures of this type were wrapped in Al foil immediately after subculture and were maintained under conditions which were otherwise identical to those used for light-grown shoots, for periods of up to 9 weeks. Both types of cultures were maintained at 23–26° and were subcultured every 4 weeks. When cultures were kept in darkness longer than 4 weeks, they were subcultured with the aid of phytochrome safe light. All tussues were harvested between 11 a.m. and 1 p.m.

Extraction. Between 10 and 20 g fr. wt was extracted with Me_2CO (100 ml) at −15° to −20°. The fibrous residue was re-extracted with Me_2CO and the filtrates were combined and analysed for chlorophyll [11]. Me_2CO was then removed under red. pres. to give an aq. residue which was acidified with 1% (w/v) HCl (0.33 ml/g fr. wt) and diluted to 0.8 ml/g fr. wt with H_2O. This soln was extracted with EtOAc (twice) to remove most non-alkaloidal material, including chlorophyll. The EtOAc phase was washed once with H_2O (1 ml/15 g fr. wt). The wash was combined with the aq. phase, which was then extracted with $CHCl_3$ (× 3), giving the first alkaloidal fraction. The aq. phase was then adjusted to pH 8.5 and extracted with $CHCl_3$–MeOH (4:1) (twice) and the extracts combined. After evaporation to dryness, both fractions were analysed according to the following procedure.

TLC analysis. Each fraction was dissolved in $CHCl_3$–MeOH (1:1). TLC was performed on pre-coated silica gel 60 F_{254} plates (E. Merck) in (a) NH_3-satd $CHCl_3$–MeOH (15:2) and (b) $CHCl_3$–MeOH–$NHEt_2$ (15:0.5:0.8). Alkaloids were visualized with diazotized *p*-nitroaniline [1]. Concns were estimated by matching the intensity of extracts' spots with those of a series of standard spots representing 0.1–1.0 μg alkaloid. Each alkaloid was analysed independently, as the sensitivity of the phenol spray varied with the structure of the alkaloid. Calibrated capillaries were used to obtain low intensity spots which could be easily compared with reference spots of pure standards.

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