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ALKALOID CONCENTRATION DURING DEVELOPMENT IN THREE *LUPINUS* SPECIES AND THE EXPRESSION OF GENES FOR ALKALOID BIOSYNTHESIS IN SEEDLINGS

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Key Word Index—*Lupinus albus*, *L angustifolius*, *L mutabilis*, Leguminosae, alkaloid content, plant development, gene expression, seedlings

Abstract—The distribution of alkaloids during development from seed germination to seed formation is described for three *Lupinus* species Alkaloid levels in seedlings decrease after germination before increasing to *ca* 0 3–0 6% dry wt in vegetative tissue prior to first flowering. The accumulation of alkaloids from vegetative parts to seeds is extremely rapid during the later maturation phase of pods and seeds At maturity the seeds contain more alkaloid than the total present in above ground tissues at 4–5 weeks before final harvest Apart from the seeds, and parts of flowers and fruits which become alkaloid rich, vegetative tissues contain alkaloid levels which are at the threshold values which differentiate between 'sweet' and 'bitter' genotypes The concentration of alkaloid during development is discussed in relation to protection against predation

INTRODUCTION

Alkaloids are synthesized in different parts of plants in different species In *Nicotiana* spp alkaloid synthesis is almost exclusively confined to root tips, whereas in *Lupinus* they are produced in green leaves and shoots [1] The concentration of alkaloids in roots and leaves of *Conium maculatum* is higher during the second flowering year of the biennial growth cycle, and fruits are characterized by increasing levels to the half-ripe stage followed by a gradual decrease to maturity [2]

The role of alkaloids in metabolism is far from clear From the evidence of rapid turnover, it would seem that they play an integral role in biosynthesis as distinct from being by-products of end- or side-reactions of other metabolic events The rapid fluctuation in the concentration of the pair of alkaloids, coniine and γ-coniceine, in fruits of *Conium* was interpreted by Fairbairn [2] as evidence that they act as co-enzymes in oxidation–reduction reactions in developing fruit Alkaloids have also been associated with a protective role against predation [3], but this is a tenuous possibility which so far lacks unequivocal supporting evidence Some of the claims such as the concentration-dependent mechanisms proposed to account for resistance in *Lupinus* species to *Glaucopsyche lygdamus* [4] are based on inadequate data and are therefore capable of alternative interpretation Of particular interest in this connection is the surprising difference that exists between species in their ability to accumulate alkaloids in seeds seeds of alkaloid-rich species of *Nicotiana* and *Papaver* [1] are almost free of alkaloid while those of leguminous species frequently show spectacular increases in concentration at the expense of other organs If alkaloids are generally important in the evolution of protection against predation, it is not obvious why some species should exclude alkaloid accumulation in seed and seedlings which are particularly vulnerable to pests and pathogens

This report describes changes in the distribution and accumulation of total alkaloid in *Lupinus mutabilis*, *L angustifolius* and *L albus*, throughout the life cycle from germination to seed formation We have also examined the relation between morphogenesis and the mobility of alkaloids between sites of synthesis and the organs in which they accumulate

RESULTS

Alkaloid concentration in relation to morphogenesis

Tables 1–3 give the total alkaloid in mg per plant and the percentage alkaloid in the dry matter at five different harvests corresponding to clearly defined morphogenetic stages

Alkaloid content in cotyledons fell rapidly, the change being particularly marked in *L angustifolius* in which the concentration in fully expanded cotyledons had fallen to less than 10% of that in the seed In *L mutabilis* and *L albus*, although both show substantial losses in the early cotyledon stage, the change in alkaloid content was proportional to the total reduction in dry wt After the cotyledons had become fully expanded (harvest 1) reduction in alkaloid in cotyledons in all species was relatively much greater than loss in dry wt These changes may simply reflect the earlier onset of dry matter accumulation and the more rapid catabolism of the

alkaloids or their distribution and consequent dilution in newly-formed tissues

Although total amount and concentration of alkaloid in vegetative tissues are not precisely comparable in the species at the same development stage, there is a consistent general trend for the levels to decrease during early seedling development (harvests 0–3) in both stems and leaves Increases in alkaloid are directly proportional to increases in dry wt and to the development of axillary branches during the rapid expansion of the canopy (Fig 1)

Of particular interest is the high alkaloid concentration in reproductive tissues, flowers, pods and seeds Apart from low values in pod-wall tissues after senescence, flowers and fruits contain substantially larger concentrations of alkaloids than the highest levels recorded in stems and leaves

The alkaloid levels between harvests 8 and 9 over a growth period of *ca* 28 days in *L mutabilis* and 35 and 48 days in *L albus* and *L angustifolius*, respectively, when seeds and pods were entering the final stages of maturation is of interest in relation to the distribution of alkaloid Total alkaloid as well as dry wt increased rapidly during this period, resulting in doubling of per plant totals and the maintenance of constant alkaloid concentrations in both *L albus* and

Table 1 Alkaloid concentration (mg) in seedlings at different growth stages (± s e)

	L mutabilis		*L albus*		*L angustifolius*	
Tissue and harvest no	Total alkaloid	Per cent	Total alkaloid	Per cent	Total alkaloid	Per cent
(a) Cotyledons						
0	2 82	1 31 ± 0 05	3 19	1 38 ± 0 04	2 58	1 27 ± 0 06
1	1 69	1 30 ± 0 07	1 07	1 04 ± 0 06	0 20	0 52 ± 0 05
2	0 36	0 52 ± 0 04	0 30	0 54 ± 0 03	0 12	0 35 ± 0 03
(b) Stems (main + side stem)						
2	1 11	0 57 ± 0 03	0 89	0 56 ± 0 04	0 27	1 26 ± 0 06
3	0 17	0 08 ± 0 02	0 40	0 12 ± 0 02	1 51	1 37 ± 0 07
4	3 25	0 18 ± 0 03	3 77	0 19 ± 0 03	2 40	0 23 ± 0 02
5	24 10	0 35 ± 0 05	15 89	0 44 ± 0 06	5 85	0 24 ± 0 05
(c) Leaves (main + side stem)						
2	0 76	0 25 ± 0 03	0 83	0 50 ± 0 04	0 15	0 26 ± 0 03
3	1 21	0 19 ± 0 04	2 91	0 13 ± 0 03	0 06	0 11 ± 0 03
4	6 40	0 19 ± 0 08	15 31	0 22 ± 0 03	4 48	0 31 ± 0 05
5	21 70	0 24 ± 0 06	63 20	0 61 ± 0 07	13 46	0 23 ± 0 02
(d) Total per plant						
0	2 82	1 31	3 19	1 38	2 58	1 27
1	2 26	1 38	2 36	1 53	0 72	0 71
2	2 24	0 40	2 01	0 51	0 55	0 48
3	1 38	0 17	3 31	0 13	1 58	0 39
4	9 64	0 19	19 08	0 23	6 88	0 28
5	45 81	0 29	88 01	0 62	19 30	0 23

Table 2 Total alkaloid (mg) at different stages of adult growth

Species and harvest no	Stems	Leaves	Flowers	Pods	Seed	Plant total
L mutabilis						
6	30 1	26 5	10 4	—	—	67 0
8	108 5	68 8	—	137 5	7 4	322 2
9	81 0	NA	—	20 1	382 2	483 3
L albus						
6	60 1	96 2	4 4	10 3	—	171 0
8	185 3	291 9	—	150 2	22 0	649 4
9	173 8	NA	—	27 5	874 8	1076 1
L angustifolius						
6	28 9	88 7	2 9	—	—	120 5
8	95 0	154 8	—	62 9	5 2	317 9
9	35 7	NA	—	4 1	737 3	777 1

NA = Not available

Table 3 Percentage alkaloid at different stages of growth

Species and harvest no	Cotyledon	Stems	Leaves	Flowers	Pods	Seed	Plant total
L mutabilis							
0	1 31 ± 0 05	—	—	—	—	—	1 31
5	—	1 35 ± 0 05	0 24 ± 0 06	—	—	—	0 29
6	—	0 30 ± 0 05	0 26 ± 0 04	1 53 ± 0 07	—	—	0 32
8	—	0 22 ± 0 03	0 29 ± 0 04	—	0 74 ± 0 06	0 60 ± 0 05	0 49
9	—	0 08 ± 0 02	—	—	0 06 ± 0 03	0 91 ± 0 07	0 26
L albus							
0	1 38 ± 0 04	—	—	—	—	—	1 38
4	—	0 19 ± 0 03	0 22 ± 0 03	—	—	—	0 23
6	—	0 50 ± 0 02	0 56 ± 0 03	1 84 ± 0 05	1 21 ± 0 07	—	0 51
8	—	0 40 ± 0 03	0 59 ± 0 04	—	0 79 ± 0 06	1 84 ± 0 09	0 55
9	—	0 21 ± 0 03	—	—	0 78 ± 0 08	1 30 ± 0 05	0 58
L angustifolius							
0	1 27 ± 0 06	—	—	—	—	—	1 27
5	—	0 24 ± 0 05	0 23 ± 0 02	—	—	—	0 23
6	—	0 26 ± 0 04	0 51 ± 0 05	0 41 ± 0 03	—	—	0 41
8	—	0 24 ± 0 06	0 34 ± 0 04	—	0 61 ± 0 04	0 60 ± 0 03	0 33
9	—	0 04 ± 0 03	—	—	0 01 ± 0 01	1 14 ± 0 06	0 40

L angustifolius The pattern in *L mutabilis* during this stage is anomalous with total alkaloid remaining constant in spite of a doubling in dry wt

The close correlation between dry wt increase and total alkaloid (see also Fig 1) might suggest that they represent end products of synthesis If they were being mobilized as active intermediates or reactants in metabolism, one would have expected fluctuations in concentration reflecting the varying growth rates at different stages of the life cycle

Undoubtedly, the most significant aspect of the distribution of alkaloids in these species is their rapid accumulation in seed tissues At the seed-ripe stage, between 80 and 95% (according to species) of the total alkaloid in the plant has been transported into seeds Since the values of alkaloids in the leaves after senescence (harvest 9) are not available it is not possible to calculate accurately the total movements of alkaloids from vegetative tissues during the ripening period However, it is likely that most loss of alkaloid from all the vegetative parts is due to transfer to maturing seeds In addition to transfer of alkaloids previously accumulated in stems, pods and leaves, it is clear that the final seed totals in *L albus* and *L angustifolius* can only be accounted for if it is assumed that the major proportion of the alkaloids synthesized between harvests 8 and 9 was directly transferred to seeds

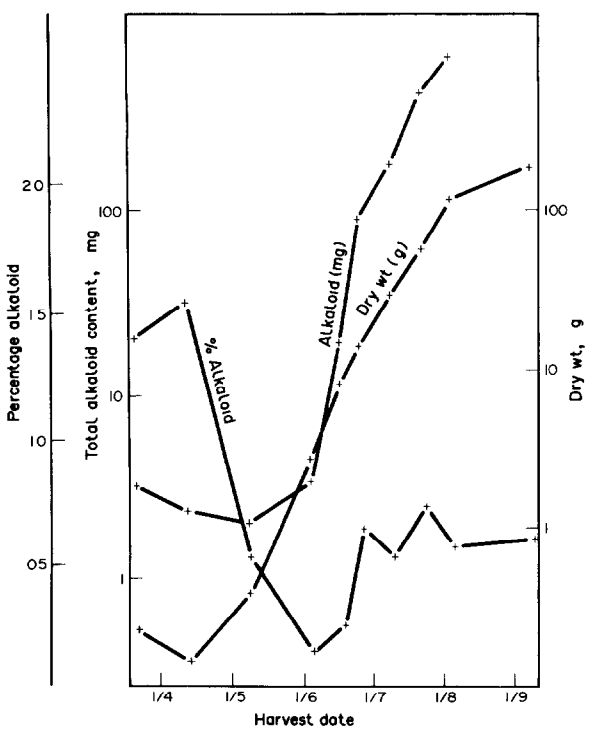

Fig 1 Changes in alkaloid level and dry wt during the life cycle of *L albus*

The expression of alkaloid-controlling genes during seedling development

Alkaloid synthesis does not occur in the seed of *Lupinus albus* and *L angustifolius* [5] so that seed alkaloids are determined by the genotype of the female parent plant, and seedlings do not express their genotype until *de novo* alkaloid synthesis has commenced In order to support further the data on alkaloid levels in the early seedling stages, tests of alkaloid concentration were made on seedlings which were genetically competent to produce alkaloids, but because of the genotype of the female parent, were phenotypically low-alkaloid forms From previous evidence [6] seedlings express their own genotype as recorded by the Dragendorff reaction within *ca* 30 days after sowing or *ca* 20–24 days after germination

In these studies two types of families were used for the test, one in *L angustifolius* in which a low-alkaloid female parent was crossed with a high-al-

kaloid male (LN 28♀ × Edelweiss ♂) and the other (*L albus*) in which both parents (Ultra ♀ × Neuland ♂) were low-alkaloid forms due to homozygosity for mutant alleles of independent, complementary genes In both, F_1 seed was 'sweet' with the seedlings becoming Dragendorff-positive (bitter) after the seedling genotype became expressed The development of the expression of low-alkaloid phenotypes was also recorded in seedlings of F_2 families segregating for alkaloid concentration These were from the crosses, Lupini Bean ♀ (bitter) × Kievskij Mutant ♂ (sweet) (*L albus*) and LN 28 ♀ × Edelweiss ♂ (*L angustifolius*)

The data given in Fig 2 are the means of total alkaloid peak areas on GC traces from two whole seedlings, including roots, sampled on different days after sowing The most striking change from the maternal to the high-alkaloid expression in seedlings was expressed by the F_1 Ultra × Neuland Alkaloid concentration increased at *ca* 16 days from germination, and after a lag phase of *ca* 5 days, production became very rapid, resulting in a 15-fold increase in total alkaloid between days 21 and 35

In *L angustifolius* (LN 28 ♀ × Edelweiss ♂), perceptible increase in total alkaloid was recorded 11 days earlier than in the *L albus* cross Production was at a slow rate for a further 21 days and seedlings achieved the level in the high-alkaloid progeny at day 30 The rate of alkaloid production in *L angustifolius* seedlings was significantly less than in the high-alkaloid progeny in *L albus* Although it is not possible to determine from these data the amount of total alkaloid synthesized in cotyledons and the first true leaf, it seems that detectable synthesis begins within 5–15 days after germination when total and percentage alkaloid are falling (Table 1) At this stage synthesis is insufficient to compensate for reduction in concentration brought about either by redistribution or catabolism

Additional evidence on timing of production and loss is given by the pattern of development of the low-alkaloid phenotype in segregating F_2 plants from high-alkaloid, heterozygous F_1 hybrids (Table 4) Alkaloid concentration in the homozygous mutant segregates fell to below the Dragendorff-positive level at between days 26 and 29 after germination, or 31–35 days from sowing the seed The high-alkaloid genotypes remained Dragendorff-positive during this period which suggests that synthesis in genetically competent seedlings compensates sufficiently for loss in alkaloid to avoid falling below the level which fails to give a positive response with the Dragendorff reagent

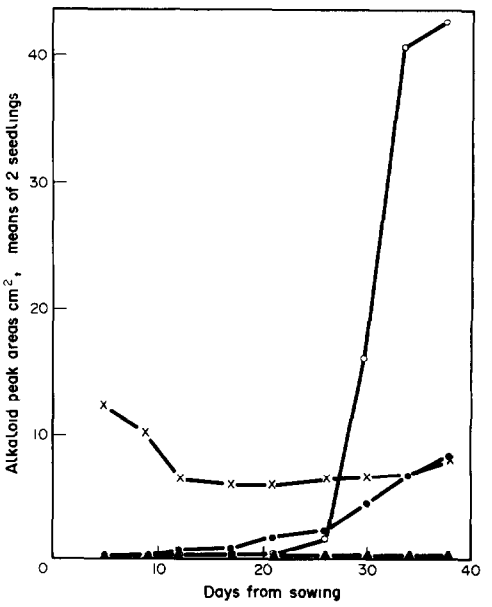

Fig 2 Total alkaloid (peak areas on GC) per seedling in high-alkaloid genotypes derived from crosses involving low-alkaloid female parents (○) Ultra ♀ × Neuland ♂, (×) Edelweiss selfed, (●) LN 28 ♀ × Edelweiss ♂, (▲) mean of all 'sweet' families

DISCUSSION

Total alkaloid in cotyledons decreased rapidly in all three *Lupinus* species during the very early stages of germination Part of the reduction may be due to distribution to the roots The lowest alkaloid levels recorded in the cotyledon (0 35–0 54%) are on the threshold of the sensitivity of the Dragendorff test and of the concentration which gives the bitter organoleptic response During the first stage of germination, loss in alkaloid parallels losses in dry matter with the result that percentage concentration of alkaloid is stable, except in *L angustifolius* in which alkaloid reduction in cotyledons was markedly more

Table 4 Dragendorff tests on seedlings of high- and low-alkaloid cultivars and on F_2 plants from crossing high-alkaloid female × low-alkaloid male

Days sowing to Dragendorff test	*Lupinus albus*			*Lupinus angustifolius*		
	Parents		F_2	Parents		F_2
	Lupini bean (A)	Kievskij mutant (B)	A♀ × B♂ + −	Edelweiss (B)	LN 28 (C)	B♀ × C♂ + −
<31	All tests positive	All tests negative	56 0	All tests positive	All tests negative	56 0
35			6 2			7 1
39			5 3			5 3

+, Dragendorff positive, −, Dragendorff negative Eight seedlings per family in each test

rapid than loss of dry matter At this stage alkaloid synthesis is at a very low rate and insufficient to maintain a constant concentration in the tissues

Increase in total alkaloid occurs at *ca* 20 days before the onset of flowering which also nearly coincides with rapid increase in dry matter [7] At flowering and subsequently, the rate of increase of alkaloid and dry matter are similar, thus the alkaloid concentration in tissues remains fairly constant right up to maturity Alkaloid concentration in stems at the earlier stages of development shows an unexpected initial peak, presumably resulting from redistribution from the cotyledons This pattern is not quite as evident in *L angustifolius* where there was an increasing trend in alkaloid concentration in stems from the date of the first harvest In leaves where the pattern is more consistent and therefore easier to interpret, the lowest alkaloid concentration was achieved during early stem expansion before the emergence of inflorescences Some of the values (< 0 20%) recorded at this stage were lower than the concentration in seed of some mutant 'sweet' genotypes [5]

It is thus clear that during the early stages of stem and leaf formation, rates of alkaloid production are significantly less than of dry matter accumulation After first flowering when plants have developed a full canopy, the relative production of alkaloid in stems and leaves increased rapidly to a relatively steady maximum of 0 3–0 6%, which at the lower end of the range is near the threshold concentration separating Dragendorff-negative (sweet) from Dragendorff-positive (bitter) phenotypes

Fruits and pods had relatively high alkaloid levels from the earlier stages of their development and like the stems, and presumably the leaves, the concentration in pods fell to insignificant amounts at the seed-ripe phase The initiation of rapid accumulation of alkaloid in reproductive tissue corresponded to the pattern of nitrogen accumulation [8] and was seen to commence when pod development was well advanced on both the primary and the later formed axillary branches

Since the seeds do not synthesize alkaloids [5], they are clearly powerful sinks for alkaloid transported from vegetative tissues The same is probably true of flowers, since their potential for synthesis is limited by the short period during which they are metabolically active before seed formation and by the small amount of tissue they contain The role of pods, which become alkaloid rich at an early stage, is not so clear, but evidence of their relatively small contribution to total dry wt accumulation in seed, suggests that they also probably contain substantial amounts of alkaloid derived by transfer from stems and leaves

It is significant that only seed tissues contain alkaloid in quantities well above the concentration required to develop the bitter phenotype, while stems and leaves, especially in *L mutabilis* and *L albus*, are near the threshold level that is associated with high-alkaloid, bitter phenotypes It is significant, however, that the alkaloid concentration in young seedlings from high-alkaloid parents does not fall below the level which is normally associated with the 'bitter' phenotypes This indicates that seedlings of the 'wild-type' of lupin species may be adequately protected against predation during early germination stages when alkaloid synthesis is still at a very low level

EXPERIMENTAL

The plant material used is listed in Table 5 The plants were grown in open ground and sampled at predetermined developmental stages during growth according to the following numbered harvests 0, seed, 1, cotyledons emerged and horizontal, 2, first leaf fully expanded, 3, 3–5 nodes visible—no laterals, 4, primary inflorescence visible, 5, first colour in petals (primary inflorescence), 6, open corolla on central flowers (primary inflorescence), 7, first colour in petals on secondary inflorescences, 8, all racemes with pods, and 9, seed ripe

For tests on early seedling stages (Table 4) samples were taken every 3–5 days after germination until the Dragendorff-negative phenotype was recorded

Quantitative analysis of alkaloid concentration was according to methods described previously [5], each harvest consisted of five plants selected at random and dried to constant wt Four replicate sub-samples were used for analysis For the study of expression of alkaloid-controlling genes during seedling development (see Results), the alkaloid content at different dates from germination was determined from total peak areas on GC traces The plants were germinated simultaneously in seedling compost in a glasshouse, and two seedlings, including roots, were harvested every 3 days after emergence above ground and the total seedling alkaloids extracted The results presented are means of the separate determinations on each of the two seedlings

The terms 'sweet' and 'bitter' which are widely used to

Table 5 *Lupinus* material examined for total alkaloid

Species	Cultivar	Genotype	Origin
Lupinus albus	Arkansas 10	Wild type	U S A
	Kievskij Mutant	*pauper* (low alkaloid mutants)	U S S R
	Ultra	*pauper* (low alkaloid mutants)	West Germany
	Neuland	*exiguus* (low alkaloid mutants)	West Germany
Lupinus angustifolius	Edelweiss	Wild type	East Germany
	LN 28	*iucundus* (low alkaloid)	?
Lupinus mutabilis	LM 24	Wild type	Peru

describe lupin phenotypes, respectively low and high in alkaloid content, are here used to denote Dragendorff-negative and -positive phenotypes [5]

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