The association of phytoecdysteroids with flowering in fat hen, *Chenopodium album*, and other members of the Chenopodiaceae

L. Dinan

Department of Biological Sciences, University of Exeter, Washington Singer Laboratories, Perry Road, Exeter, Devon EX4 4QG (England)
Received 12 April 1991; accepted 19 July 1991

Abstract. Very high concentrations of ecdysteroid are associated with flowering in *Chenopodium album*. Highest concentrations are found in anthers, but significant levels are also found in the stamens, carpels and sepals. In contrast, pollen contains only low levels. The ecdysteroid profile is the same in anthers as in whole-plant extracts, with 20-hydroxyecdysone and polypodine B predominating. The results for flowers of *C. album* are compared with the patterns determined for other chenopods (*C. capitatum, C. polyspermum, C. anthelminticum, C. giganteum, C. quinoa* and *C. foliosum*). The significance of these findings for plant-insect interactions and the relationship to the mode of plant pollination are discussed.
Key words. Chenopodiaceae; *Chenopodium album*; flowering; (phyto)ecdysteroid; plant insect interaction; pollination; radioimmunoassay.

Over 100 phytoecdysteroids have been structurally identified from a wide range of plant species[1]. Ecdysteroids have a broad spectrum of hormonal actions in insects and other invertebrates, but the functions of ecdysteroids in plants are unknown. In an attempt to resolve this question, we are undertaking a thorough examination of phytoecdysteroids in *Chenopodium album* and other species in the Chenopodiaceae. Previous results indicated that elevated levels of phytoecdysteroid are found in flowering specimens of *C. album* when compared to immature or senescing specimens[2]. In this report, I consider the relationship between phytoecdysteroid levels and floral development in *C. album* and other members of the goosefoot family. The occurrence of high levels of phytoecdysteroids during flowering in other plant species has previously been reported for *Serratula inermis*[3], *Serratula xeranthemoides*[4] and *Achyranthes fauriei*[5], but these reports have invariably dealt with the levels in intact flowers and have not compared the situation in several closely related species. While ecdysteroid levels in the two *Serratula* species are higher in the flowers than in other aerial portions and are maximal at full flowering, the levels in *A. fauriei* do not alter during the various phases of flowering and are the same as in the rest of the plant.

Materials and methods

Reference ecdysteroids were purchased from Simes, Milan, and Sigma, Poole, U.K.

Plants were grown from seed in pots containing John Innes Compost N° 2 in glasshouses at the University of Exeter. Pollen was collected by gently shaking flowering plants (40) daily over a sheet of aluminium foil. This resulted in a mixed preparation containing pollen and anthers, which were then separated by passing the pollen through a sieve (180-μm mesh).

Ecdysteroid extraction and radioimmunoassay. Plant material was freeze-dried and small samples (< 25 mg d.wt) were extracted in Eppendorf vials with methanol (3 × 0.7 ml at 55 °C for 3 h). The residues from the first two extracts were dissolved in the third extract, 0.3 ml water was added and the whole partitioned against hexane (3 × 0.5 ml). The aqueous methanol phase was then assessed for its phytoecdysteroid content by radioimmunoassay (RIA). Larger amounts of plant material were extracted in a scaled-up version of the above. Three ecdysteroid antisera have been used in the course of this work (DBL-1, H-2 and H-22), each of which recognises a different region of ecdysteroid molecules, and hence are suitable for differential radioimmunoassay[6]. The RIA procedure has been described previously[7], and results are expressed in ecdysone equivalents. The cross-reactivity factors for 20-hydroxyecdysone, makisterone A and polypodine B are 0.8, 1.8 and 5.4, respectively, with the DBL-1 antiserum, 1.7, 5.6 and 2.3 with the H-2 antiserum and 0.6, 0.7 and 3.8 with the H-22 antiserum (all values relative to ecdysone; cross-reactivity = 1).

Analysis of ecdysteroids in anthers. Phytoecdysteroids were extracted from the pollen/anther preparation (240 mg). The aqueous methanol phase contained 646 μg ecdysone equivalents (DBL-1 antiserum). The ecdysteroids were further purified on C_{18} Sep-Pak cartridges[8], where 90% of the RIA-positive material eluted in the 25–60% methanol in water fraction. The ecdysteroids were then purified by HPLC, using sequentially a semipreparative C_{18} reversed-phase (RP) column (with a linear gradient from 30% to 100% methanol in water over 40 min at a flow rate of 2 ml/min), a semi-preparative C_6 column (with a linear gradient from 20% to 100% methanol in water over 40 min at 2 ml/min) and an analytical DIOL column (4% methanol in dichloromethane isocratic or a linear gradient from 4% to 10% methanol in dichloromethane over 40 min, both at 1 ml/min).

Analysis of ecdysteroids during flowering. Flowers and seed were collected from plants at various developmental stages and extracted according to the procedure de-

scribed above. Ecdysteroid levels were determined by RIA using the DBL-1 antiserum. Organs were dissected from 30 fully open flowers of *C. album*, and these were also extracted and analysed. The identity and percentage distribution of RIA-positive material was determined by HPLC/RIA. Samples (200 ng ecdysone equivalents) were first separated by RP-HPLC on which 20OHE and PolB co-elute, to quantify the combined amount of these ecdysteroids relative to the polar and apolar RIA-positive materials. The material from the 20OHE/PolB peak was then further fractionated by NP-HPLC (DIOL column) to quantify the relative amounts of these two ecdysteroids.

Results

Ecdysteroids in anthers/pollen of C. album. The initial working hypothesis, based on the increased levels of ecdysteroid in flowering plants of *C. album*, was that the ecdysteroid was associated with the pollen, which is produced in large amounts in this wind-pollinated species. This hypothesis was influenced by the identification of brassinosteroids in pollen of some species[9], the structural resemblances between ecdysteroids and brassinosteroids, and the weak (anti)ecdysteroid activity of some brassinosteroids in insect ecdysteroid activity bioassays[10]. Consequently, pollen was collected from *C. album*, but the preparation was found to be heavily contaminated with anthers. These were separated by sieving and each was independently extracted and analysed using three ecdysteroid antisera. This clearly revealed that the initial hypothesis was wrong as, in each case, the RIA-positive material is primarily (> 97%) associated with the anthers and further, that the concentration in the anthers is very high (0.54% of the dry weight, based on ecdysone equivalents using the DBL-1 antiserum).

Separation of this extract by C_{18}-RP-HPLC and monitoring of the fractions by differential RIA revealed three regions of RIA-positive material. Most (89%) of the DBL-1-reactive material elutes at 17 min, co-chromatographing with 20-hydroxyecdysone. The material in the polar region (2.5% of the DBL-1-positive material) at a retention time of 12.5 min is recognised almost twice as well by the H-22 antiserum as the DBL-1 antiserum, but poorly by the H-2 antiserum. This would indicate that the ecdysteroid in this region is modified in the side-chain relative to ecdysone. The apolar region (eluting at 39.5 min and accounting for 9% of the DBL-1-positive material) is recognised by the three antisera in the order H-22 > DBL-1 > H-2(2.1:1:0.4). This again indicates modifications in the side-chain relative to ecdysone. The cross-reactivity ratios are similar to those for makisterone A, so the apolar RIA-positive material probably corresponds largely to ecdysteroids possessing alkyl groups at C-24 of the side-chain. This possibility has been alluded to previously[11] on the basis of isolation of one component and physico-chemical analysis. Hydrolysis of portions of each of the HPLC fractions with *Helix pomatia* gut juice enzymes and reassessment with the DBL-1 antiserum revealed no extra peaks of RIA-positive material and no alteration in the relative distribution of RIA-positive material between the three peaks. Thus, there is no evidence for the presence of *Helix*-hydrolysable conjugates in this extract.

The major peak from C_{18}-RP-HPLC remained as one peak on C_6-RP-HPLC, but separated into two UV-absorbing and RIA-positive peaks on a DIOL (normal-phase) column, the larger and smaller peaks (molar ratio, 2.5:1) co-chromatographing with 20-hydroxyecdysone and polypodine B, respectively, as expected from previous analyses[1,11].

The material in the polar region from C_{18}-RP-HPLC elutes as one peak on C_6-RP-HPLC (R_t = 21.5 min), but as two major peaks on the DIOL column (fig. 1). The amounts of material in these peaks were too small for further analysis, but the combined chromatographic and immunological data suggest that these compounds are derivatives of 20OHE and PolB, which are further hydroxylated in the side-chain.

The apolar region from C_{18}-RP-HPLC showed separation into two incompletely resolved peaks on C_6-RP-HPLC, one of which possessed a retention time similar to that of ecdysone (28 min) and the other eluting slightly before (26.5 min). The material in each of these two peaks, when separated independently on the DIOL column, yielded a complex pattern of RIA-positive material corresponding to UV-absorbing peaks (fig. 2; peaks

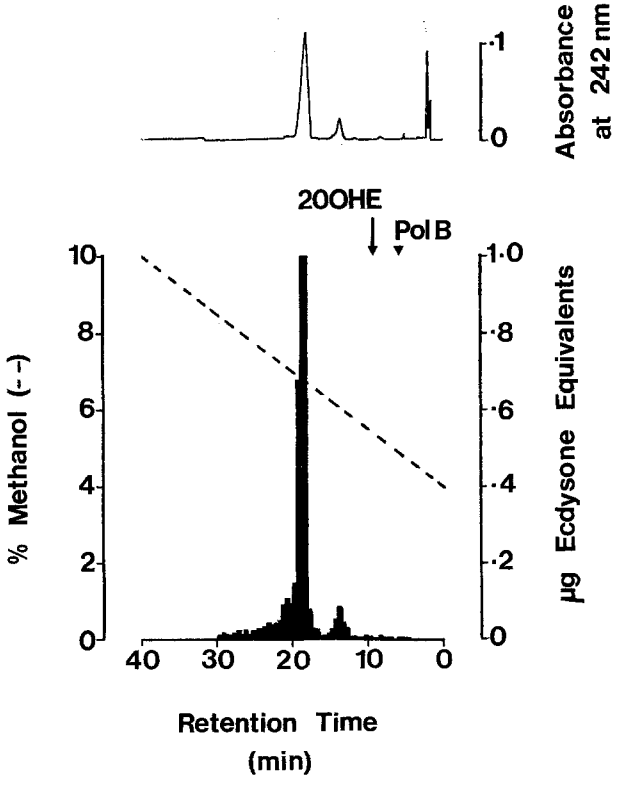

Figure 1. Separation of the polar RIA-positive material from C_6-reversed-phase HPLC by normal-phase HPLC with monitoring by UV-absorption (242 nm) and RIA (H-22 antiserum).

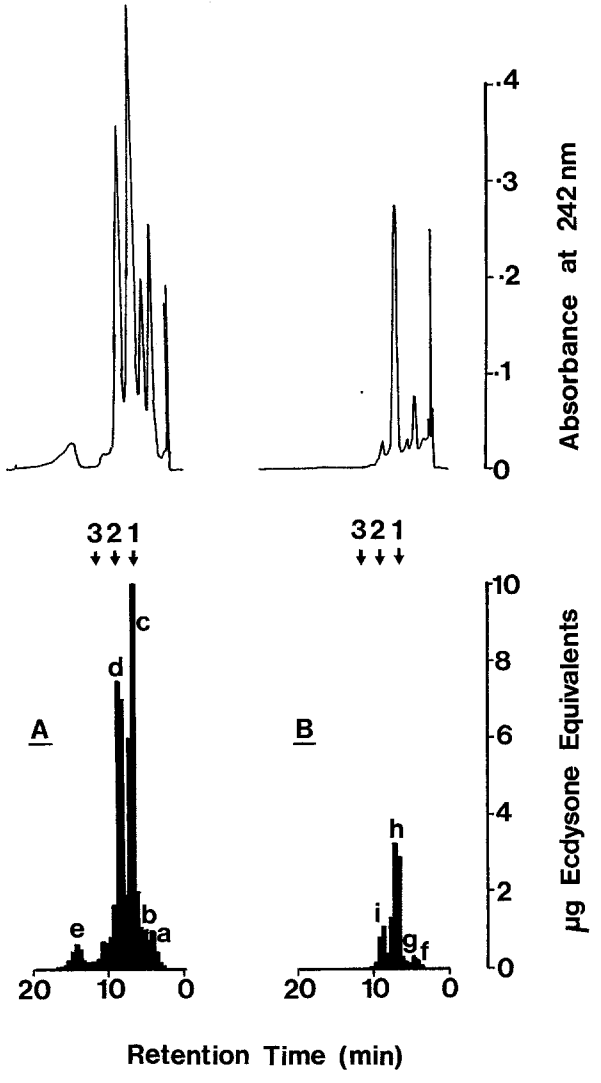

Figure 2. Separation of the apolar RIA-positive material eluting at A) 26.5 min and B) 28 min from C_6-reversed-phase HPLC by normal- phase HPLC with monitoring by UV-absorption (242 nm) and RIA (H-22 antiserum). RIA-positive peaks are designated by lower case letters. Peak g was detected at a retention time of 5.8 min (concomittant with a UV-absorbing peak) with the H-2 antiserum, but it is not detected with the H-22 antiserum. The retention times of reference ecdysteroids are indicated by the numbered arrows; 1 = polypodine B, 2 = ecdysone and makisterone A, and 3 = 20-hydroxyecdysone.

a–i), and although there is some evidence for the presence of ecdysone (peak i) which was supported by differential RIA (data not shown), the preparations were too heterogeneous for further analysis. Also, we have previously shown that only the two major ecdysteroids are biologically active in an insect bioassay system [12].

Thus, anthers of *C. album* contain predominantly 20-hydroxyecdysone and polypodine B with much smaller amounts of at least eleven other ecdysteroids.

Ecdysteroid levels during flowering in C. album. The distribution of ecdysteroids within fully opened flowers was next examined (table 1). It is clear that the highest concentration is found in the anthers (0.5%), but the levels in other portions of the flowers are not insignificant (0.1–0.15%). The developmental profile associated with flowering has been examined (table 2) and this demonstrates that the highest ecdysteroid levels are associated with the fully-open flowers and flowers just prior to opening. Levels in fruit (i.e. once the anthers have been shed) are significantly lower, as are those in the seed. HPLC/RIA demonstrated that the distributions between the RIA-positive ecdysteroids in growing tips, open flowers, seed or sepals, carpels, stamens and anthers from fully open flowers are essentially identical to those found in the anther/pollen preparation and in the majority of regions of the non-flowering plant (unpublished observations).

The association of ecdysteroids with flowering in other goosefoots. Flowers and fruits from six other members of the genus *Chenopodium* have been analysed for their ecdysteroid content (table 2). They fall quite distinctly into two classes. Flowers of *C. anthelminticum, C. capitatum* and *C. foliosum* contain no or barely detectable levels of phytoecdysteroids, whereas *C. giganteum, C. polyspermum* and *C. quinoa* possess levels equal to or exceeding those of *C. album*.

Table 1. Ecdysteroid levels in portions of fully-open flowers (30) of *Chenopodium album* determined by radioimmunoassay (DBL-1 antiserum)

	Dry wt	μg Ecdysone eq./mg d.wt	ng Ecdysone eq./flower
Anthers	0.40 mg	5.05	67.4
Stamens	0.53 mg	1.46	25.8
Carpels	0.68 mg	1.10	24.9
Sepals	1.77 mg	1.02	60.2

Table 2. Ecdysteroid levels associated with flowering in goosefoots

Species	Flowering stage	ng Ecdysone eq./mg dry wt
Chenopodium album	Growing tip	1045
	Immature flowers	1544
	Open flowers	1569
	Fruits	804
	Seed	361
Chenopodium capitatum	Immature flowers	0
	Green/pink flowers	0
	Red flowers	0
	Purple/seeded	0
	Dark purple/seeded	0
Chenopodium polyspermum	Immature flowers	2860
	Open flowers	2603
	Fruit	884
	Seed	249
Chenopodium anthelminticum	Growing tips	31
	Immature flowers	0
	Flower spikes	2
	Fruits	2
Chenopodium giganteum	Growing tips	3442
	Immature flowers	2582
	Open flowers	2775
Chenopodium quinoa	Growing tips	589
	Immature flowers	1860
	Open flowers	1135
	Green fruits	1399
	Brown fruits	252
Chenopodium foliosum	Flowers	4

Values obtained with the DBL-1 antiserum.

Discussion

One could consider the above results as supportive of either of the main hypotheses for phytoecdysteroid function. The clear correlation with flowering could indicate a developmental rôle of phytoecdysteroids within the plant. However, no physiological function in plants has ever been conclusively demonstrated to be regulated by ecdysteroids[13], and Felippe's experiment[14] on the influence of 20OHE on flower formation in *Curcurbita pepo* is unconvincing. 20OHE failed to induce flowering in *Xanthium pensylvanicum*[15].

We tend to favour the defensive hypothesis for the rôle of phytoecdysteroids. In an annual such as *C. album*, rapid meristematic growth and the production of flowers and seed can be viewed as essential attributes for the multiplication of the species. Equally, the soft tissues of the growing tips and flowers would be very attractive and nutritious to would-be insect predators, and they would need to be extensively protected. The very high concentrations of phytoecdysteroids found in these portions of *C. album* would be expected to deter all but the most adapted invertebrate predators[16]. The production of pollen represents a very high energy and nutrient input by the plant and this could explain why the organ enclosing it (i.e. the anther) contains very high concentrations of phytoecdysteroid. The low concentration in the pollen itself is somewhat enigmatic, but in a wind-pollinated species like *C. album*, a released pollen grain has only a low chance of finding a flower to fertilise. Thus, pollen grains need to be protected while developing, but there is little value in protecting individual pollen grains once released from the plant. If so viewed, the concentration of phytoecdysteroids in the anther tissue surrounding the developing pollen grains, but not in the pollen itself, has an evolutionary logic.

The goosefoots possessing high levels of ecdysteroid in their flowers possess visually insignificant inflorescences, are also the ones which produce large amounts of pollen and are presumably wind- or self-pollinated. *C. anthelminticum, C. capitatum* and *C. foliosum* produce much smaller amounts of pollen and are good candidates for insect pollination or insect-assisted self-pollination. If this is true (there is only sparse information in the literature on the modes of pollination in the different members of the Chenopodiaceae), one would expect high levels of phytoecdysteroid in the flowers of these species to be detrimental to the success of pollination. We are currently following this avenue of research and will examine the distribution of phytoecdysteroids in other members of the Chenopodiaceae including the oraches (*Atriplex* spp.) which are monoecious and thus bear separate male and female flowers.

These studies together with those on other plants show that the occurrence of phytoecdysteroids is not universal and seem to suggest that the distribution of phytoecdysteroids within the plant (if present) is species-dependent and determined by the growth pattern of the plant and the nature of the predatory or beneficial invertebrates it encounters.

Acknowledgments. Seed was generously provided by the Botanic Gardens of the Universities of Hull, London, Oxford and Sheffield. I should like to express my thanks to Mr P. Goodson for growing the plants, Dr J. Roddick and Ms A. L. Rijnenberg for helpful advice and discussion, and C. Y. Clément for his comments on the manuscript. The antisera were very generously provided by Profs L. I. Gilbert, J. D. O'Connor (Chapel Hill, NC, USA) and J. Koolman (Marburg, FRG.)

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0014-4754/92/030305-04$1.50 + 0.20/0