Comparison between the effects of 20-hydroxyecdysone and phytohormones on growth and development in plants

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Abstract. Insect moulting hormone, 20-hydroxyecdysone or ecderosterone (E), was tested in a series of bioassays that are commonly used for evaluation of the activity of phytohormones. The assays included: auxin coleoptile bioassay in wheat, gibberellin bioassays in dwarf maize and dwarf rice; cytokinin bioassay on tobacco callus; brassinolide related ethylene formation assay in dwarf maize, alfalfa and Chenopodium, flowering assay on Chenopodium and special assay on somatic embryogenesis in cell cultures of alfalfa.

The 20-hydroxyecdysone has no biological activity in tests used for detecting phytohormone activity, excepting for slight gibberellin-like activity in rice. Moreover, E does not have any synergistic or antagonistic activity. In tissue cultures of alfalfa, E affected differentiation of the embryos. It is concluded that, in spite of its widespread occurrence in plants, E has no direct action on growth and proliferation of plant tissues (which is the main action of E in arthropods). However, E and other ecdysteroids could provide a source of easily available, slightly polar, polyhydroxylated sterol, required for growth and cell proliferation in plants.

INTRODUCTION

Ecdysteroids are “polyhydroxylated steroids with insect moulting hormone activity, characterized by an α,β-unsaturated 6-keto group” (Sláma, 1993). The first ecdysteroid with known chemical structure, ecdysone, was isolated from insects in 1965 by screening for insect moulting hormone activity (Karlson et al., 1965), but almost immediately there were reports on extremely large quantities of similar compounds being present in plants (for review see Bergamasco & Horn, 1983; Lafont & Horn, 1989). A present around 200 types of ecdysteroids have been shown to occur in various plants, randomly distributed among various families of both the lower and higher plants. However, in spite of their widespread occurrence in the plant kingdom, the true physiological significance of ecdysteroids in plants remains unknown (cf. Lafont & Horn, 1989).

Recent studies show that the content and translocation of ecdysteroids may be in certain cases linked with plant growth (Dinan, 1992). The random distribution of ecdysteroids among species as well as among various parts of the plant system led earlier to the conclusion that ecdysteroids could not be considered as the growth hormones of plants (Sláma, 1979). The occasional accumulation of ecdysteroids in hibernating storage organs, with their simultaneous accumulation in the fast growing parts suggested that these
slightly water-soluble steroids could be perhaps used for mobilization and transport of steroids within the hydrophilic milieu of the plant system (Sláma, 1979). Superficially, such a claim that an insect hormone could be used for transport of steroids in plants, looks strange and has been always taken with caution. It remains that the exact physiological role of ecdysteroids in plants is still obscure. The only reasonable argument for ecdysteroid presence in plants thus far appears to be for defense against insect herbivores (for more details and references see Sláma, 1969, 1979, 1993). Among all hitherto known animal hormones, ecdysteroids occupy special position. In invertebrates, their biosynthesis is not strictly localized into a single endocrine gland, which is the case with other hormones, but they can be secreted into the haemolymph by a number of peripheral target cells, e.g. situation similar to plant hormones. Finally, in the vertebrates, ecdysteroids cause a number of biochemical, physiological and pharmacological changes whose nature is not hormonal (for review see Sláma & Lafont, 1995).

In this report we employ a comprehensive experimental approach which has, in part, been used by several authors (Hendrix & Jones, 1972; Dreier & Towers, 1988). The ecdysteroids were subjected to a series of standard plant bioassays, which were commonly used for determinations of biological activities of certain phytohormones. Although many of previous results were minimally active or negative, it is difficult to reconcile that such an enormous accumulation of ecdysteroids in certain plants (30 mg or more per g of dry mass; see Jizba et al., 1967) would have no physiological significance.

MATERIAL AND METHODS

Ecdysteroid material

In most of our bioassays we used 20-hydroxyecdysone (E), natural product isolated from the roots of Lesceae (Rhaponticum) carthamoides Iljin. In most instances we used the product containing 96% E and 4% Polypodine B (5β,20-dihydroxyecdysone). The stock solution (10⁻³ M) was prepared in 10% ethanol-water, large dilutions were made with water or with the respective nutrient solutions.

The auxin coleoptile bioassay

Segments of 10 mm in length were cut from wheat coleoptiles 3 mm below the tip (Triticum aestivum L., cv. Kavka, 19–22 mm long) as described by Nitsch & Nitsch (1957). The variety Kavka was chosen from 36 available varieties due to the best responses to auxin (Pavlová et al., 1977). The segments were washed in distilled water for 2 hours prior to their incubation in the buffered solutions of the tested substances (20 h at 29°C in darkness). Changes in length of the segments were evaluated by means of an optical projecting device.

Gibberellin bioassays

The dwarf maize test was made with the d₄ mutant of maize, as previously described (Phinney, 1956). The seeds were at first germinated for 5 days in darkness at 25°C and then the plantlets were grown in the nutrient solution under constant light at 24°C. After 2 days, the tested solutions were applied in 50 μl amounts into the still rolled primary leaf. The effects were evaluated according to the length of the second leaf 6 days thereafter. The dwarf rice test was made with seeds of the dwarf rice mutant (Oryza sativa L., mutant Tan-ginbozu) according to Sembdner et al. (1988). The seeds were germinated for 3 days at 28–30°C in darkness. The seedlings were transferred into test tubes containing 1 ml of the tested solutions. The effects were evaluated after 7 days at 28–30°C under constant light, according to changes in length of the seedlings.

Cytokinin bioassay

This test was made with the cytokinin dependent tobacco callus (Nicotiana tabacum, cv. Wisconsin). The callus was grown on agar MS medium supplemented with pyridoxine (0.25 mg l⁻¹), nicotinic acid (0.25 mg l⁻¹), 3% sucrose (Murashige & Skoog, 1962), α-naphthylacetic acid (NAA, 1 mg l⁻¹).
benzyaminopurine (BAP, 0.285 mg l⁻¹) and various concentrations of ecdysterone. The callus growth took place in darkness at 25°C for 4–5 weeks, the effects were evaluated on basis of differences in the fresh mass.

**Ethylene formation assay**

Experimental plants (Chenopodium rubrum, dwarf maize and alfalfa suspension cultures) were tightly closed inside special vessels. They were incubated for 1–3 hours at 25°C in light (in case of Chenopodium at 20°C) in the presence or absence of E. After incubation a sample of air was withdrawn by means of an air-tight syringe and the content of ethylene was determined by GC method (Macháčková et al., 1986).

**Flowering assay**

These assays were made on a short-day plant, Chenopodium rubrum, as previously described (Ullmann et al., 1985). After germination the plants were kept for 5 days under constant light at 20°C. After this they were subjected to flower induction by two complete cycles of 12:12 hour, light:dark (full induction) or only one such photoperiodic cycle (partial induction). Solutions to be tested were applied to the plumule in form of a 3 μl droplet 2 hours before the onset of the scotophase. Partial induction was used for testing stimulatory effects on flowering, whereas full induction served for testing possible inhibitory effects. After termination of the photoperiodic treatment, the plants were maintained under continuous light for 6 days; the induction of flowering was analysed under stereomicroscope.

**Effects on somatic embryogenesis**

Embryogenic cultures of alfalfa (Medicago sativa L., cv. G 13) were cultivated in liquid medium (see Blaydes, 1966), with the addition of 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μM kinetin (K), using rotary shaker at 25/20°C day/night temperature and 16-hour photoperiod. The cultures were sieved through 104 μm gauge sieves. The material separated into single cells, smaller cell clumps and embryo fraction, previously kept in medium without growth regulators were used as experimental material. At the end of the experiments, the cultures were sieved again and fresh weight of the pre-embryonic mass or newly developed embryos was determined.

In all the experiments, each treatment included 20 plants (in the case of tobacco callus 3 flasks; in alfalfa embryonic culture 4–6 flasks); all experiments were repeated 2 to 3 times. The values of S.E.M. show variation within each experiment. The control experiments are obvious from the design and results in Figs 1 to 7.

**A abbreviations**: 2,4-D – 2,4-dichlorophenoxyacetic acid, BAP – benzyaminopurine, E – ecdysterone (20-hydroxyecdysone), EMB – embryonic fraction, GA – gibberelic acid, IAA – indole-3-acetic acid, K – kinetin, NAA – α-naphthalicetonic acid, PEM – pre-embryonic mass, PGR – plant growth regulators.

**RESULTS**

In arthropods and other invertebrate animals, ecdysteroids function as special growth hormones, that stimulate cell divisions and proliferation in the epidermis as well as other tissues. If analogous responses in plants could be reliably determined in one or other bioassay for phytohormones, a hormone based developmental model could be hypothesized. We began our experiments testing for auxin-like biological activity in the wheat coleoptile assay. The results presented in Fig. 1 show that E has no auxin-like activity. The largest concentration of E (10⁻⁷M) produced in fact a slight inhibition. There were no remarkable synergistic or antagonistic relationships between the effects of E and IAA.

The gibberellin assay in dwarf maize (Fig. 2), demonstrated that E exhibited a slight, but definite, dose-dependent stimulatory action. With the use of 5 μg doses of E the second leaf grew 31.1% over the control length, whereas the same dose of GA produced as much as 120.6% increase. Quite consistent results were obtained in the second gibberellin assay performed on dwarf rice (Fig. 3). Here the 10⁻⁷M concentrations of E caused 68.5% stimulation of growth while the same concentration of GA produced 189.2%. In addition, the
results in Fig. 3 indicate a possibility of slight synergistic action between E and GA (compare the values of GA-5 with GA-5+E-5 in Fig. 3).

Being encouraged by slightly positive responses of E in the above gibberellin assays, we performed also the cytokinin tests on tobacco callus. The results in Fig. 4 demonstrate that E is inactive in this bioassay. The highest concentration of E (10^{-5} M) actually produced some necrotization of the callus, but this may be a side effect due to the presence of some ethanol needed for better solubilization of high concentrations of E (not included in Fig. 4). The combined concentrations of E with NAA did not show any special interactions, but there was potentiation of the effects of BAP.

Fig. 5 shows the results of our assays with ethylene production in Chenopodium and dwarf maize. In both cases E produced a slight stimulation of ethylene formation within 3 to 5 hr after the application but later this effect disappeared (data not shown), suggesting that E would have a very small, if any, effect on ethylene production in the tested systems. Accordingly, Fig. 6 shows that 50 M concentrations of E in the liquid medium also failed to affect the rate of ethylene production by cultures of somatic embryos of the alfalfa.

In animals, E chiefly affects growth of the immature larval or pupal stages, while it may be completely ineffective in the fully differentiated tissues of an adult. With this observation in mind, we performed bioassays including the effects of E on growth, differentiation and ethylene formation in the pre-embryonic and embryonic tissue cultures of alfalfa. The
results are presented in Fig. 7. There was no effect of E on growth of the alfalfa somatic embryos (in the heart-torpedo stage of development), although E (10⁻⁴ to 10⁻³ M) evidently stimulated growth in the nondifferentiated pre-embryogenic mass (PEM, see the differences between the series with and without the PGR in Fig. 7). In this respect we have also found the stimulatory effect on ethylene production in the cultures of pre-embryonic alfalfa cells, which were maintained in the medium containing PGR and 50 M E. The results described in Fig. 7 reveal some further relationships related to possible effects of E on plant growth. Namely, that E actually inhibited or delayed the induction of somatic embryos, both in the absence and in the presence of 2,4-D and kinetin (cultivation of PEM with ecdysterone did not change the pH of the media). As a consequence of such altered growth, the proportions between growth of the PEM and formation of the embryos (PEM/EMB ratio in Fig. 7) were remarkably affected by E. These results obtained with the PEM of alfalfa indicate for the first time some remote analogies in the effects of E on animal and plant growth, although they still need to be verified on some other systems.

In insects, there are species where E profoundly affects reproduction while it is virtually ineffective on reproduction in many other species (Sláma et al., 1974). In this work we have also attempted to investigate possible effects of E on flowering in 5-day-old Chenopodium rubrum. These plants are easily induced to full flowering by 2 cycles of 12 : 12 hr light : dark illumination. However, E had no effect on flowering in the range of concentrations between 10⁻² to 10⁻¹ M. Moreover, there were no effects of E on flowering even

Fig. 3. Gibberellin test showing the effects of ecdysterone (E; 10⁻⁴ to 10⁻³ M conc.) and gibberellic acid (GA; 10⁻⁴ to 10⁻³ M conc.) on growth of the seedlings of dwarf rice Tan-ginbozu. The seedlings were cultivated in the tested solutions for 7 days at 28°C in constant light.

Fig. 4. Cytokinin test showing the effects of ecdysterone (E; 10⁻⁴ to 10⁻³ M conc.), benzylaminopurine (BAP; 0.285 mg.l⁻¹) and α-naphthylacetic acid (NAA; 1 mg.l⁻¹) on growth of the tobacco callus. The calli were grown for 4–5 weeks at 25°C in darkness.
Fig. 5. Ethylene production test showing the effects of ecodysterone (E; $10^{-3}$ to $10^{-6}$ M conc.) on ethylene production in the 5-day-old Chenopodium rubrum (black columns) and in dwarf maize plants used in the gibberellin assays above (lined columns; 0.1 to 5.0 μg/plant).

when the plants were subjected to a subinductive treatment (only one 12 : 12 hr light cycle).

**DISCUSSION**

We were unable to show any "growth hormone-like" activity for ecodysteroids and their role in plants remains obscure. The first studies performed two or three decades ago claimed the presence of gibberellin-like activity in some ecodysteroid-containing extracts from insects or plants (Carlisle et al., 1963; Matsuoka et al., 1971). Later, with more advanced techniques, there was no evidence for gibberellin-like activity of ecodysteroids in several assays on different plants (Hendrix & Jones, 1972; Felipe, 1980). More recently, Dreier & Towers (1988) observed slightly positive gibberellin-like effects in one assay system while they found no gibberellin activity in another plant assay. Our results confirm the slight gibberellin-like effects of E, especially in rice (Fig. 3) which is the plant used by earlier researchers (Carlisle et al., 1963; Matsuoka et al., 1971; Dreier & Towers, 1988).

The inability of E to mimic the effects of other phytohormones is consistent with some previous results related to cytokinin (Dreier & Towers, 1988). Our failure to affect flowering in Chenopodium by E corroborates the findings of Jacobs & Suthers (1971) who found no effects of E on flowering in Xanthium. According to all these equivocal responses of

Fig. 6. Growth of alfalfa somatic embryos and ethylene production showing the combined effects of ecodysterone (E; 50 μM in liquid medium) and plant growth regulators (PGR; 5 μM 2,4-D and 1 μM kinetin).
plant bioassays, we are in agreement with the earlier conclusions of Sláma (1979), that ecdysteroids (phytoecdysones) cannot be regarded as being the growth hormones of plants.

In analogy with the situation occurring in brassinosteroids (which are closely related by chemical structure to ecdysteroids), we have assumed that any more pronounced effects of E on growth in a plant should be associated with an increase of ethylene production (see Artec & Schlaghauf, 1984). However, the obtained results show no direct effect of E on the rate of ethylene production in intact plants. Only in the case of alfalfa pre-embryonic culture, we have recorded some increase in ethylene production, which was also related to E effects on growth. When compared with the effects of a true brassinolide, however, the effects of E are very weak (Dreier & Towers, 1988).

The increased ratio between growth of the PEM and the formation of embryos is generally claimed to be a good criterion for determination of altered growth conditions in plant systems. Our findings, suggesting that E increases the PEM/EMB ratio in alfalfa, show that E might somehow affect cell differentiation in this assay on plants. This may indicate existence of certain analogies between ecdysteroid action on immature cells of plants and arthropods. This observation, however, can hardly justify the presence of very large amounts of ecdysteroids in certain plants. In invertebrates, ecdysteroids play a role of real growth hormone, regulating moulting and developmental cycles. In vertebrates, they cause a number of nonspecific, biochemical and pharmacological, vitamin-like effects (Sláma & Lafont, 1995). Our results demonstrate that in plants, ecdysteroids might also represent rather a favourable growth condition, but do not directly regulate growth (see Sláma, 1993).

The investigations of ecdysteroid action in higher plants are still complicated by the fact that their transport is not well understood. In addition, isolated plant tissues like calluses, for example, can endogenously synthesize ecdysteroids (Lev et al., 1989), which may render the exogenous artificial supply ineffective. These, and other problems of ecdysteroid action in plants are the subject of further studies in our laboratories.

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