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Hormonal Physiology of Flowering in Xanthium strumarium.

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Hormonal Physiology of Flowering in Xanthium strumarium" submitted by Gregory F.W. Gocal in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Hormonal physiology in Xanthium strumarium was studied in relation to floral evocation. The florigenic signal (from photoperiod induction through floral evocation) for this short-day plant (SDP) may involve a net change in the balance of floral-inhibitory and floral-promotive substances.

Endogenous Gibberellins (GAs) identified by gas chromatography-mass spectrometry (GC-MS) in Xanthium included $GA_{1,3,4,8,9,19,20,29}$. Quantitation by GC-MS-selected ion monitoring, showed levels of GA_1 and 2ß-hydroxy GA_1 (GA_8) decreased in both leaf and apical region tissue following 16 hour (h) long night floral induction. Conversely, GA_4 levels increased in induced leaves and decreased more slowly in induced apical region tissue, both relative to these tissues from non-induced long-day (LD) plants.

In a Xanthium seedling flowering bioassay, chlorogenic acid and less-polar GAs, including $GA_{4/7/9}$ mixture >GA_7 >GA_4 >2,2-dimethyl GA_4 >GA_9 >GA_5, listed in order of decreasing efficacy, were all promotive of floral evocation when applied before a marginally-inductive long night (LN). The order of GA efficacy with regard to promoting epicotyl growth was similar. Application of two GA biosynthesis inhibitors reduced flowering response to 1 inductive 16 h LN, and one of these (uniconazole; 3 g/L) completely inhibited flowering with its inhibition reversed by the application of $GA_{4/7/9}$.

Applied indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC), and (s)-abscisic acid (ABA) all decreased the level of floral evocation induced by a single 16 h LN. In fact, 100 µg/seedling of IAA and ACC totally inhibited flowering. Ethylene production by the apical region, 4 h after ACC application to the leaf, was 5-fold higher than that after IAA application, which in turn was 10- to 20-fold higher than that from the apical region of untreated (LD) control plants. Simultaneous application of silver thiosulfate prevented (or reversed) ACC-, but not IAA-induced inhibition of flowering.

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ABBREVIATIONS

ABA ..... abscisic acid

ACC ..... 1-aminocyclopropane-1-carboxylic acid

ANOVA ..... analysis of variance

BSTFA ..... bis-(trimethyl-silyl) trifluoroacetamide

C_{18}-PC ..... C_{18} preparative column

CCC ..... 2-chloroethyltrimethylammonium chloride

CGA ..... chlorogenic acid

d.w. ..... dry weight

DMSO ..... dimethyl sulfoxide

EtOAc ..... ethyl acetate

f.w. ..... fresh weight

GA ..... gibberellin

GA_n ..... gibberellin #

GC-MS-SIM ..... gas chromatography-mass spectrometry-selected ion monitoring

HOAc ..... acetic acid

HPLC ..... high performance liquid chromatography

IAA ..... indole-3-acetic acid

KRI ..... Kovat's retention index

LD ..... long day

LDP ..... long-day plant(s)

Me ..... methyl

MeOH ..... methanol

MSD ..... mass selective detector

NB..... night break

PAR..... photosynthetically active radiation

R_t ..... retention time

SD ..... short day

SDP ..... short-day plant(s)

SiO_2-PC ..... silica gel partition column

SNK ..... Student-Newman-Keuls statistical test

STS ..... silver thiosulfate

TCMS..... trimethylchlorosilane

TIBA..... 2,3,5 triiodobenzoic acid

CHAPTER 1

INTRODUCTION

In higher plants a critical developmental event is the evocation of flowering, the transition of the apex from a vegetative to a reproductive state. This transition and subsequent seed development represents the pinnacle of the life of an angiosperm. Photoperiodic induction is the term used to refer to the inductive events occurring in the leaves, whereas, evocation is used to describe the transition of the apex from a vegetative to a reproductive state (Evans, 1971). Evocation is thus an obvious and important transition to study. In addition to its importance with regard to the propagation of plant species, success and failure in flowering of higher plants also heavily impacts the world economy due to the consumption of fruit and seeds, both directly and indirectly, in the human diet. Thus, studies probing the physiological, biochemical and molecular bases of flowering may have substantial consequence. In fact, basic research in these areas may permit manipulation and control of the reproductive process in higher plants. Such manipulations could result in increased crop yields with accompanying economic gains. Even weed control might be accomplished via manipulation of flowering.

Daylength is an environmental variable which enables plants to approximate, among other things, the number of days remaining in their growing season. Their ability to sense this variable is essential in environments with a growing season of finite length. Since temperatures may be unseasonable, it is more advantageous for plants to respond to an inaementally changing photoperiodic cycle, although there are many examples of low temperature being used as a flowering signal (e.g. vernalization). Annual plants which are unable to complete their life cycle (e.g. dispersal of desiccated seed) in a single growing season will fail to leave progeny to the next generation and would naturally be selected against. The further north or south a plant grows from the equator, the shorter is its growing season and thus the more important photoperiodic measurement becomes. Plants

which must complete their growth prior to a dry-season are under similar pressure to utilize photoperiodic changes as a signal in flowering.

1.1 Photoperiodism.

Plants which flower under certain daylength conditions are said to be photoperiodic. Short-day plants (SDP) flower only when the photoperiod is less than some critical length (i.e., Xanthium strumarium (cocklebur) (Mukherjee, 1974) and Pharbitis nil (Japanese morning glory) (Nakayama, 1958). In actual fact, SDP measure night length, which must exceed a critical value to initiate the transition from a vegetative to a reproductive state (Zeevaart, 1978). For this reason SDP could be more appropriately termed long-night plants. Xanthium is a classic absolute SDP (Salisbury, 1982). Plants which flower when the daylength is greater than a certain length are termed long-day plants (LDP) (i.e., Nicotiana sylvestris (tobacco)) (Lang, 1965) and Lolium temulentum (rye grass) (Evans, 1969a). The requirements for SDP and LDP can be obligate or facultative (Zeevaart, 1978). Plants which are facultative will eventually flower even under unfavorable photoperiods, although much later in their development. Although SDP and LDP comprise the majority of photoperiodically inducible species, two additional sensitive classes exist: short-long-day plants (SLDP) (i.e., Coreopsis grandiflora) and long-short-day plants (LSDP) (i.e., Bryophyllum diagremontianum) (Zeevaart, 1978). These two classes require a sequence of short and long days to initiate flowering. Plants which lack photoperiodic sensitivity are termed day-neutral plants (DNP) (i.e., Zea mays (corn) and Glycine max (soybean)) (Bernier, 1988), although flowering in plants classed as DNP species may often be hastened by either SD or LD (Salisbury, 1982).

In the experiments discussed herein the obligate SDP Xanthium strumarium has been chosen as the experimental material. The obligatory SD (long night) requirement for flowering of this plant is the aspect which makes it so interesting as a system to test various treatments which might be promotive of floral evocation (Mukherjee, 1974). Xanthium is not "leaky" as are so many other photoperiodically inducible species (e.g. Brassica spp. and Nicotiana sylvestris). That is, no background flowering occurs in the absence of the inductive long night (Salisbury, 1985). An additional advantage of Xanthium with respect to floral evocation is its inducibility by a **single** long night (16 h dark), which creates an appropriate time zero. Few plants exhibit both these characteristics.

1.2 Phytochrome.

Perception of daylength occurs in the leaves via a photochemical reaction between photons and the phytochrome protein (P) (Nakayama, 1958). Simplistically, there are two interconvertible forms of phytochrome. One, P_{fr} absorbs far red light (FR) (725-735 nm) and becomes P_r, the red light (655-665 nm)-absorbing form of phytochrome. The P_{fr}/P_r ratio thus acts as a photoperiod perception system (Vince-Prue, 1975). In SD plants (such as Xanthium) which sense night length, the perception of the dark period is hypothesized to occur as follows: At day's end, when the dark period begins, a substantial portion of the total phytochrome is in the P_{fr} form (Smith and Whitelam, 1990). As the dark period progresses P_r is de novo synthesized while P_{fr} is degraded, thus causing the relative level of P_r to rise in the plant during the dark period (Smith and Whitelam, 1990). It appears that the synthesis and degradation pathways of P_r and P_{fr} stabilize soon after the dark period begins, thereby allowing the P_r level to rise in a linear fashion during the dark

period (Smith and Whitelam, 1990). A critical level of P_r, or a critical P_r/P_{fr} ratio must be attained in order to engage the floral evocation sequence (Zeevaart, 1978). In Xanthium, the leaves are the unit of perception, and the apex is the unit of morphological change (Mukherjee, 1974).

1.3 Florigen.

For almost three decades plant physiologists have struggled in their attempt to determine the hormonal events responsible for evocation. The term **florigen** was coined in 1937 by Chailakhyan (1968) as a substance or complex of substances which could move through living tissue. Various experiments have shown florigen to be graft-transferable from an induced donor leaf to a non-induced host, thereby causing the non-induced host plant to flower (secondary induction). Throughout this thesis florigen, florigenic stimulus and flowering hormone will be used interchangeably. Various experiments have shown the ability of the florigenic stimulus to be transferred across a graft union, traveling at the same rate as sugar in phloem conduits, (Chailakhyan, 1968; King and Zeevaart, 1973; Lang, 1965; Ogawa and King, 1990). The florigenic stimulus is not only graft transferable within a class, within a species and between species, but also between certain LDP and SDP of unrelated families (ref. cited in Zeevaart, 1978). In an extreme example, a leaf from an induced SDP (Xanthium) grafted to a vegetative LDP (Silene armeria) caused the latter to flower, although these two species belong to unrelated dicot families (Lang et al., 1977). This type of experiment implies that the florigenic stimulus produced by both types of photoperiodic plants **should be** physiologically similar, if not chemically identical. The fact that the stimulus is transferred through living tissue suggests it might be hormonal in nature. By definition a hormone is an organic molecule, produced in minute amounts and

transported to cause biochemical, physiological and/or morphological responses (Salisbury and Ross, 1978).

Numerous studies have attempted, unsuccessfully to date, to isolate and characterize a florigen. One cause of these failures may well have been the lack of a rapid and reliable bioassay. The development of such a flowering assay was one goal accomplished by this investigation. Once developed, this bioassay was used to detect and attempt characterization of the florigenic stimulus.

1.4 Previous gibberellin application studies.

There are many experiments in which plant hormones and related growth regulators have been applied (in a pharmacological approach) so as to assess the hormonal basis of floral evocation. Several examples for LDP are papers by Pharis et al. (1987) and Evans et al. (1990). These have not led to any conclusive evidence as to the identity of "florigen". However, for LDP (almost all of which respond to the GA class of molecules by flowering under non-inductive SD) GA structure:function experiments which vary the presence and location of A-ring double bonds, and location and number of hydroxyl groups in the C- and D-rings can certainly describe those GA structures which **might** be important in floral evocation of a LDP. This was accomplished, specifically in the Lolium temulentum system (Evans et al., 1990 and 1993). Such experiments have also been conducted in the Pharbitis (SDP) system, although on a much-reduced scale (King et al., 1987). Interestingly, of all the substances which have been applied to a wide variety of plants species under strictly non-inductive or marginally-inductive conditions, only the GAs have been generally shown to effectively promote floral induction, initiation and/or early floral developmental processes.

Thus in general, LDP, and many of those plant species with a cold requirement (the latter usually exhibit a rosette growth habit) bolt and flower in response to exogenous GAs (usually GA_3 was tested), while SDP are not usually (as a class) responsive or are even inhibited in their flowering by GAs (Cleland and Zeevaart, 1970; ref. cited in Pharis and King, 1985). There are exceptions, however, and some GAs and related compounds have been successful at enhancing the flowering response under non-inductive LD [e.g. Chrysanthemum and other Asteraceae (Pharis, 1972; and refs. cited in Pharis and King, 1985)] or under marginally-inductive long nights in SDP (King et al., 1987). Whether the GA is promotive or inhibitory in this system depends on GA type, dose, and time of application relative to the inductive long night, as well as on the plant variety (King et al., 1987).

One component of this study will analyze this "enhancing" response of several natural and synthetic exogenously applied GAs under marginally-inductive long night conditions (ca. 10-12 h dark periods). Separation of the events regulating floral evocation and early floral differentiation/development is not possible in such an experiment since the effect of exogenously applied GA is determined by dissection of the apex 9 days later. From here forward the evocation and/or early differentiation/development events will be referred to as **floral evocation**. In view of the large number of plants in which applied GAs promote floral evocation, it is logical to conclude that certain of the GAs may have a critical role in (at least) this process. However, of the 90 known GAs only a small number are **highly** bioactive in growth and/or floral induction assays (Pharis and King, 1985; Evans et al., 1990).

For one Graminae species, Lolium temulentum, a LDP, an unusual relationship has often been observed between the ability of applied GA to induce flowering versus cause

internode elongation (Evans et al., 1990). The GAs most effective at inducing flowering under either totally non-inductive SD conditions or under partially-inductive (1 LD) conditions are often relatively ineffective at causing internode elongation (Evans et al., 1990). Exceptions to this, however, would be GA_3, 15ß-hydroxy GA_3 and 2,2-dimethyl GA_4 (Evans et al., 1990) which are very good florigens, while very effectively promoting stem elongation. In this study, the GAs and other substances are applied to the base of the primary leaf blades. Thus, the relationship between floral evocation and petiole and internode growth can readily be assessed for this SDP (Xanthium) to see if this precedent for Lolium (highly florigenic GAs are poor promoters of internode growth) holds true for Xanthium.

The main objective of this research project was to examine the process of flowering from a hormone physiology perspective, with an aim toward better elucidating the florigenic stimulus that causes floral evocation in the apex. Thus, the process of floral evocation (initiation and/or early differentiation/development) will be examined through: (i) the analysis of endogenous GA levels in apical and leaf tissue following long night (16 h dark) floral induction in physiologically mature (6-week-old cocklebur plants), (ii) the analysis of deutero-GA_{20} metabolism during floral evocation in physiologically mature plants, (iii) the exogenous application of partially purified extracts, of highly florigenic GAs and of highly florigenic substances utilized in other systems, and (iv) the application of known flowering inhibitors from other systems, including indole-3-acetic acid (IAA), abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC) in a young-seedling Xanthium floral induction bioassay.

While this study will by no means provide a holistic view of the process of flowering, it will probe one of the many independent, yet coordinated aspects united in the process of floral induction and evocation.

CHAPTER 2

DEVELOPMENT OF A SEEDLING BIOASSAY FOR FLORIGENIC SUBSTANCES

Introduction

Many plants cannot be induced to flower until a certain size or age has been achieved, even if subjected to an inductive photoperiod. This phenomenon is termed juvenility and is (presumably) a means whereby plants prevent flowering until they are of a size large enough to adequately supply the developing reproductive tissues (floral buds, seeds and fruits) with assimilate (see ref. cited in Zimmerman et al., 1985 and Pharis et al., 1989). A mature (large) plant usually has an advantage in competition for light, thus size and not age is usually the determining factor in the duration of this juvenile phase, especially for herbaceous species (e.g. Xanthium). The duration of this juvenile phase can be as short as a few days or weeks in some herbaceous plants, to as long as 40+ years in some tree species (Lang, 1965 and Zimmerman et al., 1985). Pharbitis nil does not have a juvenile phase since its cotyledons are photoperiodically sensitive from germination (Nakayama, 1958), whereas, Xanthium cotyledons can not be induced, so this species does not become photoperiodically responsive until after the primary leaves develop (Mukherjee, 1974). Thus, in Xanthium, as in many other plants, the leaf is the organ type, which upon attaining "adult status," becomes capable of responding to inductive signals and producing the floral stimulus.

The development of a bioassay for florigenic substances was a necessary first step towards the success of this study. The necessary qualities of such a bioassay are: (1)

sensitivity, (2) reliability and (3) rapidity. All these goals were met by the Xanthium seedling bioassay for florigen which was developed in this study.

Methods

2.1 Growth of Seedlings

Plant material was germinated and grown as per Mukherjee (1974). Essentially burrs of an obligate photoperiodically sensitive cultivar of Xanthium strumarium (obtained from Drs. Frank Salisbury, Manfred Ruddat and I.D. Mukherjee) were soaked in water overnight at room temperature. Burrs were then cut carefully to remove the seeds, which were imbibed in water in a Petri-dish at room temperature for 16 h. The testa was then removed and the imbibed seeds were planted in rows in wet sterile vermiculite in perforated plastic trays (60 X 30 X 8 cm). The seedlings were grown under a 20 h LD photoperiod with illumination provided by high intensity mercury lamps ($250\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation (PAR) at plant height) in a growth chamber (Controlled Environment Systems model PGV 36LT Winnipeg, Man.). The day and night temperatures were 24 and 20°C respectively and the relative humidity (RH) 60%. Dates of 50% and 100% germination were recorded for each batch of seedlings to enable a comparison of the relative developmental time courses between experiments. Seedlings were watered with Peter's Professional™ fertilizer every 3 days to field capacity throughout the experiment.

One week after planting the imbibed seeds (3 days post-germination), individual seedlings were transplanted into 2.5" pots containing 2:1 greenhouse mix:Terragreen™.

In a preliminary trial, bare root Xanthium seedlings were transplanted into culture tubes containing either sterile vermiculite soaked with ¼ strength Hoagland's solution, or ¼ strength Hoagland's solution. However, in both cases severe nutrient deficiency resulted, despite the fact that this solution was exchanged every third day. In Xanthium, nutrient stress reduces photoperiodic induction and subsequent development of the floral apex (Naylor, 1941; Neidle, 1939; Wada and Totsuka, 1982). For this reason all subsequent bioassay attempts were performed with soil-grown seedlings watered twice weekly with Peter's Professional™ fertilizer solution. This latter scheme successfully prevented nutrient deficiency from occurring during the growth period following transplanting.

To determine the optimal age for photoperiodic sensitivity a pilot study was performed. For each of five days after transplanting, groups of five seedlings were induced with a single long night (16 h dark period). The inductive dark period was provided by transferring plants at 1600 h for induction from the growth chamber to a dark room (23°C), then transferring them back to the LD (non-inductive) chamber at 0800 h the following day. Thereafter the induced seedlings were allowed to resume growth under 20 h LD photoperiod. Nine days post-induction (all under LD) the floral stage of each seedling was assessed by dissection of the shoot apex and scoring the terminal staminate flower according to Salisbury's (1955) flowering stage system (see figure 2.1).

This method of quantifying the flowering response divides the Xanthium floral development continuum into eight equally spaced stages (1 to 8). Salisbury's floral stage system was initially determined via the analysis of mature induced plants. In this study, where seedlings are being scored, floral development was found to lag behind that of the mature plants by 1 to 2 stages (i.e., maximum floral stage observed after induction, in the seedling assay after a single long night (SD) was stage 7). Salisbury showed that apices

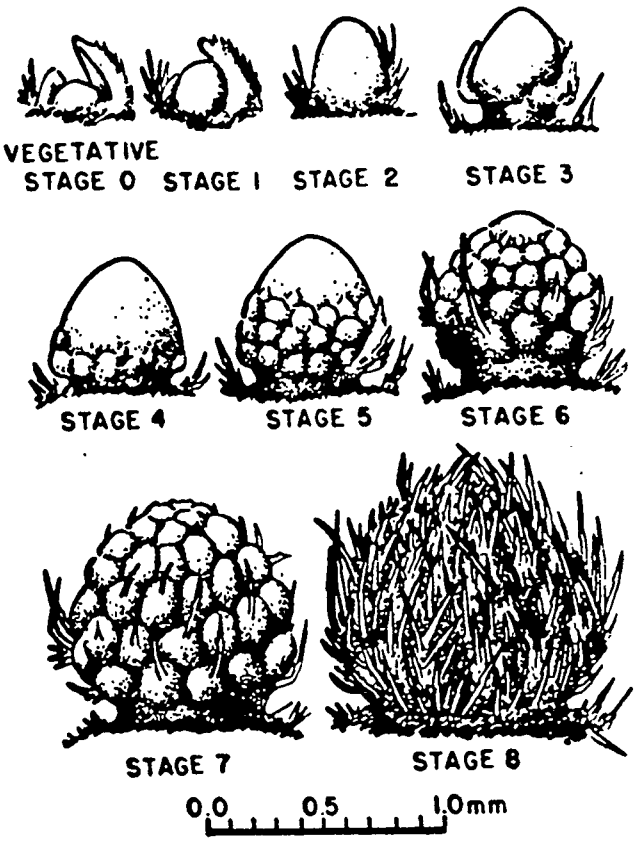


Figure 2.1. The system of floral stages used in many studies with Xanthium strumarium (From Salisbury, 1955).

with a stage ≥3 can not revert back to the vegetative condition (Salisbury, 1955). At this stage a constriction has formed below the apex. Using this as the precedent, apices achieving stage 3 or greater were considered as being induced to flower. For consistency, the term floral evocation will be used throughout this thesis to describe the floral promotion observed in the Xanthium seedling bioassay under marginally-inductive or fully-inductive long nights.

Following determination of the stage at which each seed source was ripe-to-flower, seedlings (5 per treatment) were given dark periods ranging from 8 to 16 h. The dark period was given by transferring groups of five seedlings at the appropriate time to the dark room. Due to the extreme sensitivity of Xanthium seedlings to night break (NB) (i.e., threshold for NB being 10 times the illumination of the light of a full moon), extreme caution in eliminating **all** external lighting (with a series of black curtains) was imperative when transferring later groups (those after the first group) of seedlings to the dark room. All plants were taken out of the dark room at 0800 h the following morning. Thereafter the plants were allowed to resume their growth under a non-inductive 20 h LD photoperiod. Nine days later the degree of floral evocation was assessed by dissection (as above).

Results

During the course of this study the three Xanthium seed sources were employed, designated by the name of the scientist who donated them. As was stated by Habjorg (1978) sensitivity of plants to SD will change with latitude and altitude in different ecotypes. In this study each seed source differed slightly in its degree of juvenility as well

as in its photoperiodic sensitivity. Length of the juvenile phase ranged from 9 days post-planting (5 days post-germination) in the Salisbury source to 14 days post-planting (10 days post-germination) in the Ruddat source. The juvenile stage of the Mukherjee source (11 days post planting) was intermediate in length. As mentioned above, Xanthium cotyledons are not photoperiodically inducible, but the primary leaves are. On day 9 the Salisbury source had two primary leaves, each with an area of 1 cm^2. By comparison, the less sensitive Mukherjee source only became sensitive when its primary leaves had fully expanded. The least sensitive Ruddat source required fully expanded primary leaves as well as an additional two leaves, each with an area of 1 cm^2, for full sensitivity. One reason for the difference in leaf area required for the various sources may be due to differences in synthesis of, or sensitivity to the floral stimulus. It could thus be postulated that the Ruddat source is less sensitive and thus requires a higher level of florigenic stimulus for induction than does the Salisbury source. Once attained, inducibility was retained for all older seedlings. All sources developed to stage 5-6 during the 9 day period after induction with a single 16 h long night.

Seed stocks also varied with respect to their critical night lengths, with the length of the critical long night being proportional to the photoperiodic sensitivity of each source. A typical induction profile for the Mukherjee source is shown in figure 2.2. The Salisbury source had the shortest critical long night at 11 h, whereas the Mukherjee and Ruddat sources had critical nights of 12 h and 12.5 h respectively. The critical night length of the seedlings was extended with respect to mature plants. For the Salisbury source mature, 6-week-old plants have a 8.25 h critical dark period (Wareing and Phillips, 1986), however, the critical dark periods for mature plants ranges from 7.5 h in ecotypes from northern latitudes to 11 h in ecotypes from Mexico (Salisbury, 1985).

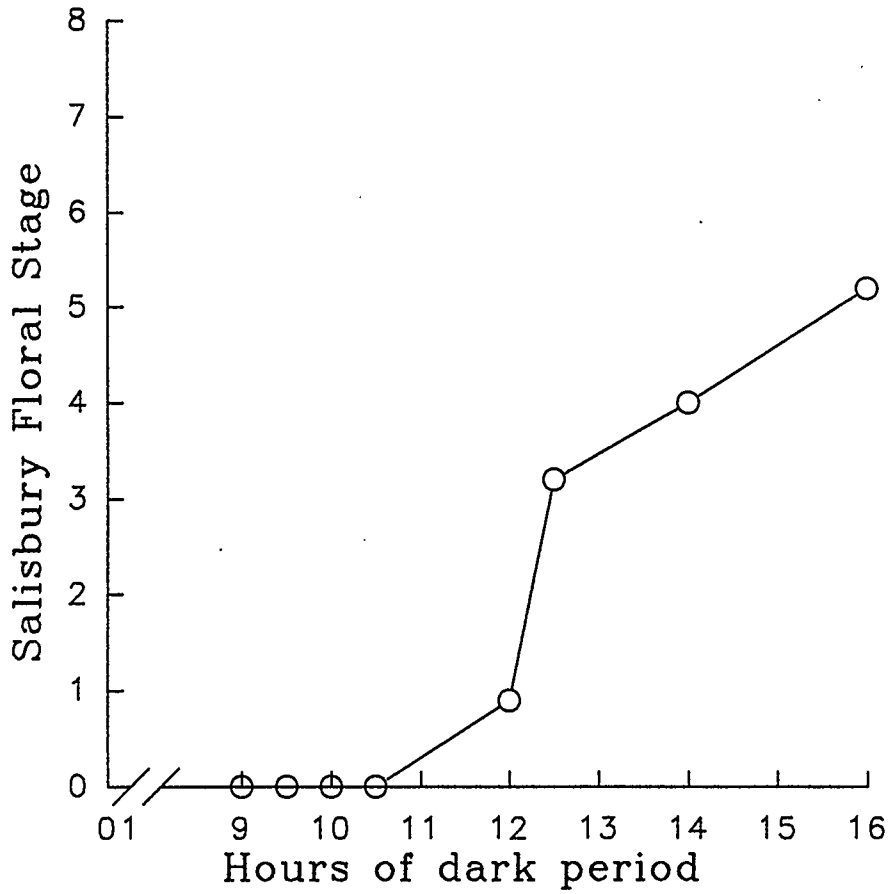


Figure 2.2. Floral stage as a function of night length in 2-week-old Xanthium strumarium (Mukherjee source) seedlings.

Discussion

The qualities of rapidity, reliability and sensitivity were all addressed in the design of the Xanthium bioassay. The brief juvenile phase in Xanthium is one aspect which makes it as useful as Pharbitis for a bioassay system. Both require 5 days post-germination to achieve maximum sensitivity (Wareing and Phillips, 1986). In addition, through exogenous application of highly florigenic GAs and extracts, as well as inhibitors of flowering (e.g. as shown from experiments with Pharbitis and other systems), the Xanthium seedling bioassay makes it possible to compare the nature of the florigenic stimulus between these two SDP systems (e.g. Xanthium and Pharbitis) as well as others. Such comparisons may possibly lead to the development of a general set of rules as to the nature of florigen within the angiosperms. Thus, Xanthium seedlings which had achieved maximum sensitivity (as detailed above) were used to assess the photoperiodic sensitivity of the assay, as well as to determine the effect of applied extracts and exogenous growth substances on floral evocation.

Two factors enhanced the repeatability of this bioassay. First, the use of young seedlings resulted in very uniform test plants. Such uniformity is not easily obtainable with fully-grown plants. Second, even though there were differences in sensitivity between seed sources, these differences were stable between experiments. In addition, results between repeat experiments, even using different seedling sources, always yielded comparable results.

The fact that batches of seedlings differed in the length of their juvenile phase, as well as in their critical night length, reinforces the fact that each seed source should be tested for its inducibility requirements prior to use in a bioassay. These differences in source sensitivity could also be useful as a window into the mechanisms of critical night

length perception and juvenility. Such facets should be further explored from physiological, biochemical and molecular botanical standpoints.

There are several other advantages of the seedling bioassay for florigenic substances. Since seedlings can be grown in small pots, the seedling bioassay also allows for increased replication relative to the use of large plants. In addition, since a larger numbers of plants can be grown simultaneously, the efficacy of several test substances can be compared within a single experiment, rather than as cross comparisons between experiments. This may allow small differences in efficacy to be determined with greater certainty. Also seedlings, due to their reduced size (but comparable sensitivity) have the advantage of requiring a lower dose of test substance. This is highly desirable since many of the substances which are potentially florigenic are difficult to synthesize and/or extract and purify in large quantities. In this Xanthium seedling bioassay experiment repeated 3 times over a large range of doses required <4 mg of test substance. It is obvious that the seedling bioassay system presents a number of useful advantages over that using larger plants.

CHAPTER 3

GIBBERELLINS, EXOGENOUSLY APPLIED, AND THE USE OF APPLIED GIBBERELLIN BIOSYNTHESIS INHIBITORS IN RELATION TO FLOWERING AND THE PROCESSES OF EPICOTYL AND PETIOLE ELONGATION IN XANTHIUM

Introduction

As summarized in the introductory chapter, GAs are strongly implicated in the initiation/early development events of floral evocation, with LDP being more responsive to exogenously applied GA than SDP. Since flowering in Xanthium, as well as in many other plants is accompanied by a decreased rate of epicotyl and petiole elongation, these parameters were measured throughout the application studies. Most of the GAs applied in the present study were chosen as a result of their high florigenicity in other systems including the SDP Pharbitis nil, as well as the LDP Lolium temulentum and Brassica spp. (Evans, 1969b; Evans et al., 1990; King et al., 1987; Mandel et al., 1992; Pharis et al., 1990; Pharis, 1991; Pharis et al., 1992).

If GAs control (promote or inhibit) the events of floral evocation in Xanthium, then inhibitors of their biosynthesis might be expected to modify long night-induced flowering; indeed there is evidence for this in the literature (Graebe and Ropers, 1978; Pharis and King, 1985). For this reason several such inhibitors were applied, first as a foliar spray and later as a soil drench.

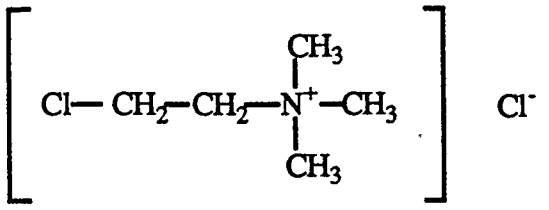
Presently, three different groups of GA biosynthesis inhibitors exist. These include (1) "onium" compounds, (2) compounds which are N-containing heterocyclics and (3) acyclohexanetriones. In the present study, only compounds from the first two classes

were utilized, e.g. CCC of the "onium" class, and ancymidol (a pyrimidine), uniconazole (a 4-pyridine) and BAS 111..W (a 4-pyridine) of the N-containing heterocyclic class (Figure 3.1).

"Onium" type compounds possess a positively charged quaternary ammonium, phosphonium or sulphonium moiety at physiological pH (6.5 to 7.0) (Rademacher, 1991). These compounds obtain their effect by interfering with biosynthetic steps directly before ent-kaurene, inhibiting the cyclization of geranylgeranyl pyrophosphate (GGPP) to ent-kaurene by the enzyme ent-kaurene synthase (see figure 3.2) (Hedden, 1990). It is suggested that the "onium" compounds, may mimic carbocationic high-energy isoprenoid intermediates during cyclization. Such transition state intermediates are expected to bind more tightly to the enzyme than the substrate and consequently some of the inhibitors have K_i values several orders of magnitude lower than the K_m for the substrate oxidosqualene (Hedden, 1990). Hedden (1990) concludes that, except at very high concentrations, the primary action of CCC is probably also via the GA biosynthetic pathway at steps directly before ent-kaurene (Figure 3.2).

By comparison, the N-containing heterocyclic class is structurally related to fungicides. These inhibit the oxidation reactions leading from ent-kaurene to ent-kaurenoic acid by way of the lone electron pair of the sp^2-hybridized nitrogen binding to the protoheme of a cytochrome P-450, thus blocking the monoxygenase which catalyzes the oxidation of ent-kaurene (Rademacher et al., 1984). Ancymidol treatment of plants has been shown to cause a dramatic decrease of extractable GA-like activity (Shive and Sisler, 1976; Coolbaugh and Hamilton, 1976), but its effect could be reversed by GA_3 (Coolbaugh and Hamilton, 1976). In the present study, reversal of the inhibitory action of uniconazole was attempted by the application of $GA_{4/7/9}$ mixture (62:30:8 by weight respectively).

A



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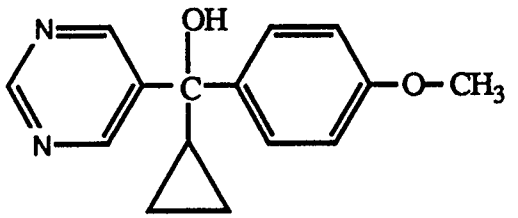

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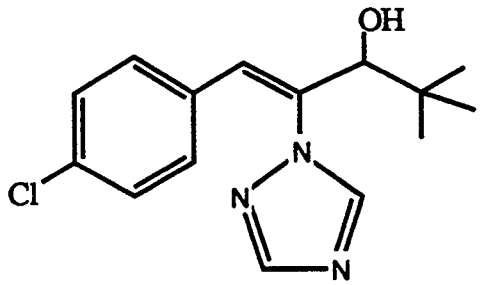

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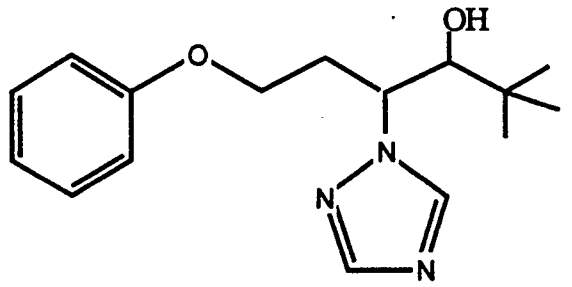


Figure 3.1 Structures of : A; CCC, a plant growth retardant of the "onium"-type, and B; plant growth retardants of the pyrimidine and 4-pyridine types: B(i) ancymidol, B(ii) uniconazole and B(iii) BAS 111..W.

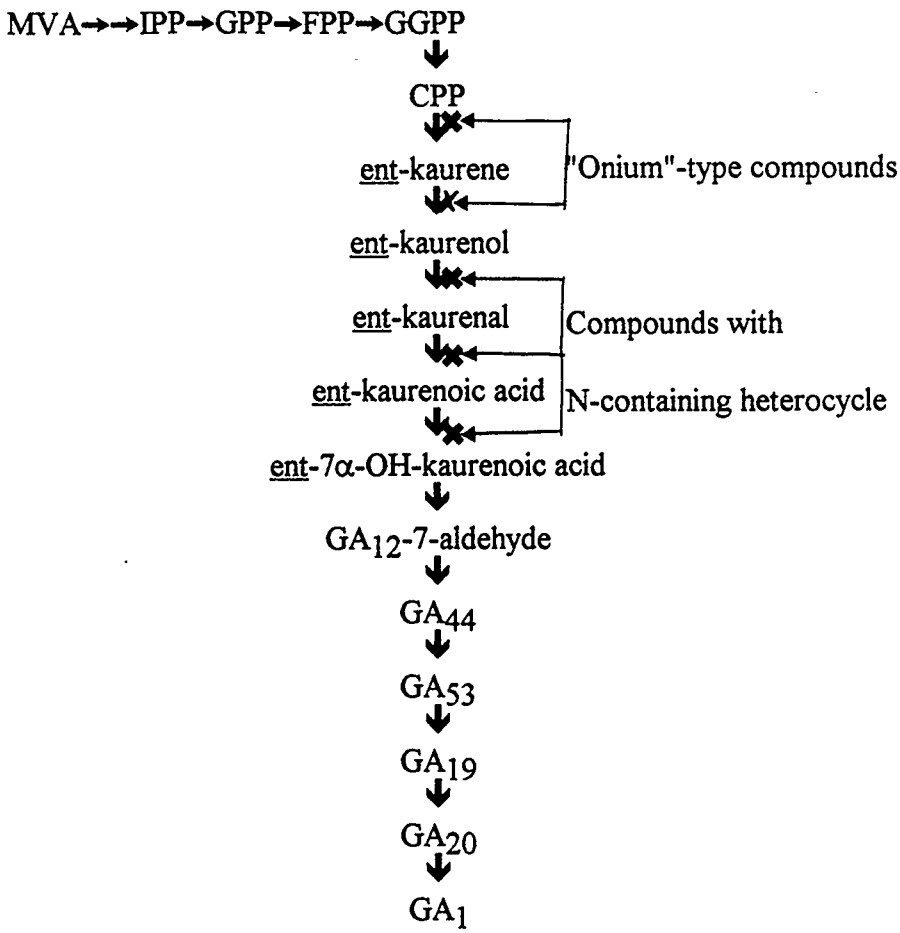


Figure 3.2 Principal steps involved in the biosynthetic formation of GA_1 from mevalonic acid (MVA) via the early C-13α-hydroxylation pathway, showing points of inhibition by plant growth retardants (lower degree of activity indicated by thinner X). Adapted from Rademacher (1991). (Legend: IPP, isopentenyl-pyrophosphate; GPP geranyl-pyrophosphate; FPP, farnesyl-pyrophosphate; GGPP, geranylgeranyl-pyrophosphate; CPP, copalyl-pyrophosphate)

Methods

3.1 Gibberellin biosynthesis inhibitors

Seedlings were grown as described above (Methods 2.1). Two days following transplanting seedlings were treated with known GA biosynthesis inhibitors including uniconazole (20 mg/L), CCC (1 g/L active ingredient), BAS 111..W (4.5 mg/L) and ancymidol (132 ppm), all prepared as aqueous solutions with 0.1% Activator™ as the surfactant. Gibberellin inhibitors were initially applied twice, at an interval of one week, as a foliar spray. Induction with a single 16 h long night occurred two days subsequent to the final growth retardant treatment. The initial spray application experiments failed, e.g. seedlings were not dwarfed nor was the growth of their petioles reduced. Thus, only the two inhibitors (CCC and uniconazole) which tended to inhibit floral evocation were applied in subsequent experiments.

Hedden (1990) also noted that foliar application of GA biosynthesis inhibitors was less effective than root application. Therefore CCC was applied as a soil drench (50 mL/ seedling) at a rate of 10 g/L as was uniconazole at 3 g/L. These concentrations gave the desired morphological changes with few signs of apex necrosis. In a third trial, an application rate of 2 g/L (50 mL/plant) of uniconazole was used. Additionally reversal of the uniconazole (3 g/L) inhibition of flowering was attempted by applying $GA_{4/7/9}$ mixture (100 µg/seedling). Experiments where inhibition of flowering was obtained via use of GA biosynthesis inhibitors were repeated three times with similar results.

3.2 Pharmacological applications of GAs

Seedlings were grown as described above (Methods 2.1). For all exogenous applications, initial measurements of epicotyl (e.g. height above the primary leaves, the latter being the point of application) and petiole lengths (both primary leaves) of ripe-to-flower seedlings were recorded. All leaf-applied growth regulators were prepared as 95% EtOH solutions. For all experiments the dose range of GAs and all other substances was 1 to 100 μg per seedling, with half of each dose being applied to the base of each primary leaf blade as a 10 μL droplet. Within this range each growth substance was applied at the rate of 1, 3.3, 10, 33 and 100 μg/plant. A dose range within the linear part of the overall response curve is considered most desirable.

For one trial an alternative solvent, DMSO, was used due to its more polar nature. This solvent made it easier to dissolve some of the applied substances (especially leaf extracts). However, application of growth substances in this solvent caused toxicity (e.g. necrotic lesions on the leaves and often death of the shoot apical meristem). Its use was thus abandoned.

Although the seedling bioassay system yields near-uniform plants, within each experiment control versus "to-be-treated" seedlings were carefully matched using the criteria of primary leaf size and seedling height. This was done to enable the inhibitory or "building" responses [e.g. ability of an applied substance under marginally-inductive photoperiod (10-12 h dark) to promote floral evocation] to be compared more precisely in Salisbury floral development stage between levels of a single growth regulator, between growth regulators and against the control (zero growth regulator). Each treatment (EtOH control or exogenously applied growth regulator) consisted of 5 ripe-to-flower seedlings (as described in chapter 2). Applications were always made at 1200 h prior to initiation of

the fully-inductive long night 1600 h or prior to marginal induction between 2000 and 2200 h that same day. This inductive or marginally-inductive photoperiod was provided in a dark room as described above (Chapter 2). Following this inductive dark period the plants were allowed to continue their growth under 20 h LD until dissection 9 days later. In my hands, the only stimulus which could evoke flowering was a fully-inductive photoperiod alone or a marginally-inductive photoperiod and exogenous growth regulator. Floral stages were scored and final epicotyl and petiole lengths measured as above, 9 days following treatment. All applications were repeated in at least three experiments with similar results.

In each experiment at least two types of standardized treatments were included: (1) vegetative seedlings held under a 20 h LD photoperiod and (2) photoperiodically-induced seedlings given a single 16 h dark period at 1600 h, post-growth regulator application, the day of the experiment. The 20 h LD control seedlings were required to guarantee that floral induction of the test plants resulted specifically from induction during the long night, rather than to growth chamber failure. The long night (SD)-induced seedlings were given 16 h of darkness to ensure that each seedling batch was indeed photoperiodically inducible. This latter SD treatment was also used to gauge the maximal level of **photoinduction** which could be achieved within each seedling batch. Additionally (when required), control seedlings were marginally-induced (10 to 12 h dark depending on the seedling source) to gauge the basal level of flowering resulting from partial photoinduction. Such a "control" enabled me to assess the floral apex-"building" effect of various florigenic substances. Seedlings used to assess the inhibitory nature of certain growth substances (such as IAA, ACC and ABA) were photo-induced with a single 16 h long night, whereas, seedlings used to assess promotory (floral apex building) substances (such as GAs) were photo-induced with a marginally-inductive long night. Use of these

two regimes allowed for the effective assessment of inhibitory as well as promotory substances.

3.2 Pharmacological application of floral-promotive growth substances under marginally-inductive long night (SD)

Eight GAs were tested for their efficacies in (a) epicotyl and petiole growth and (b) flowering. These included GAs native to Xanthium such as $GA_{3,4 \text{ and } 9}$, other natural GAs [including GA_5 and GA_7 and a $GA_{4/7/9}$ mixture (62:30:8)], as well as certain GA derivatives [2,2-dimethyl GA_4 and its 3α-hydroxy epimer (see figure 3.3 for GA structures)]. All were C_{19} GAs and were chosen by merit of their florigenicity in other systems.

In each study, GAs were tested in groups of three or four over a log range of five test doses. Since it was determined that optimal epicotyl and petiole growth, as well as floral evocation responses occurred at the dose of 100 µg/plant (2,2-dimethyl GA_4 and GA_7 excepted), a final large experiment was used to directly compare the efficacy of each GA on these three parameters at the 100 µg/plant dose. This single cumulative experiment employed 10 replicate seedlings for each GA dose.

Several preliminary experiments determined that none of the applied GAs (at any dose) could substitute for an inductive long night by evoking flowering (e.g. no GA could induce under LD 20 h daylength treatment). This was also the case for Pharbitis nil (King et al., 1987). Therefore, all future applications were conducted using a single marginally-inductive long night, at 8-10 h following GA application at 1200 h. The inability (to date) of exogenously applied substances to negate the requirement for photoperiodic

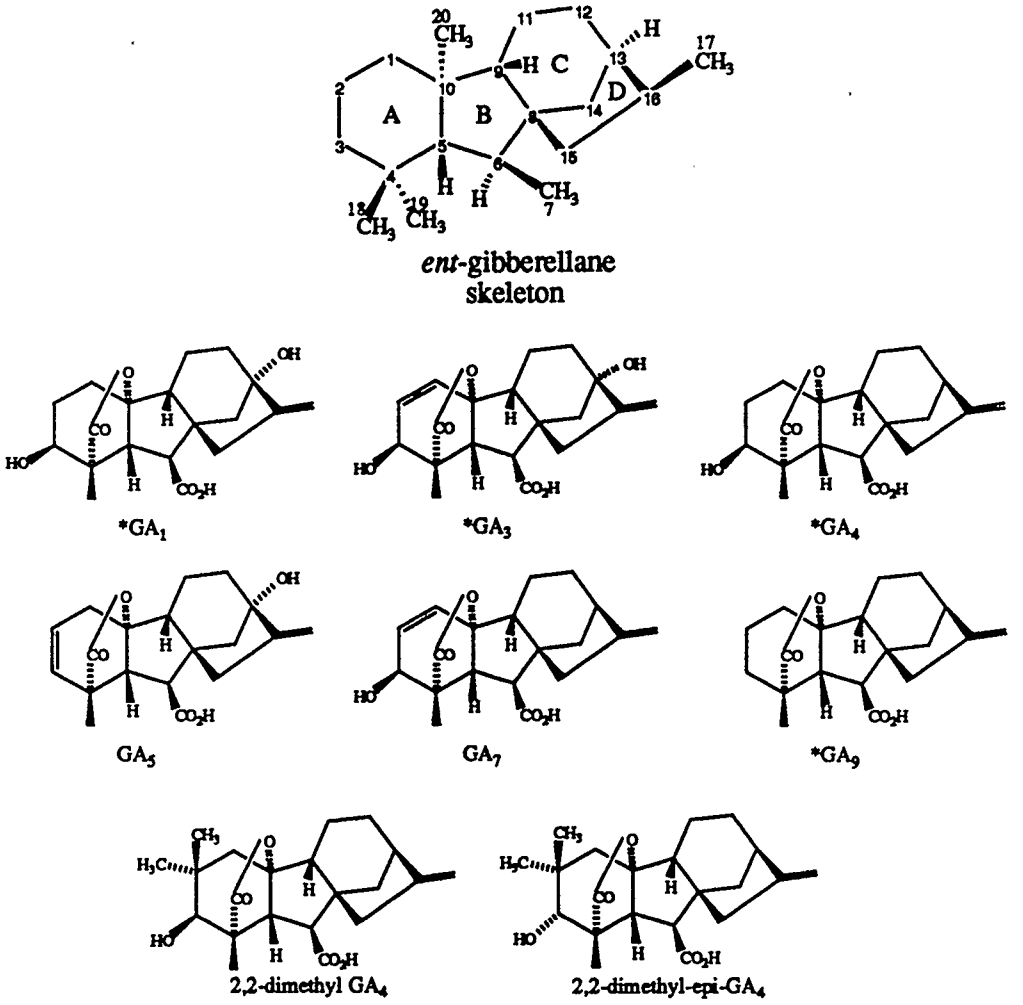


Figure 3.3 Structures of the GAs applied in Xanthium seedling bioassays. Gibberellins identified from Xanthium leaves or apices are denoted with an asterisk.

induction is the most interesting characteristic of the Xanthium system. The lack of success at inducing flowering by any of the broad range of GAs which were applied, exemplifies just how strict the inductive requirement is in this species and also emphasizes the power of this system as a floral evocation bioassay tool. Due to a relatively low flowering stage in the marginally-inductive control treatment (generally between stages 1 and 3), the promotory effect of exogenously applied GAs on floral evocation could be readily assessed in seedlings under marginally-inductive photoperiod. Marginally-inductive photoperiods thus allow promotive effects of growth substances on floral evocation responses to be examined (Pharis et al., 1990) in systems where it would otherwise be impossible.

3.3 Calculations.

For each seedling, petiole and epicotyl growth was calculated and the mean values of each treatment were subsequently compared between treatments by the Student Newman-Keuls (SNK) ANOVA using the Statistical Analysis Software For Personal Computers program. The Salisbury (1955) floral stages were averaged for each treatment and the percentage flowering (flowering quotient) was calculated. Graphs for each of the three measured parameters were prepared, and expressed as the mean ± a 95% confidence interval.

Results and Discussion

Effect of GA biosynthesis inhibitors on floral evocation

If GAs are essential for any of the several events of evocation, then it would be expected that GA biosynthesis inhibitors should successfully repress this process. Inhibition of other processes, such as stem and petiole growth which are (presumably) controlled by GAs were previously studied using these inhibitors as tools (Graebe, 1987; Graebe and Ropers, 1978).

Of the GA biosynthesis inhibitors I tested, only CCC and uniconazole affected floral evocation, while BAS 111..W and ancymidol, at the concentrations tested, were ineffective at preventing or delaying this process. At a dose of 3 g/L (0.15 g/plant) uniconazole completely inhibited the transition from vegetative to floral development and at the 2 g/L dose (0.10 g/plant) slowed development by 4 floral stages (see figure 3.4). At 20 mg/L (1 mg/plant), a 2-stage inhibition of development was obtained. The $GA_{4/7/9}$ mixture successfully reversed the uniconazole (3 g/L) inhibition of flowering. This suggests that, even at this high dose, the uniconazole-mediated inhibition of floral evocation resulted specifically from inhibition of GA biosynthesis.

In comparison, CCC did not completely inhibit floral development at either concentration tested, it simply slowed development (figure 3.4). At both doses of CCC, 1 g/L (0.05 g/plant) and 10 g/L (0.5 g/plant), floral development was inhibited by only a single floral stage, even though at 10 g/L of CCC quite severe dwarfing and even chlorosis was observed. Interestingly, the effect of CCC on floral evocation in other systems ranges

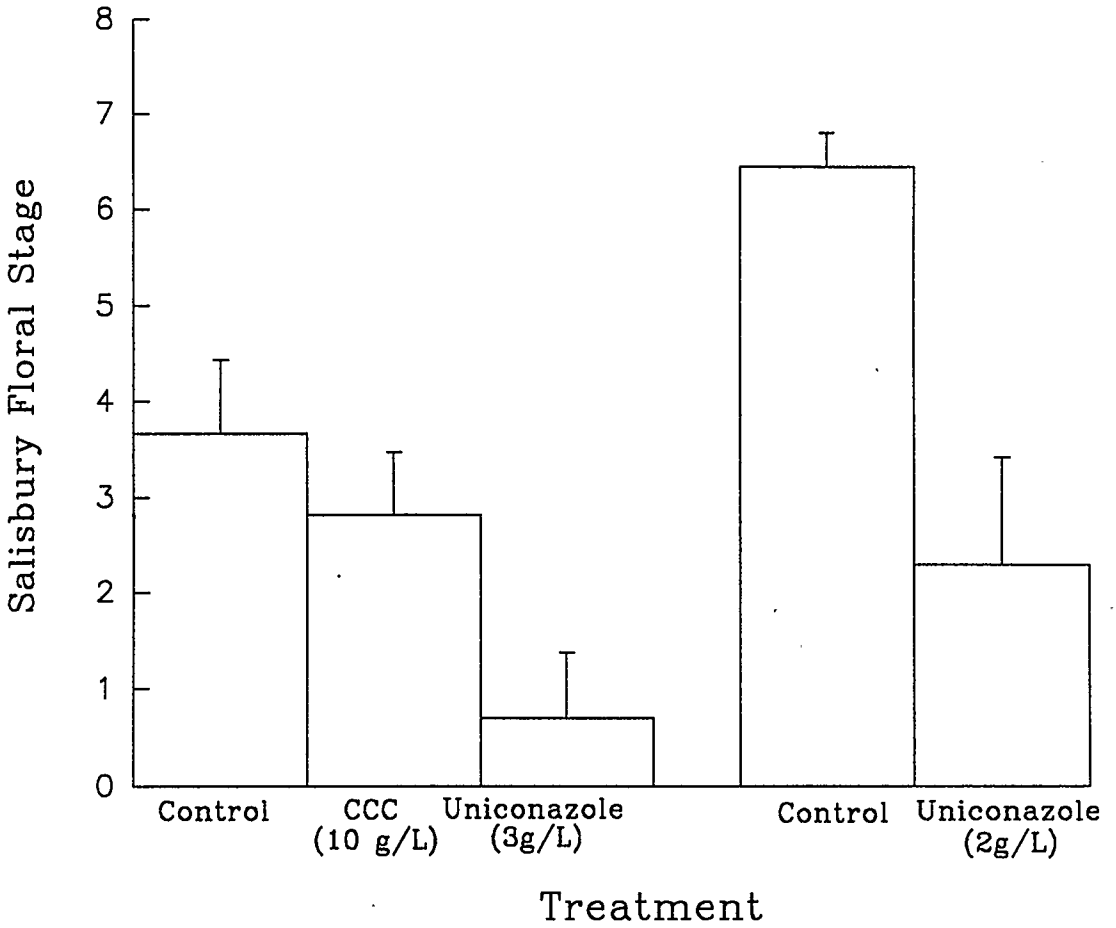


Figure 3.4 Effect of GA biosynthesis inhibitors, CCC (10 g/L) and uniconazole (2 g/L and 3 g/L), on Salisbury floral stage upon dissection 9 days following a single (16 h dark) inductive long night. Values are the mean of 10 seedlings. Error bars represent 95% confidence intervals.

from promotion to absolute inhibition. However, these unique effects of CCC are strongly dependent on the application rate. When applied to Lolium (with GA_3) and Blitum (both LDP) CCC enhances floral evocation (Evans, 1964; Evans, 1969b; Jacques, 1970), possibly through its "black magic" effect (at low dose) of increasing rather than decreasing GA levels in some systems (ref. cited in Graebe and Ropers, 1978 and Pharis and King, 1985). Such a floral promotory effect of CCC on floral evocation was not observed in Xanthium at either dose tested. Nor did CCC alone promote Lolium flowering either (marginally-inductive LD was **not** tested) (Evans, 1964). The effect of CCC in my study was similar to that of AMO-1618 (a quaternary ammonium compound, as is CCC) in spinach where Gianfagna et al (1983) determined that AMO-1618 delayed, but did not prevent, inflorescence appearance under inductive (LD) conditions. However, CCC was an effective inhibitor of flowering in the LDP Samolus (Baldev and Lang, 1965). Although CCC delayed, but did not prevent floral evocation in Xanthium, it was an effective inhibitor of shoot elongation.

Differences in the effectiveness of the applied GA biosynthesis inhibitors may be explained by: (1) differences in the penetration and uptake of each inhibitor to the tissue, (2) biosynthetic step which is inhibited, and (3) ability of the plant to metabolize or detoxify the inhibitor. With regard to (2), it is known that the levels of GAs close to the point of inhibition are more reduced than those further away (Hedden, 1990) although the basis for this result is not clear. From results which will be presented later in this chapter (i.e., C_{19} GAs are florigenic), it would be expected that the N-containing-heterocyclic class of inhibitors should be more effective than the "onium" class (See figure 3.4). Indeed this was the case, with uniconazole being a more effective inhibitor of flowering than CCC.

The fact that both CCC and uniconazole (at low dose) reduced, rather than completely inhibited, floral evocation implies, for this SD species at least, that (endogenous) GAs are involved in (and are probably essential for) either the floral apex initiation or the control of early floral apex differentiation/development events. However, based on their inability to induce flowering under strict non-inductive LD, it seems unlikely that GAs are the florigenic substance per se that is **induced** by long nights which travels from the leaf to the apex. This supposition is supported by the exogenous $GA_{4/7/9}$ reversal of the uniconazole-mediated (3 g/L) inhibition of floral evocation. Thus, some other factor(s) probably regulates the earliest events of floral induction, or under LD overrides the promotive aspects of endogenous (or applied) GAs. Further discussion of GAs in relation to floral evocation will be addressed as part of the effect of exogenously applied GAs on flowering.

Effect of exogenous GAs on epicotyl elongation

As described in the general introduction (Chapter 1), flowering in many SDP (e.g. Pharbitis nil), is accompanied by a reduced rate of epicotyl elongation. This is logical, since stem growth is nearly always reduced by SD (Salisbury, 1982). This precedent of reduced epicotyl growth under inductive SD (16 h dark) was confirmed for the Xanthium system, e.g. epicotyls of plants treated with a single long night were 20% shorter ($P \leq 0.05$) than either non-flowering seedlings grown throughout the entire experiment under continued 20 h LD, or flowering seedlings receiving a single marginally-inductive long night with subsequent development under LD (see figure 3.5). A decreased epicotyl growth response indicates either a reduced rate of synthesis of elongation "effector", an

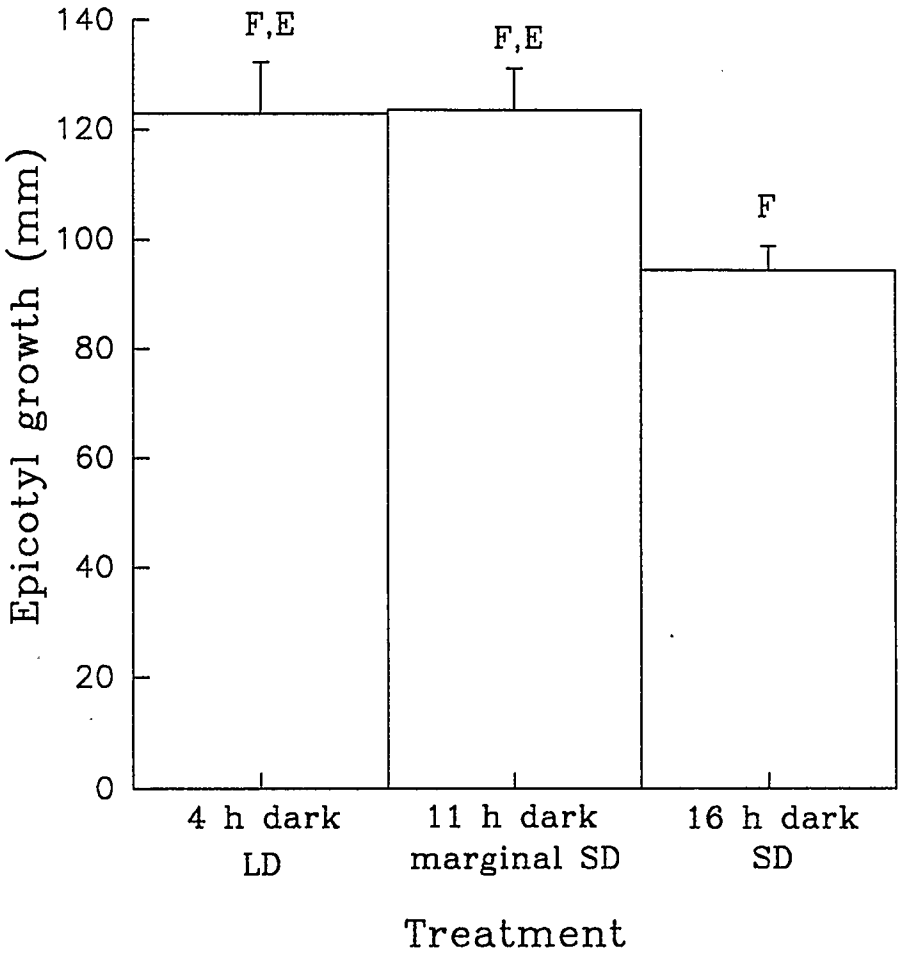


Figure 3.5 Effect of a single inductive long night (16 h dark) and a single marginally-inductive (12 h) long night on epicotyl growth of Xanthium seedlings measured 9 days following induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

increased rate of inactivation of said effector, or an attenuation of the system by which the plant responds to the elongation effector.

Since GAs are proven effectors of shoot elongation growth in many higher plant species (Phinney et al., 1990), and it has been postulated that GAs have a role in floral evocation, then it might be hypothesized that the structural motifs important for each response should differ. The Lolium system seems to be a case where those GAs which are relatively ineffective at inducing stem elongation (i.e., 2,2-dimethyl-epi GA_4, and low doses of GA_5 and GA_{32}) are the ones which are most effective at inducing flowering. By comparison, in Pharbitis the GA level necessary to promote flowering under marginal long nights is 10-100 fold lower than that required for stem elongation (King et al., 1987). In Pharbitis, when flowering is considered, there is a strong relationship between GA type and that dose range which actively promotes floral evocation. Gibberellins possessing a 13α-OH (i.e., GA_3 and GA_5) are highly active in promoting floral evocation at low dose, but become inhibitory at high dose (King et al., 1987). The GAs which possess a 3ß-OH (e.g. GA_4 and 2,2-dimethyl GA_4) have intermediate activity promoting floral evocation, but are effective over a broad range of doses (King et al., 1987). One GA with both 13α- and 3ß-OH, but no double bond in ring A, (e.g. GA_1) promotes floral evocation only at quite high doses (King et al., 1987). Given these precedents, it was postulated that GA types and/or doses which are ineffective in stem elongation might be most effective in floral evocation. To assess this postulate, 8 GAs were exogenously applied in the Xanthium seedling assay.

In Xanthium, as was the case in the Pharbitis and Lolium systems (Evans et al., 1990; King et al., 1987), exogenously applied GAs caused varying degrees of stem elongation, depending on GA type and dose. With the exception of 2,2-dimethyl-epi-

GA_4, all applied GAs promoted epicotyl growth. It is likely that the 3α-OH of 2,2-dimethyl-epi-GA_4 is the basis for its inability to promote elongation since for several other GAs the α-hydroxy epimer is usually less effective than its 3ß-OH form in elongation (Brian et al.. 1967). For the remaining GAs response increased up to 100 µg/plant, except for GA_7 and 2,2-dimethyl GA_4 both of which yielded optimal growth promotion at 33 µg/plant (the 100 µg/plant dose was slightly inhibitory to epicotyl elongation). The order of efficacy for epicotyl elongation of Xanthium was: $GA_{4/7/9}$>GA_7>GA_9>GA_4>2,2-dimethyl GA_4>GA_3>GA_5>2,2-dimethyl-epi-GA_4≈control (see figure 3.6 and top graphs of figures 1 to 24 in Appendix I).

Several trends can be discerned in these results. First, the GAs that were most effective in the epicotyl elongation response ($GA_{4/7/9}$, GA_7, GA_9, GA_4, and 2,2-dimethyl GA_4) have relatively non-polar structures, (e.g. GA_9 has no hydroxyl group, the others have only a single hydroxyl group at C-3ß). The above GAs may either be effective in their promotion of epicotyl growth (as well as other processes) by merit of their primary structure, or as a result of their metabolism to more elongation-active GAs. There is good evidence that GA_1 and GA_3 are both potent effectors of elongation in many dicot and monocot systems [e.g. Pisum sativum (Reid, 1990), Pharbitis nil (King et al, 1987), maize (Murakami, 1972), Lolium telemulentum (Pharis et al., 1987) and Tan-ginbozu dwarf rice (Takahashi and Kobayashi, 1991)]. Thus, GA_1 could arise as a product of the metabolism of GA_9→GA_4→GA_1 (Sponsel, 1988) or GA_9→GA_{20}→GA_1 (Sponsel and MacMillan, 1977) or GA_{19}→GA_{20}→GA_1 (Smith et al., 1991; Kamiya and Kwak, 1991), whereas GA_3 could be produced in vivo by the metabolism of GA_{20}→GA_5→GA_3 (Smith et al., 1991). It has also been postulated that GA_4 and GA_7 are effective at mediating the elongation response in the absence of their metabolism to the above mentioned effectors, although this point has not been unambiguously demonstrated (Nakayama et al., 1991;

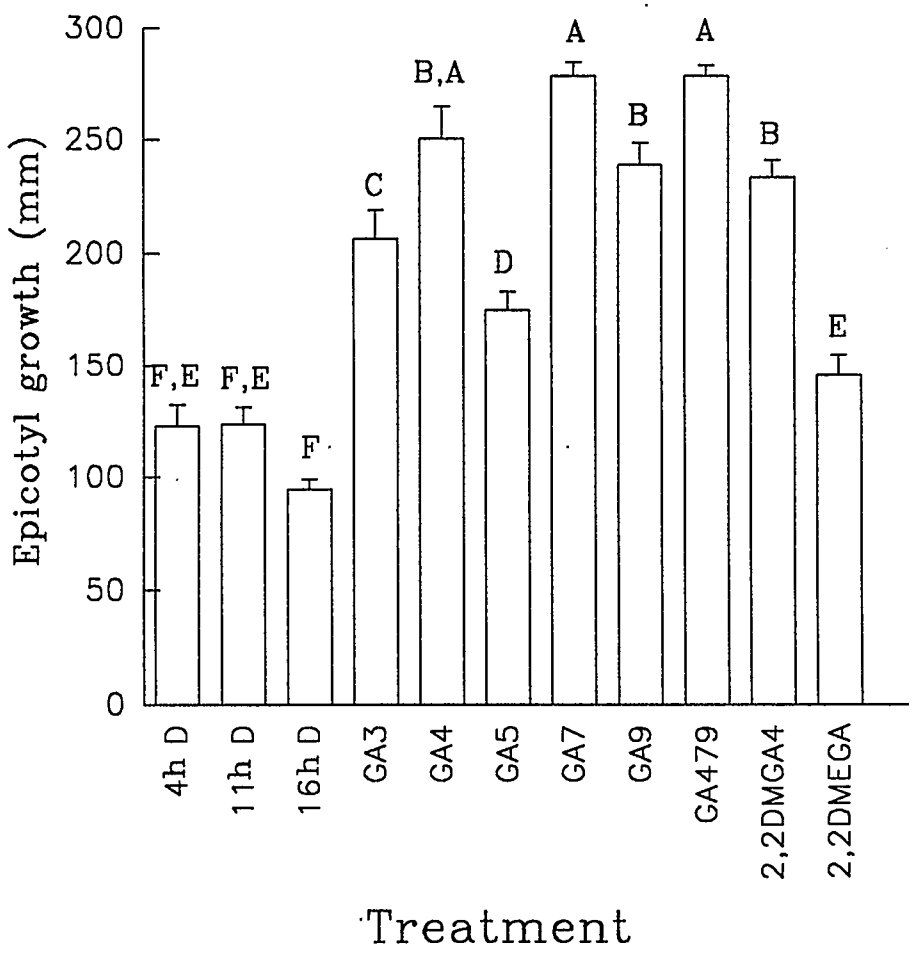


Figure 3.6 Direct comparison of the efficacy of 8 GAs on epicotyl growth in Xanthium. Note that a dose of 100 µg/plant was applied at 1200 h, 9 h prior to floral induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 10 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test. The abbreviation "DME" stands for dimethyl-C-3-epi-.

Takahashi and Kobayashi, 1991). Differential uptake may also be important in analyzing the effect of an exogenously applied GA on elongation and other processes.

For the exogenously applied GAs, two types of epicotyl elongation responses were observed. Gibberellins $A_{3,4,5,9}$ and 4/7/9 mixture generally exhibited a log-linear growth response to increasing log dose of applied GA (example seen in figure 3.7 and top graph of figures 1 to 9 and 13 to 18 in Appendix I). However, a biphasic response was observed for GA_7 and 2,2-dimethyl GA_4 with a burst of epicotyl growth at 1µg, followed by a plateau or slight depression of growth over the intermediate concentrations (3.3 to 33 µg/plant) and another promotion of growth at the highest dose tested (100 µg/plant) (figure 3.8 and top graph of figures 10 to 12 and 19 to 21 in Appendix I). The difference in these response types could to be due to differences in the metabolism of the GAs. Gibberellins typifying a linear response type would be expected to be rapidly metabolized to an effector, and/or inactivated (by 2ß-hydroxylation and/or conjugation), thus preventing a saturation of the elongation "response system". However, those GAs which exhibit a biphasic response type might be expected to be metabolized at a lower rate, with high doses, perhaps, leading to substrate-induced synthesis of GA glucosylating enzymes, or 2ß-hydroxylase enzymes. If the above hypothesis is correct, then the Xanthium 2ß-hydroxylation enzyme system does not appear to be able to recognize and/or inactivate GA_7 and 2,2,-dimethyl GA_4 [or GA_3 for that matter, see top graphs (at high dose) in figures 1 to 3 in Appendix I], possibly due to the presence of the C-1,2 double bond in GA_7 and GA_3 and the two C-2 methyl groups in 2,2-dimethyl GA_4. These two motifs should thus foster biological persistence and GAs possessing them presumably have a long biological half life. Enhanced activity of 2,2-dimethyl GA_4 could also result from increased uptake and transport, as speculated by King et al. (1987), rather than from

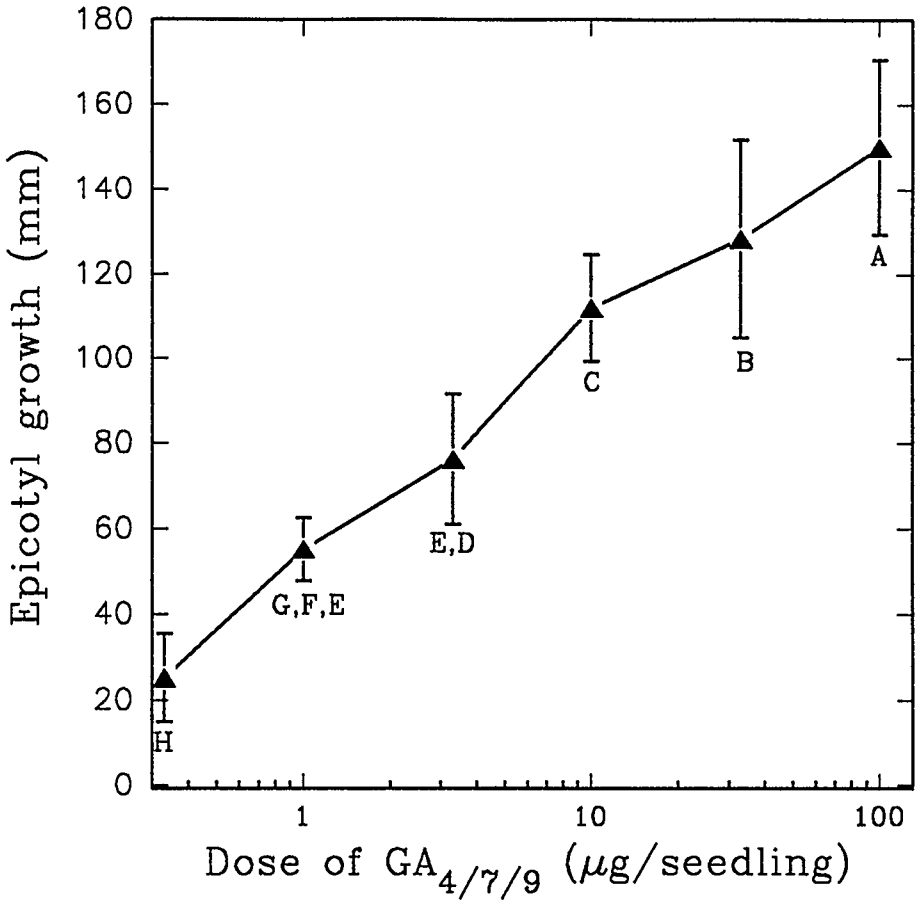


Figure 3.7 Typical linear epicotyl growth response to an increasing log GA dose, observed for exogenously applied $GA_{3,4,5,9}$ or 4/7/9 mixture. The dose of $GA_{4/7/9}$ mixture was applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

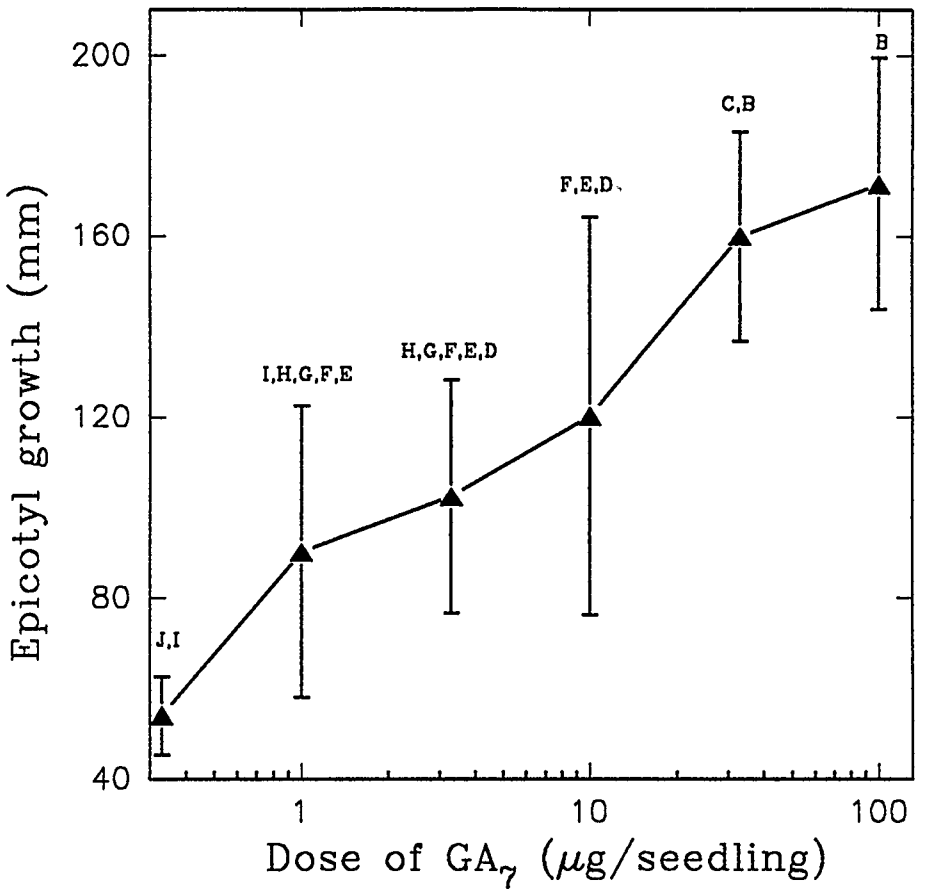


Figure 3.8 Typical biphasic epicotyl growth response to an increasing log dose, observed for exogenously applied GA7 and 2,2,-dimethyl GA4. The dose of GA7 was applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

hindrance to 2ß-hydroxylation. It must be noted that neither GA_7 nor 2,2-dimethyl GA_4 are known to be endogenous to Xanthium, and that fact might also explain the absence of an effective inactivation system.

Effect of exogenous GAs on petiole elongation

Just as inductive SD reduced epicotyl growth, petiole growth was also reduced for seedlings treated with a single fully-inductive 16 h long night (SD), or marginally-inductive 11 h long night, compared to seedlings maintained under 20 h LD (however, the differences between the three photoperiods are not statistically significant) (see figure 3.9). Although reduced petiole growth under SD was not as substantial as was epicotyl growth, this difference was observed in every experiment between SD and LD control seedlings. As mentioned above, this reduced growth could result from reduced GA synthesis, increased inactivation (conjugation or 2ß-hydroxylation), or even due to decreased sensitivity of the petiole extension "response system".

Analogous to the epicotyl elongation response, 2,2-dimethyl-epi-GA_4 did not significantly ($P \leq 0.05$) promote petiole elongation beyond that observed in the marginally-induced controls. This indicates that promotion of petiole elongation by a GA also requires the presence of a 3ß-hydroxyl. In addition, GA_5 did not significantly increase petiole growth, possibly due to its lack of hydroxylation at the C-3 position or poor metabolism to a 3ß-hydroxylated GA (e.g. GA_3). On average the effect of exogenous GA application (even for the most effective GAs) on petiole growth was much less noticeable than on epicotyl growth. The order of efficacy for the applied GAs on petiole growth was 2,2-dimethylGA_4>GA_7≈GA_4>GA_3≈$GA_{4/7/9}$>GA_9»GA_5≈2,2-dimethyl-epi-GA_4≈control

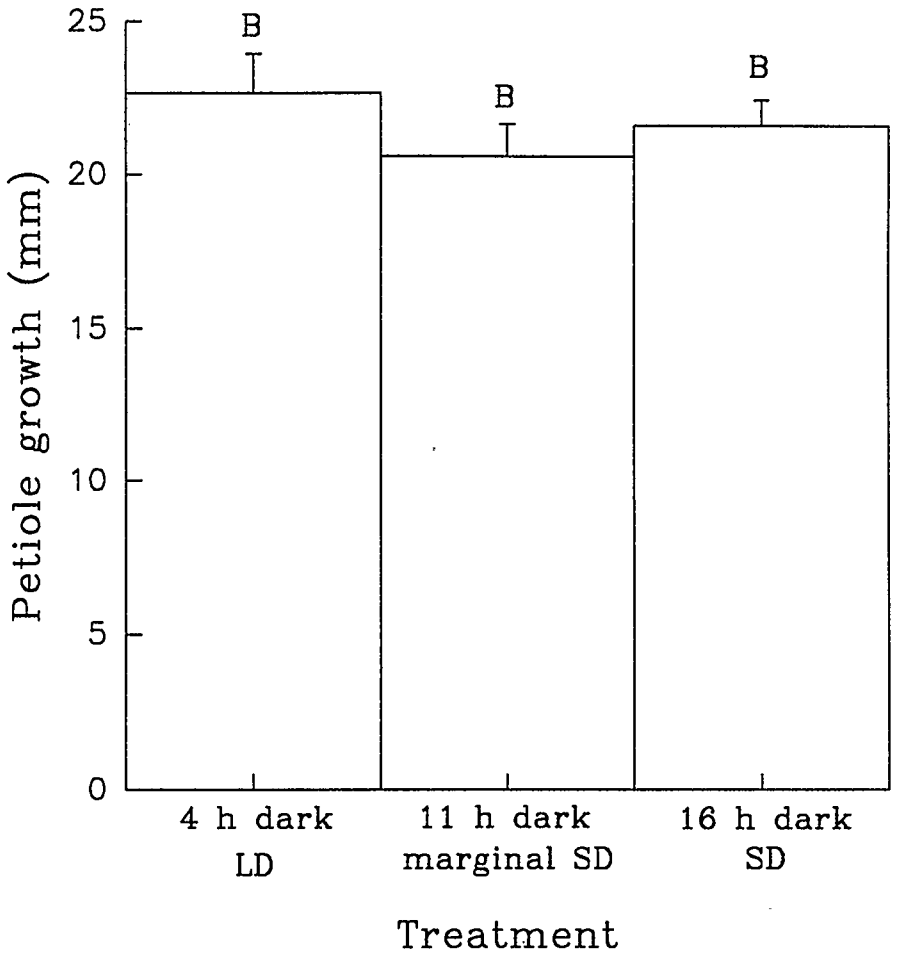


Figure 3.9 Effect of a single inductive long night (16 h dark) and a single marginally-inductive long night (12 h dark) on petiole growth of Xanthium strumarium seedlings measured 9 days following induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

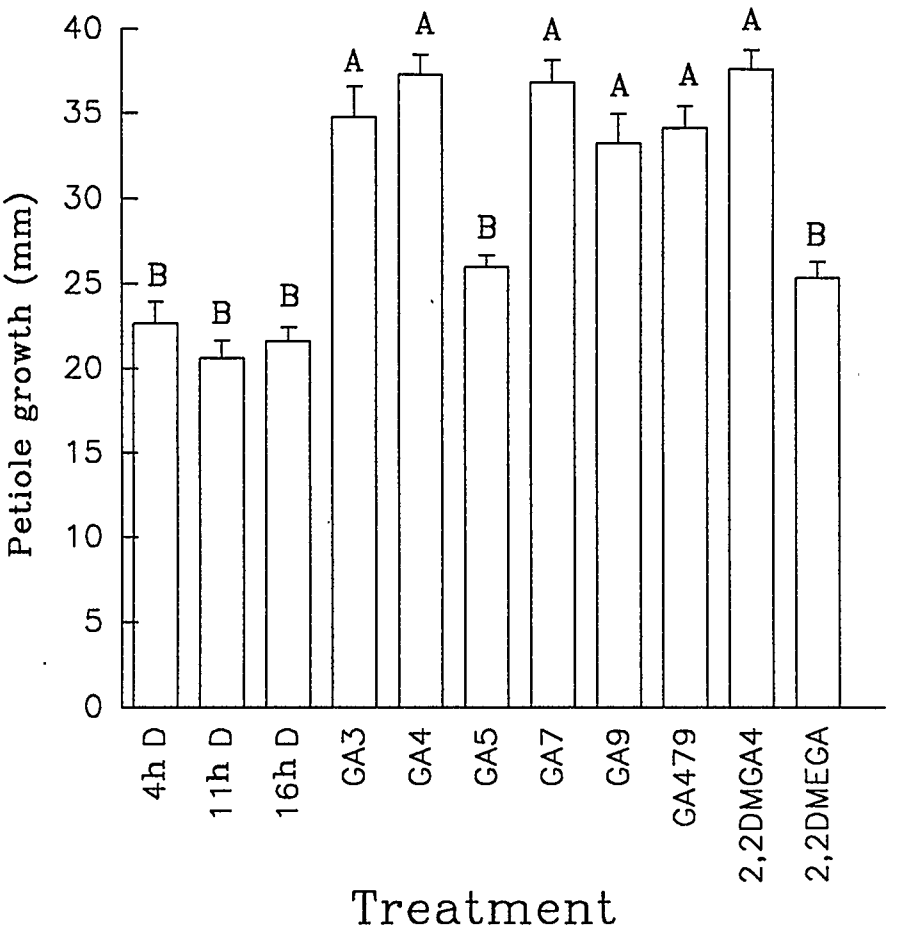


Figure 3.10 Direct comparison of the efficacy of 8 GAs on petiole growth in Xanthium. A dose of 100 µg/plant was applied at 1200 h, 9 h prior to floral induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 10 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test. The abbreviation "DME" stands for dimethyl-C3-epi-.

(see figure 3-10 and bottom graphs in figures 1 to 24 in Appendix I). As was the case for epicotyl elongation, the GAs which were most effective in petiole elongation possessed a 3ß-OH and were quite non-polar. Additionally, by comparison to epicotyl growth, the three most effective GAs in petiole elongation were those which may be the most difficult to inactivate by 2ß-hydroxylation (2,2-dimethyl GA_4, GA_3 and GA_7). This reinforces the likelihood that Xanthium is unable to inactivate such GAs. Unlike the epicotyls, however, GA_9 and $GA_{4/7/9}$ were of relatively low efficacy in the petiole elongation system. Petiole growth in Xanthium thus seems to have not only a strong requirement for a 3ß-OH, but also for motifs (such as a double bond at C-1,2) which may foster biological persistence.

Also analogous to epicotyl elongation two curve types were observed. The first was a log dose:linear response over the entire range of doses tested. This was characteristic of GA_3 and GA_9 (see figure 3.11 and bottom graphs in figures 1 to 3 and 13 to 15 in Appendix I). The remaining GAs which promoted petiole growth (GA_4, GA_7, $GA_{4/7/9}$ and 2,2-dimethyl GA_4) had a curve in which a burst of petiole growth was observed at 1 µg/plant followed by a plateau of elongation for **all** higher doses (figure 3.12 and bottom graphs in figures 4 to 12 and 16 to 24 in Appendix I). Thus the petiole elongation response may become saturated by the lowest dose of applied GA. Further, GA_7, $GA_{4/7/9}$ and 2,2-dimethyl GA_4 all exhibited a depression of petiole elongation at 100 µg/plant, relative to the elongation observed at 1 µg/plant. For these GAs, at least, the petiole elongation response appears to become saturated at a 100-fold lower dose than does the epicotyl elongation response (compare figures 3.7 and 3.12). Given these results it is possible to speculate that the "receptor" for epicotyl elongation may be quite different from that for petiole elongation. It also seems possible that GA metabolism of less polar GAs by the petiole may be quite reduced, at least relative to the epicotyl.

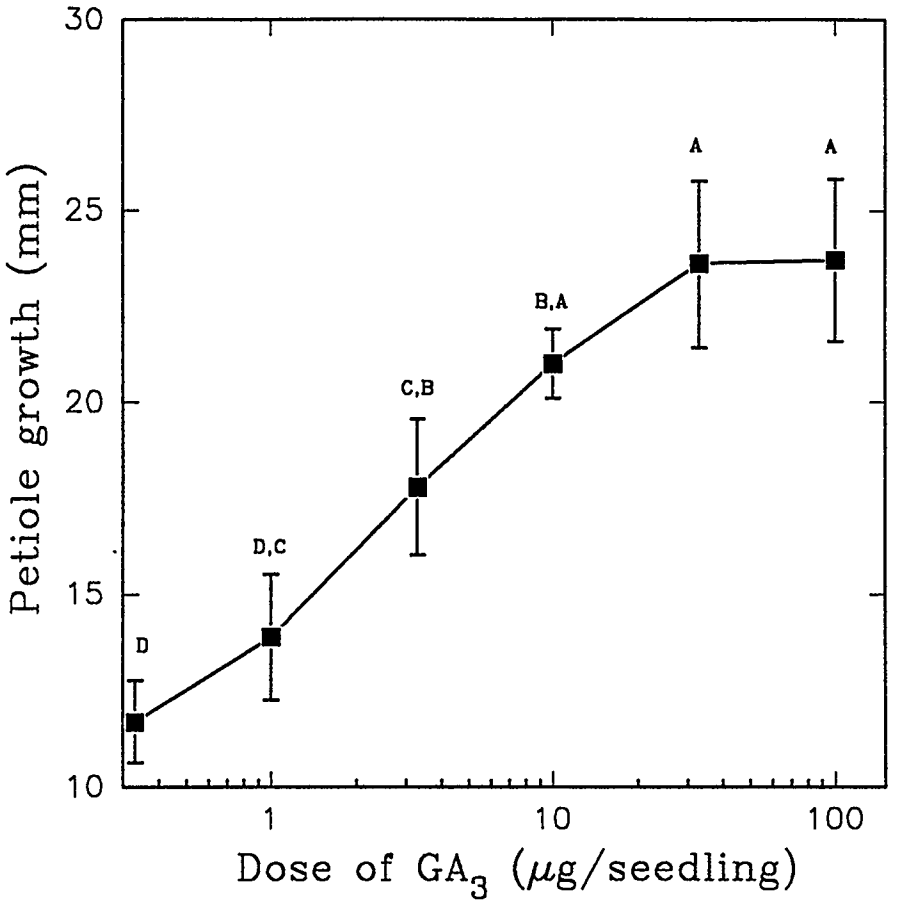


Figure 3.11 Typical linear petiole growth response to an increasing log GA dose, observed for exogenously applied GA_3 and GA_9. Dose of GA_3 was applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

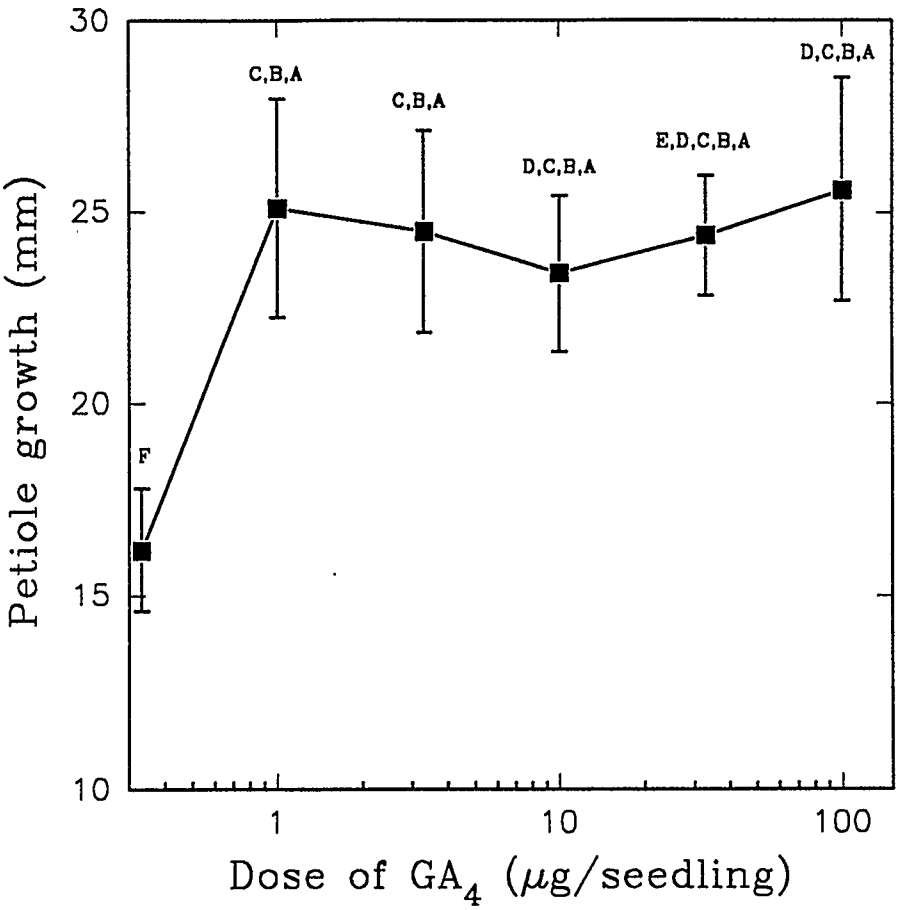


Figure 3.12 Typical "burst" of petiole growth in response to an increasing log GA dose, observed for exogenously applied GA_4, GA_7, $GA_{4/7/9}$ and 2,2-dimethyl GA_4. Dose of GA_4 was applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

Effect of exogenous GAs on flowering

All applied GAs exhibited at least a low level of promotion of floral evocation (or subsequent early floral differentiation/development) in marginally-induced Xanthium. However, depending on the level of marginal-induction (typified by the marginally-induced control seedlings) the order of efficacy of the applied GAs was variable. Thus, floral evocation in the Xanthium system was not as strongly promoted by GAs as it was in the SDP Pharbitis or the LDP Lolium. Unlike Pharbitis though, high doses of GA were not inhibitory to flowering in Xanthium (see figures 1 to 24 from Appendix 24). The order of efficacy of each applied GA was determined specifically by its effect on promotion of the process of **evocation** (the transition of the apex from a vegetative to a floral state). In determining this scale I weighted experiments in which controls were marginally-induced to a low floral stage (on average, Salisbury floral stage 1) more heavily than those induced to a more advanced stage of development (Salisbury floral stage 3 or 4). Comparison of GA efficacy in flowering was done primarily by a comparison of average Salisbury stage of flowering with more rigorous comparison by flowering quotient. Thus, for the process of floral evocation the order of efficacy for GAs was $GA_{4/7/9}$>GA_7≈GA_4≈2,2-dimethyl GA_4>GA_9≈GA_5≈2,2-dimethyl-epi-GA_4>GA_3>control (figure 3.13 and figures 1 to 24 in Appendix II). Interestingly, the GA structures which were most effective at causing stem elongation (e.g. $GA_{4/7/9}$, GA_7 and GA_4) were also most effective at evoking flowering. The time of application (relative to the marginally-inductive long night) was not analyzed in this study, but was studied by Mukherjee who determined that optimum promotion of floral evocation in mature plants was achieved with applications of GA_3 between 4 and 8 h prior to marginal-induction (unpublished results). Application after the marginally-inductive long night was ineffective at promoting floral evocation (Mukherjee,

unpublished results), and King et al (1987) noted for Pharbitis that exogenous application of GA after the marginally-inductive long night was actually inhibitory to floral evocation.

Floral evocation curves (for floral stage) for several applied GAs (e.g. GA_4, 2,2-dimethyl-epi-GA_4, and $GA_{4/7/9}$) showed a weak promotion at low dose, with a plateau over the mid-range of doses tested (3.3 to 33 µg/seedling), followed (usually) by a large increase in floral stage at 100 µg/plant (see figure 3.14 and figures 4 to 6, 16 to 18 and 19 to 21 in Appendix II). One intriguing explanation for this increased promotion at the highest dose may be the possibility that highly florigenic contaminants are present within the applied GA. At the 100 µg/plant dose, these may become significant enough to increase (synergize?) the flowering response promoted by the main GA.

One similarity between the GAs effective at promoting floral evocation in Lolium, Pharbitis and Xanthium is that they are all C_{19} GAs (however, in the present study C_{20} GAs were not tested). In addition it might be expected that a free carboxyl group at C-7 is required in Xanthium as it is in Lolium and Pharbitis (Evans et al., 1990; King et al., 1987). Although GA methyl esters were not tested in the present study on Xanthium they are inactive in Lolium (Evans et al., 1990). Past this point similarities break down and the efficacy order is very different between Xanthium and Lolium (especially) and Xanthium and Pharbitis. Thus, floral promotive features of GAs vary considerably depending on order, family, photoperiod or cold-requirement type.

For the Xanthium system, with few exceptions, those GAs which have a large promotory effect on floral apex development (Salisbury floral stage) are also those most effective for stem elongation. An almost parallel situation occurs for Arabidopsis (Goto and Pharis, 1993) and for Pharbitis (SDP), although in Pharbitis low doses of exogenously applied GA (which did not promote elongation) were much more efficacious at inducing

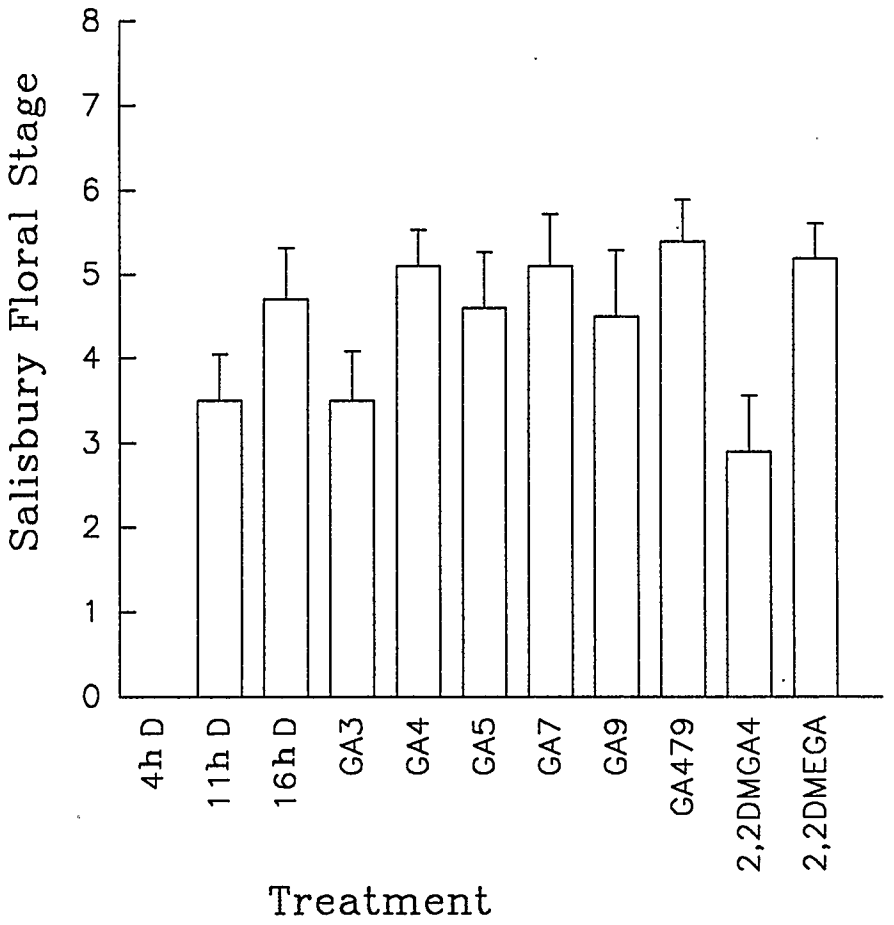


Figure 3.13 Direct comparison of the efficacy of 8 GAs on floral evocation in Xanthium. A dose of 100 µg/plant was applied at 1200 h, 8 h prior to induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 10 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test. The abbreviation "DME" stands for dimethyl-C3-epi-.

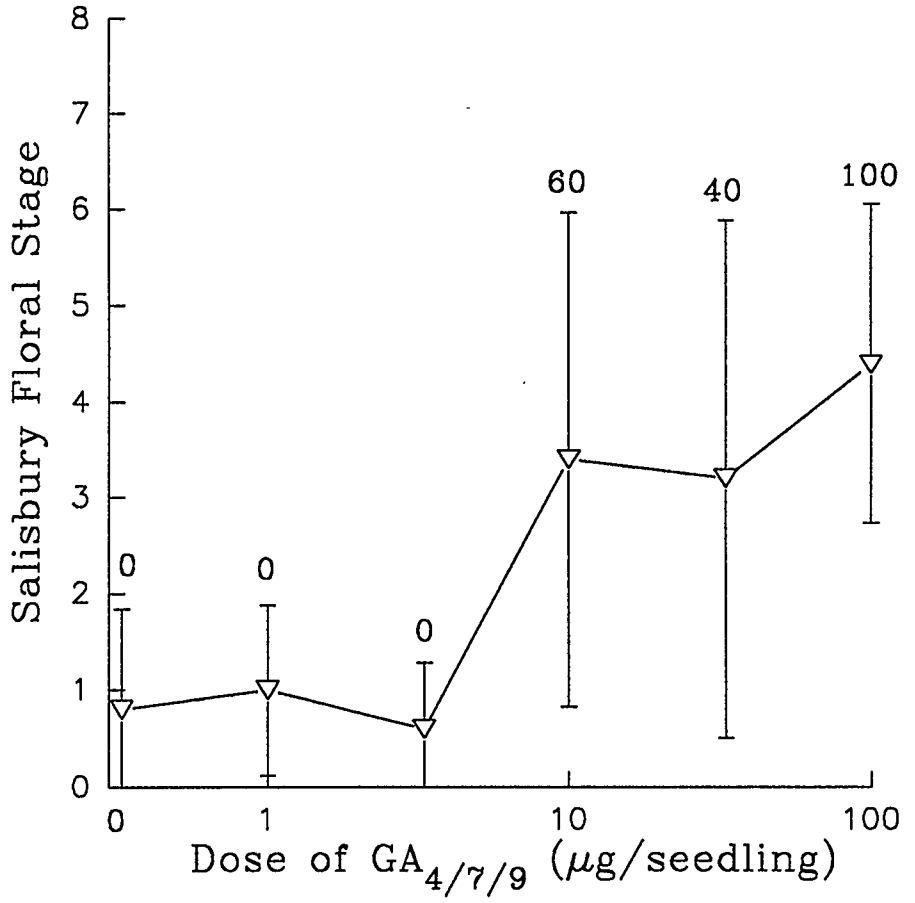


Figure 3.14 Efficacy of $GA_{4/7/9}$ on floral evocation in the Xanthium seedling bioassay. Doses of $GA_{4/7/9}$ were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury floral stages) dissected 9 days post-induction. Error bars represent 95% confidence intervals. Numbers above the bars indicate flowering quotients.

flowering, whereas higher doses which significantly promoted stem elongation, actually inhibited flowering (King et al., 1987). Unlike Pharbitis, the highest dose (100 µg/seedling) was in all cases the most effective dose for stimulating both floral evocation and growth in Xanthium. Is the efficacy of elongation-promoting GAs on promotion of floral evocation in these two SDP (and Arabidopsis) a general characteristic for herbaceous dicots? Although GA efficacies for epicotyl growth and flowering are strongly correlated in both Pharbitis and Xanthium studies, the orders of structural efficacies cannot be directly compared. For instance, GA_7 and GA_4 were the most **ineffective** GAs at promoting evocation in Pharbitis at low doses (King et al., 1987), but in Xanthium these were two of the three best. Thus, when dose is taken into account the order of efficacies for flowering, (2,2-dimethyl GA_4 excepted), is reversed between Pharbitis and Xanthium. Xanthium seems to have a strict requirement for a 3ß-hydroxyl, but unlike Pharbitis where GA_3»GA_1, Xanthium does not appear to exhibit a preference for a double bond in ring A (e.g. witness the equal efficacy of GA_4 and GA_7 in figures 4 to 6 and 10 to 12 in Appendix II). There exists almost a parallel order of efficacy between Xanthium and the florigenicity of several GAs in some lines of Brassica (i.e., Westar, WW1033 and Glacier) (Mandel et al., 1992) and Arabidopsis thaliana (Pharis and Goto, 1993). In essence, order of efficacy is 2,2-dimethyl GA_4>GA_5>GA_3 in all three systems.

Strong differences in GAs effective in promoting flowering were also noted between Xanthium and the LDP Lolium. In LD-requiring plants, of which Lolium is a good model system, there is (generally) a very distinct structure/function relationship for GAs effective in elongation compared to GAs effective in floral induction. Evans et al. (1990) commented for Lolium that if there is a GA receptor involved in floral induction, it would seem to have different structural requirements than the GA receptor involved in stem elongation. Such a difference in "receptors" for elongation and flowering may also

be evident in red clover, where GAs with a C-1,2 double bond (i.e., GA_3 and GA_7) or C-2,3 double bond (i.e., GA_5) are most effective in both flowering and stem elongation, whereas those lacking a double bond (i.e., GA_4) are only effective in the elongation response. In Lolium, GAs with low efficacy (at low dose) with respect to inducing stem elongation (GA_5, C-12ß-OH GA_5, and 2,2-dimethyl-epi-GA_4 to name a few) are often those which are most effective at inducing flowering (Evans et al., 1990; Evans et al., 1993). In Lolium, 3ß-hydroxylation is actually a detrimental feature for flowering (Evans et al., 1993), even if a double bond is present (GA_7). The most important structural features for Lolium are hydroxyls on rings C and/or D and the presence of a ring A double bond. By comparison, florigenic GAs in the Xanthium system (i.e., $GA_{4/7/9}$ and GA_7) seem to be those best able to promote epicotyl extension growth (at least under a marginally-inductive photoperiod), in contrast to the GA efficacies in Lolium. One argument which may reconcile this apparent anomaly is the fact that the marginally-inductive photoperiod, although it does enhance the degree of flowering, in Xanthium does not reduce the epicotyl growth relative to the LD controls (figure 3-5). This indicates that in Xanthium GA metabolism and/or response still differs between the marginally-inductive long night and the fully-inductive 16 h long night regimes. Marginally-inductive long night, although it may be an effective tool for preliminary screening of florigenic substances (since it partially activates transitional events sufficiently to induce flowering) may be insufficiently long enough to significantly alter GA metabolism and/or response (although see chapter 5 on endogenous GAs and chapter 6 on GA_{20} metabolism).

Conclusions

The results of this chapter provide evidence which suggests that it is unlikely that GAs alone mediate the total evocation response, although at least one component of floral evocation may be promoted by GAs (e.g. GAs are not florigen but may be a component of it). Thus, in Xanthium, GAs may act in exerting their building effect on a marginally-induced apex by means of promoting later floral developmental processes, rather than early evocation processes. Evidence which supports this supposition is three-fold. First, none of the applied GAs were effective at inducing flowering under a totally non-inductive 20 h LD photoperiod. However, for most SDP, it is well known that GAs can rarely substitute for an inductive long night. Is this simply a question of sensitivity? The hypothesis that GAs are specifically involved in the floral development process is supported by the fact that many GA "deficient" mutants from several species including rice, maize (Pharis and King, 1985), Arabidopsis (Wilson et al., 1992; Goto and Pharis, 1993), pea and tomato (Nester and Zeevaart, 1988; Sekhar and Sawhney, 1990) all initiate flowering readily under normal growth conditions, but the flowers do not develop, or show various structural defects. This indicates that GAs may be involved in the steps which follow induction. Thus, delayed floral development results when GAs regulating the later steps in floral development are limiting. This is seen in a few GA "deficient" mutants including Arabidopsis thaliana (ga1-6 mutant), red clover, Brassica rapa and Thlaspi arvense which do flower normally, though flowering is delayed (Jones and Thomas, 1993; Jones, 1989; Wilson et al., 1992). In "leaky" mutants or, as was determined in this study, with GA biosynthesis inhibitors which were less effective (CCC) or applied at lower doses (due to the less drastic reduction of endogenous GA level) floral evocation occurs, but is also delayed. Zeevaart (1971b) also determined for spinach that AMO-1618 delayed but did not inhibit floral development. It could be argued that all the

above mentioned mutants are too leaky to completely inhibit flowering. In this vein, the ga1-3 mutant of Arabidopsis thaliana, the most deficient mutant used by Wilson et al. (1992), did not flower under SD (but did eventually die) unless GA was exogenously applied. An analogous situation to the ga1-3 mutant may be seen in Xanthium plants treated with high levels of GA biosynthesis inhibitor. Thus, application of uniconazole (at 3 g/L) inhibited floral evocation, even under 16 h inductive nights, as was the case for the LDP Samolus parviflorus (Baldev and Lang, 1965), treated with AMO-1618 and held under inductive photoperiod. In each case, applied GA could reverse the inhibition, suggesting that the biosynthesis inhibitors were acting specifically by reducing the level of endogenous GAs. This implies that flowering may be inhibited when GAs reach a critical low level, a conclusion supported by the fact that applied GA could reverse the inhibition in both Samolus and Xanthium. However, since in Xanthium, applied GAs alone will not **induce** floral evocation, it seems likely that this (and other?) SDP require GAs for the later steps of floral evocation. The question which still remains is: Why can GAs promote the floral response in Xanthium and many other SDP, yet not evoke it?

CHAPTER 4

INHIBITORY EFFECT OF EXOGENOUSLY APPLIED ABSCISIC ACID, 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID AND INDOLE-3-ACETIC ACID ON EVOCATION FLORAL IN XANTHIUM

Introduction

In several systems GAs and IAA have been shown to have antagonistic effects in growth and development processes, including flowering. For example, the differentiation of male flowers in several dicot species, including cucumber and Xanthium, is enhanced by exogenous GA (e.g. $GA_{4/7}$ and GA_3 respectively) and with femaleness being enhanced by exogenous IAA (Leonard et al., 1980; Fuchs et al, 1977). This situation is reversed in conifers (see refs. cited in Pharis and Kuo, 1977; Ross et al., 1983) where exogenously applied auxin in the presence of $GA_{4/7}$ disproportionately promotes the differentiation of male cone buds, whereas $GA_{4/7}$ alone mainly promotes the differentiation of female cone buds. The situation is similar in tomato where auxin promotes the differentiation of anthers and GA_3 promotes the differentiation of ovules (Singh et al., 1992).

With respect to floral evocation, exogenously applied auxin usually inhibits flowering in SDP, including Xanthium (Hamner and Bonner, 1938; Bonner, 1949), Chenopodium rubrum (Pavlová and Krekule, 1990), Kalanchöe (Harder and van Senden, 1949), Pharbitis nil (Nakayama and Kikuchi, 1956), soybean (Hamner and Nanda, 1956) and petunia (Green and Fuller, 1948), while in LDP such as barley (Leopold and Thimann, 1949; Salisbury, 1955) and tobacco (Lozhnikova et al., 1990) IAA hormone can promote floral initiation. Also, in pineapple auxin (Clark and Kerns, 1942; Cooper, 1942) or

ethylene (Lewcock, 1947) can promote flowering, and ethephon is commercially used for this purpose. Auxin, however, can also be inhibitory to floral initiation in LDP (e.g. Lolium) (Evans, 1964). In the present study I sought to repeat and extend the work of Hamner and Bonner (1938), who first determined that applied IAA was inhibitory to floral evocation in Xanthium when applied during the inductive period until the translocation of the floral inductive stimulus to the apex was complete (e.g. 44 h after induction) (Hamner, 1958). Bonner (1949) previously determined that auxin applied to the induced leaf during photoinduction inhibited induced plants (under SD) from exporting the stimulus by using a secondary induction experiment, e.g. a vegetative graft partner maintained under LD (Bonner, 1949). This indicates that auxin could exert its inhibition by preventing the synthesis and/or transport of the floral inductive stimulus to the apex (Pavlová and Krekule, 1990) or even by reducing the effectiveness of the light period preceding the inductive dark period (Hamner and Nanda, 1956). But since, inhibition by exogenously applied auxin occurs not only when applied to the leaves (Pavlová and Krekule, 1990), but also when applied to the apex (Krekule and Privratsky, 1973), the mechanism by which auxin inhibits floral evocation is unclear. The following set of experiments sought to gain additional insight into this Pandora's Box.

Indole-3-acetic acid (IAA, but also auxin in general) is known to stimulate ACC synthase activity (Yoshii and Imaseki, 1981; Yu, et al., 1979). Therefore it was postulated that the IAA-mediated inhibition of floral evocation might actually be caused by ethylene production which was enhanced through the stimulation of ACC synthase activity by the IAA. To test this hypothesis ACC was applied to determine if this ethylene precursor could inhibit floral evocation. Indeed it did (see figure 4.1), and I thus postulated that ethylene, acting as a common secondary messenger, may be mediating the inhibition for both of applied IAA and ACC. Two approaches were then pursued: (1) ethylene

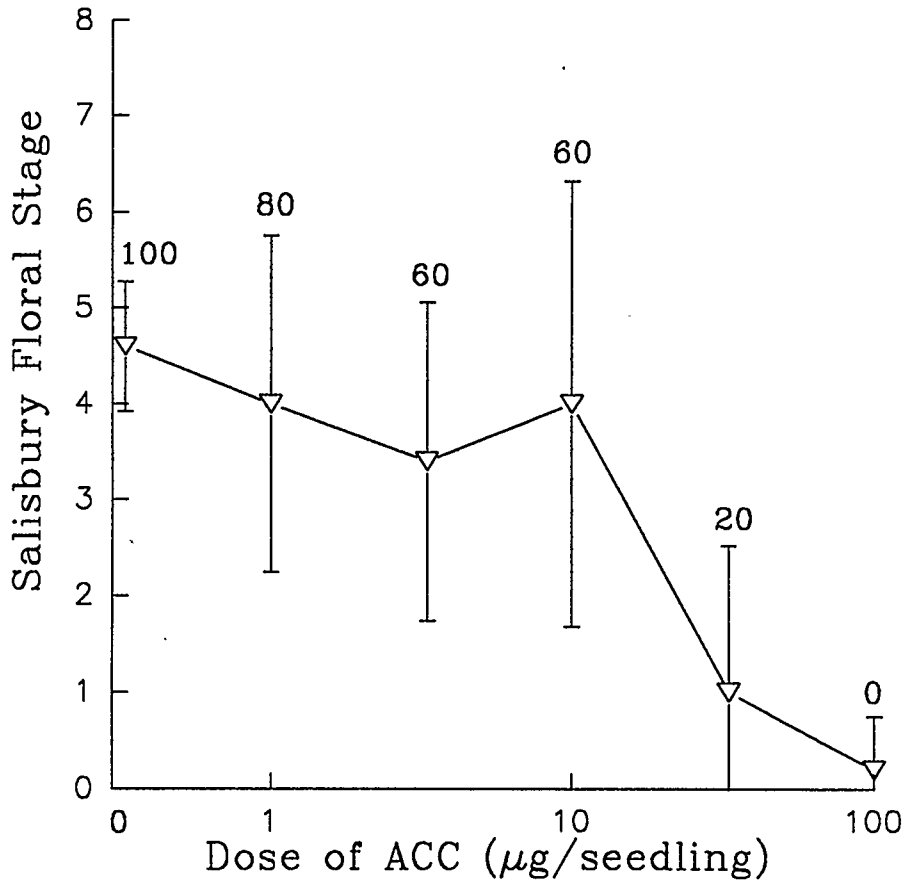


Figure 4.1 An example of ACC inhibition of floral evocation in the Xanthium seedling bioassay. The doses of ACC were applied to leaves at 1200 h, 4 h before induction with a single fully-inductive 16 h long night. Values represent the mean of 5 seedlings (Salisbury floral stages) dissected 9 days post-induction. Error bars represent 95% confidence intervals. Numbers above the bars indicate flowering quotients.

evolution following the application of each of IAA and ACC was assessed to determine if either or both substances, when applied to the leaf, cause an increased rate of ethylene production by the apex, and (2) STS, a known inhibitor of ethylene action (which possibly acts by competing with an iron atom at the active site of an ethylene receptor) (Beyer, 1976), was applied to the leaf prior to treatment with ACC or IAA to determine if the inhibition of floral evocation obtained by either or both IAA or ACC could be prevented (or reversed).

King and Evans (1977) and Evans, (1969a) determined that severe water stress could inhibit flowering in Lolium. Additionally, Aspinall and Husain (1970) reported that water stress inhibited long night-induced flowering of both Xanthium and Pharbitis. Such inhibition could be mediated through endogenous ABA, which in Lolium was found to increase 4- to 30-fold within 8 h (King and Evans, 1977). Also, when exogenously applied in several systems, ABA has been shown to inhibit floral bud initiation in Kalanchöe, Pharbitis and Spinacia (El-Antably and Wareing, 1966; Schwabe, 1972; Kamuro et al., 1990). In the Pharbitis system s-ABA (which is the natural form in higher plants) completely inhibited flower bud initiation, but the racemic mixture did not, even when applied at high doses (Kamuro et al., 1990). Given these precedents, it was postulated that s-ABA should also inhibit floral evocation in Xanthium and it was thus tested in the Xanthium seedling bioassay. However, it should be kept in mind that there is very little evidence that endogenous levels of ABA increase under normal (non-drought) circumstances enough to be a major determinant in the evocation of flowering (Bernier, 1988).

In both tobacco (Macháckova et al., 1988) and Chenopodium rubrum (Lozhnikova et al., 1988) the endogenous level of IAA decreases during induction, due to the presence

of increased levels of IAA-oxidases (Zársky et al., 1990). Based on their reasoning and on Japanese literature showing promotion of floral evocation by certain phenolics and flavenoids (Umemoto, 1971; Shonozaki et al., 1987; Shonozaki et al., 1988), a cofactor (*p*-coumaric acid) and a competitive inhibitor for the cofactor site (CGA) of the IAA oxidase class of enzymes (which degrade IAA by oxidation) were applied.

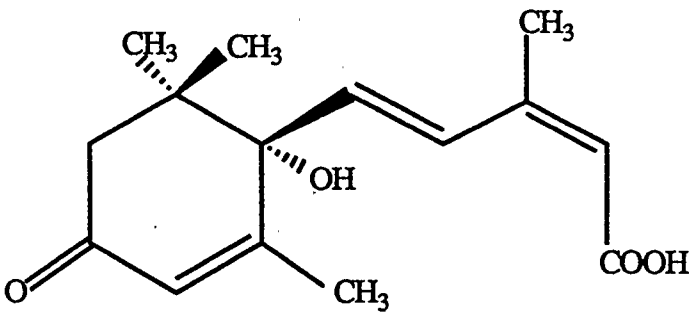
Methods

4.1 Application of flowering inhibitors

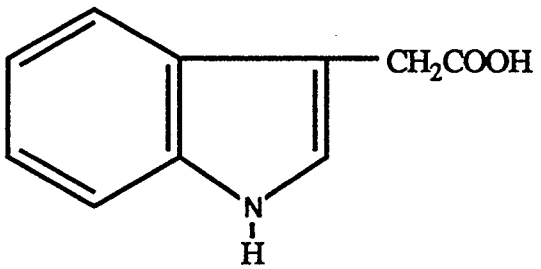
Seedlings were grown and applications of IAA, ACC, *p*-coumaric acid, CGA and s-ABA made as detailed in chapter 3 (3.2). The IAA, ACC, *p*-coumaric acid, CGA and s-ABA were dissolved in 95% EtOH (see figure 4.2 and 4.3 for structures), then applied at 1, 3.3, 10, 33 and 100 µg per plant with half of each dose being applied to the base of each primary leaf blade as a 10 µL droplet.

4.2 STS treatment of seedlings

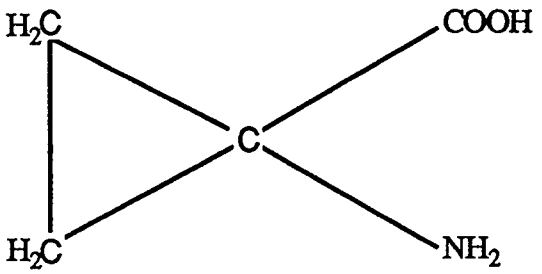
The STS was prepared from fresh stock solutions of aqueous 2.5 mM $AgNO_3$ in 0.1% Activator™ (surfactant) and 10 mM $Na_2S_2O_3$ in each experiment. At 1200 h, 4 h prior to the inductive dark period, seedlings (entire epicotyl) were inverted, dipped in this solution for 30 s and subsequently allowed to dry. Control seedlings were dipped in a 0.1% Activator™ aqueous solution. Once seedlings were visibly dry (10 min later), the primary leaves were treated with either ACC, IAA or EtOH (control) as described above



s-(+)-Abscisic acid
(ABA)



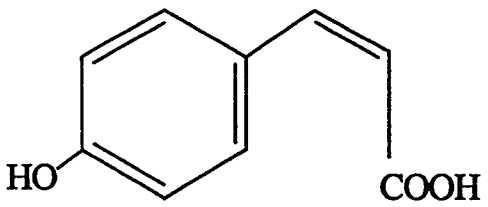
Indole-3-acetic acid
(IAA)



1-Aminocyclopropane-1-carboxylic acid
(ACC)

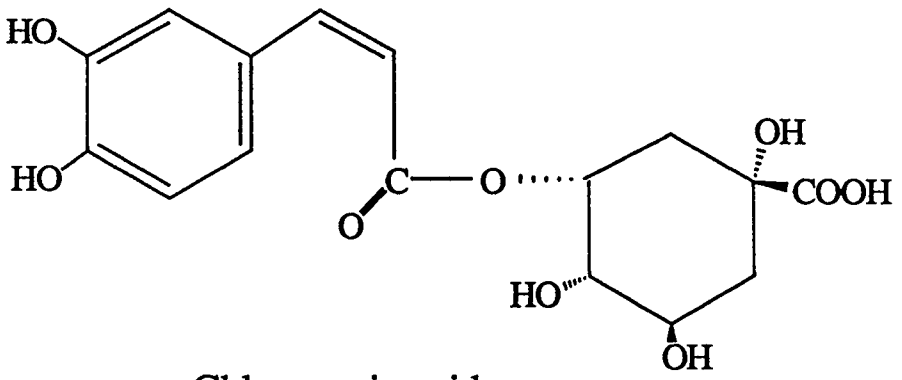
Figure 4.2 Structures of s-abscisic acid, 1-aminocyclopropane-1-carboxylic acid, and indole-3-acetic acid.

A i



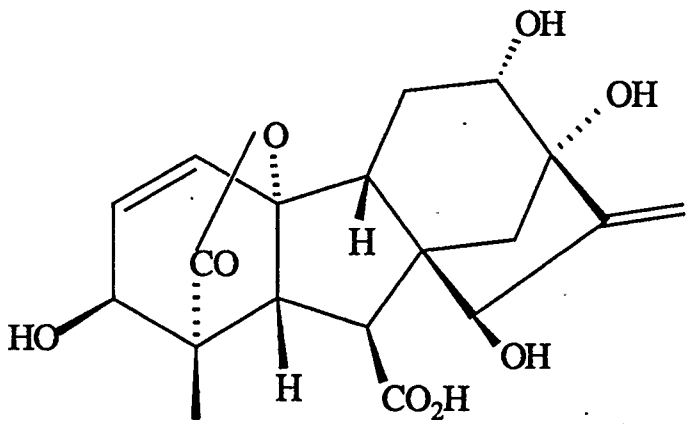
p-Coumaric acid

ii



Chlorogenic acid

B



GA_{32}

Figure 4.3 A. Structures of an IAA-oxidase cofactor (i) (*p*-coumaric acid) and an IAA-oxidase competitive inhibitor (ii) (CGA). B. Structure of GA_{32}, a highly florigenic GA in Lolium and Pharbitis.

and the plants placed back into the lighted growth chamber until the long night induction (16 h dark).

4.3 Quantitation of ethylene evolution following treatment with ACC or IAA

At time zero (1200 h, 4 h before the fully-inductive 16 h long night), the primary leaves of seedlings were treated with either IAA or ACC at a dose of 100 µg/plant. In all repeat experiments this dose was inhibitory to floral evocation. Ethylene evolution from the apices of treated plants was analyzed by the method of Finlayson et al. (1991). Essentially ethylene samples were obtained using a device made by connecting two syringes together with a 3-way valve. Both syringes were flushed with ambient air prior to sampling tissue. At times between 0 and 21 h following treatment, apical tissue (6 buds per sample) was cut and quickly placed into a 2.5 mL syringe (sample syringe) with the plunger adjusted to 1.4 mL. After precisely 10 min, a 1 mL gas sample was withdrawn from the sample syringe into a second gas-tight® syringe through the three-way valve. A preliminary study had determined that wound-induced ethylene cannot be detected in this system for at least 30 min. The 1 mL gas sample was analyzed for ethylene using a Photovac 10S10 gas chromatograph with a 3.2 mm X 2.45 m 60/80 Carbopack B column (1.5% XE-60/1% H_3PO_4; Supelco Canada, Oakville, Ontario, Canada) by photoionization detection. Quantitation was performed against an external standard curve, subtracting for ethylene in the ambient air (by means of triplicate air samples collected during the sampling time). Bud fresh weights were quickly determined immediately after sampling. Each experiment was repeated at least twice, with four replicate bud samples being analyzed for ethylene evolution within each experiment.

Results and Discussion

4.1 Inhibition by IAA, ACC and ethylene

The results of this study (see figure 4.4) confirmed Hamner and Bonner's (1938) earlier work that IAA inhibits the process of floral evocation in Xanthium. Depending on the experiment, partial inhibition by IAA was apparent in the dose range of 3.3 and 10 µg/plant, with complete inhibition of floral evocation occurring between 33 and 100 µg/plant. In all repeats a 10-fold dose range between partial inhibition and total inhibition of floral evocation by IAA was evident (figure 4.4). The broad range of IAA doses resulting in total inhibition of flowering in the present study may in part be due to the fact that IAA was more inhibitory (i.e., had effects at lower levels) in younger seedlings (13 days after planting) than in older seedlings (21 days after planting). In Xanthium, IAA (at 100 µg/seedling) only partially inhibits flowering in mature plants (I.D. Mukherjee, personal communication).

The effect of IAA on the gross morphology of the plant was striking. At the lower doses tested (1 and 3.3 µg/plant) a slight bending of the petioles was apparent 21 h after application. However, at the higher doses (33 to 100 µg/plant) the effects were more severe, yielding very epinastic plants with extremely thickened epicotyls and highly curled petioles. These morphological effects occurred at the same doses that inhibited flowering. However, none of IAA, ABA nor ACC significantly affected epicotyl or petiole elongation growth within the dose range tested.

Since IAA is known to stimulate ethylene synthesis by increasing ACC synthase activity, and acts in a dose-dependent manner (Yoshii and Imaseki, 1981; Yu et al., 1979; Saki and Imaseki, 1971), it was hypothesized that the inhibition of the floral evocation

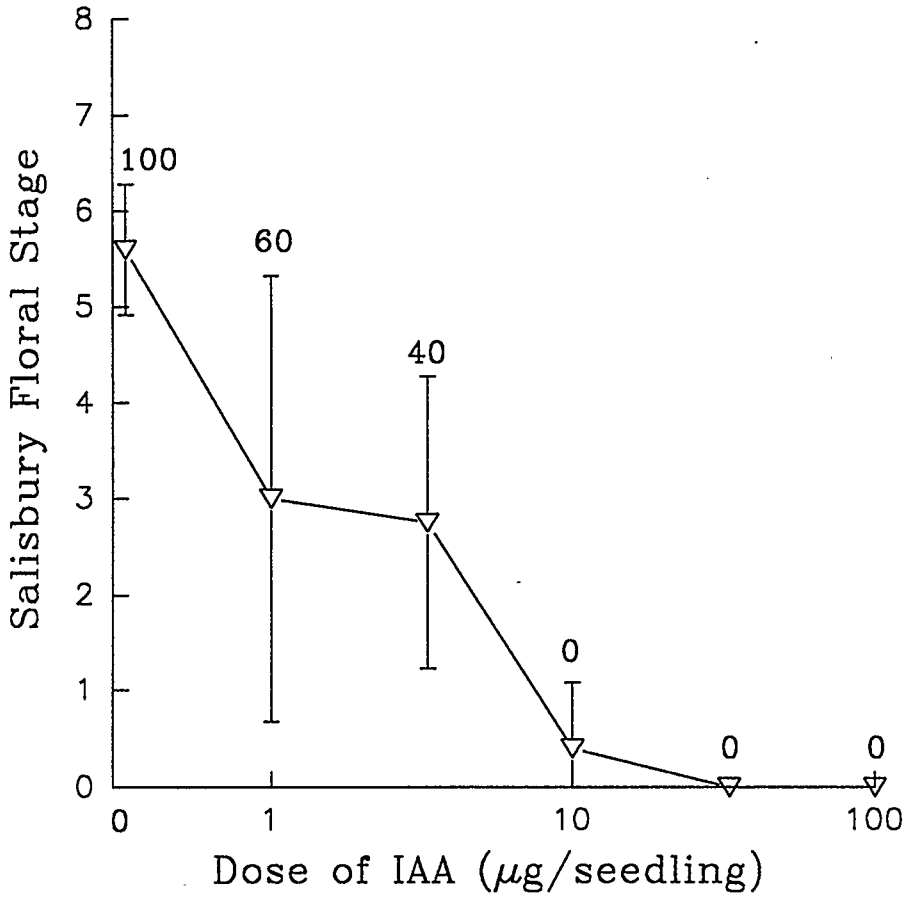


Figure 4.4 An example of IAA inhibition of floral evocation in the Xanthium seedling bioassay. Doses of IAA were applied 1200 h, 4 h before a single inductive long night (16 h dark). Values represent the mean of 5 seedlings (Salisbury floral stages) dissected 9 days post-induction. Error bars represent 95% confidence intervals. Numbers above the bars indicate flowering quotients.

obtained by IAA treatment might be mediated via increased ethylene production. This postulated mechanism can also be used to explain the current model for apical dominance whereby high IAA acting via increased ethylene, prevents axillary bud development (Gocal et al., 1991; Russell and Thimann, 1990). A mechanism connecting the processes of floral initiation and apical dominance is possible since one of the first signs of the induced state in Xanthium is decreased apical dominance (i.e., growth of axillary buds). In mango, decapitation of a main shoot causes flower initiation in the lateral branches which in the control trees remain vegetative (Reece et al., 1946). This was the rationale for testing ACC on inhibition of floral evocation induced with a single long night (16 h dark).

Indeed ACC was found to inhibit floral evocation, with partial inhibition apparent at 10 µg/plant, and complete inhibition occurring at 100 µg/plant in all repeats (see figure 4.1). However, unlike the effect of IAA on seedling morphology, ACC did not elicit curling of the petioles nor thickening of the epicotyl. Seedlings treated with ACC appeared morphologically similar to the control seedlings. The absence of an effect of ACC on seedling morphology seems to indicate that although some IAA effects on seedling morphology may be mediated through ACC-derived ethylene, that this secondary messenger (ethylene) does not elicit all IAA-produced effects.

To further explore the hypothesis that IAA and ACC inhibition of flowering were not mediated by the same mechanism, two further experiments were conducted. In the first, the rate of ethylene evolution was measured from apical tissue upon termination of the inductive dark period, 21 h following application of each of ACC or IAA to the primary leaf bases. At this time, the rate of ethylene evolution by the apical tissue from plants treated with IAA was similar to that of the controls (figure 4.5). From this result it was postulated that the IAA-induced ethylene evolution was so transient that it became

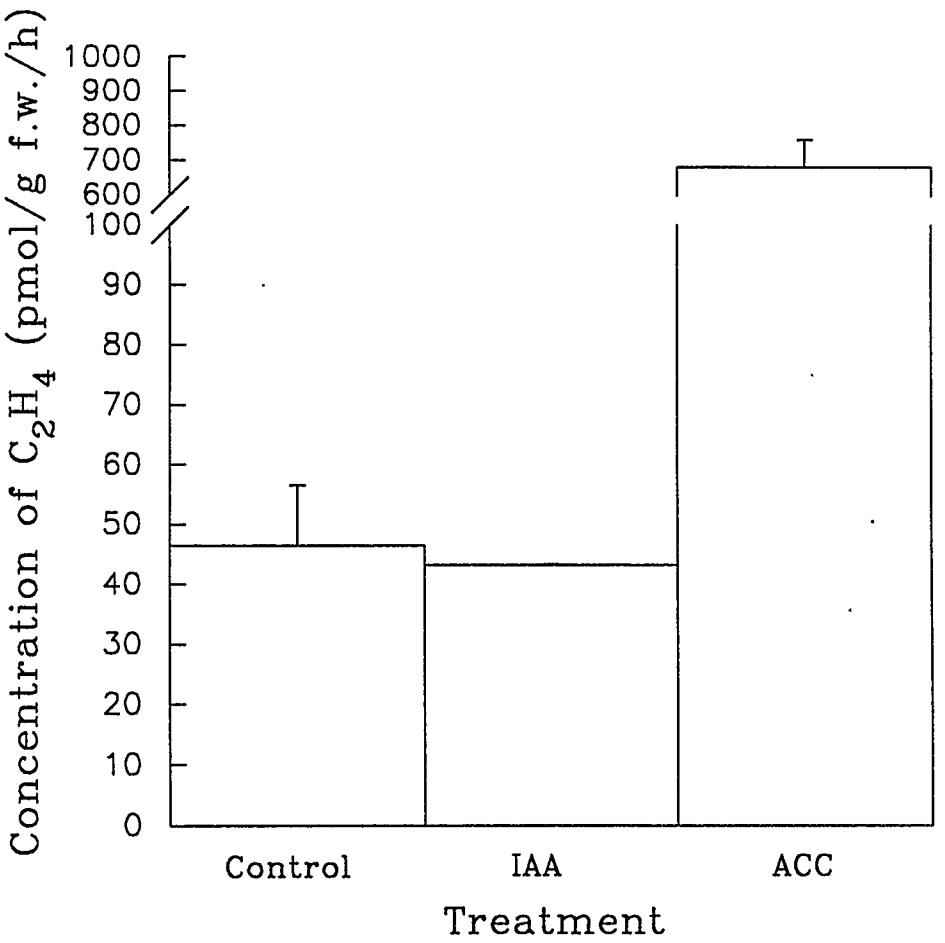


Figure 4.5 Effect of a dose of 100 µg/plant of IAA or ACC applied to primary leaf bases of Xanthium seedlings on ethylene evolution by apical tissue 21 h later. Growth substances were applied at 1200 h (4 h prior to the beginning of the long night) and ethylene evolution was measured at 0800 h the following day (21 h post-application) by GC with photoionization detection. Data represent the mean of four replicate samples (3 apical buds in each sample). Error bars represent 95% confidence intervals.

undetectable within 21 h of exogenous IAA application. In comparison, exogenous application of ACC caused a sustained production of ethylene over at least 21 h (see figure 4.5). In a subsequent experiment the effect of IAA on ethylene production was studied over a 15 h period following exogenous application of IAA. Here it was determined that exogenous application of IAA resulted in a transient increase of ethylene production by the apical tissue, which appeared to peak between 3 and 6 h after treatment (fig 4.6). The IAA-induced ethylene production had diminished to control level by the end of the 16 h inductive dark period.

For all future ethylene measurements the beginning of the dark period (4 h after hormone treatment) was chosen as a convenient sampling point. Both IAA or ACC application to the leaf blade 4 h earlier resulted in a high level of ethylene evolution at the beginning of the dark period by the apical tissue, relative to the control apical tissue (see figure 4.7). Thus, ACC-induced ethylene evolution was 5-fold higher than IAA-induced ethylene evolution, which in turn was 10-20-fold higher than ethylene evolution by untreated control seedlings (figure 4.7).

In a second experiment, seedlings were treated with STS 10 min prior to exogenous IAA or ACC application (which was at 1200 h, 4 h before the beginning of the long night). Flowering was scored 9 days later. Silver is one known inhibitor of ethylene action, possibly acting by competing for an iron atom at the active site of the ethylene receptor (Beyer, 1976). Although silver in the form of STS is quite mobile, and has fewer toxic side effects than other silver-containing inhibitors, in this study it did produce a few small necrotic lesions on the stems. This obvious wounding effect did not, however, inhibit floral evocation. The ACC-induced inhibition of floral evocation was prevented (or restored to the control level) for STS-pre-treated seedlings dosed with ACC (figure 4.8).

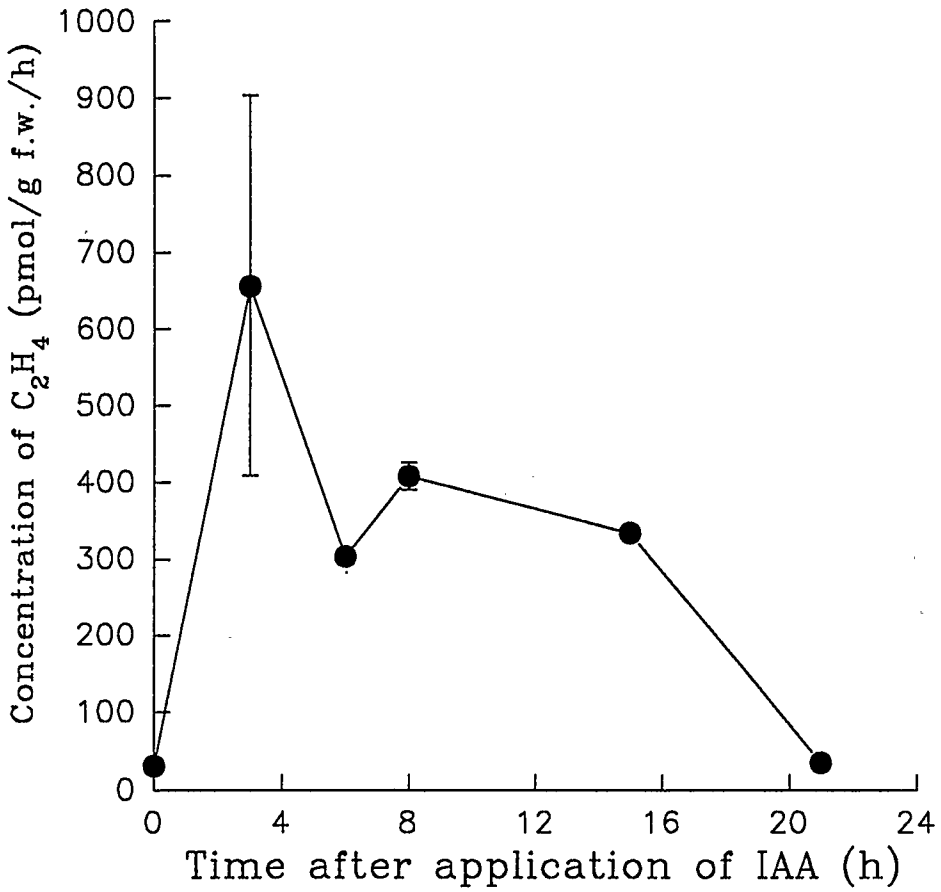


Figure 4.6 Effect of a dose of 100 µg/plant of IAA, applied to the primary leaf bases of Xanthium seedlings, on ethylene evolution by apical tissue measured as a time course. Growth substances were applied at 1200 h and the inductive long night began at 1600 h. Ethylene evolution was measured at selected time points by GC with photoionization detection. Data represent the mean of four replicate samples (3 apical buds in each). Error bars represent 95% confidence intervals.

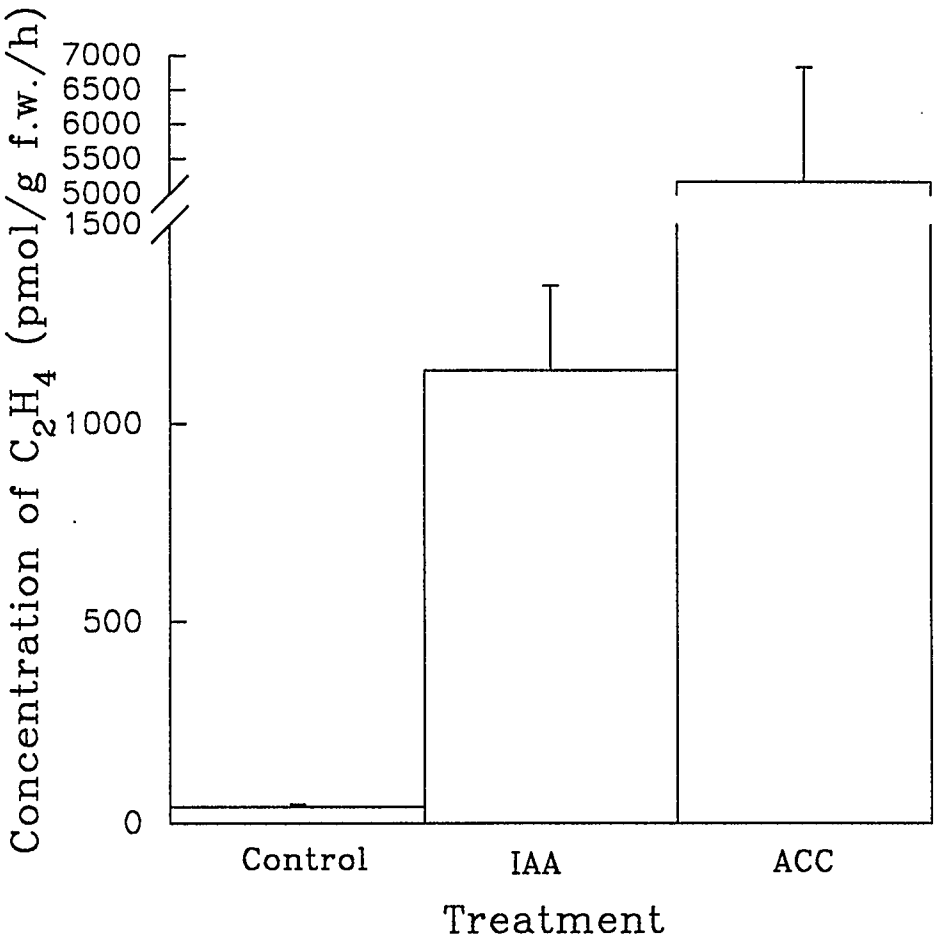


Figure 4.7 Effect of a dose of 100 µg/plant of IAA or ACC applied to the primary leaf bases of Xanthium seedlings on ethylene evolution by apical tissue 4 h later. Growth substances were applied at 1200 h and ethylene evolution was measured at 1600 h (the time when the inductive long night would have begun) the same day by GC with photoionization detection. Data represent the mean of four replicate samples (3 apical buds in each). Error bars represent 95% confidence intervals.

However, the IAA-induced inhibition of floral evocation was not reversed by STS treatment (figure 4.8).

It would thus appear that the floral inhibitory mechanisms of IAA and ACC are not the same, and are likely mediated by separate mechanisms. Three results tend to corroborate this conclusion: (1) IAA inhibition of floral evocation is not prevented (or reversed) by the presence of STS (a known inhibitor of ethylene action) while the floral inhibition induced by ACC is prevented (or reversed) by STS. This indicates that although IAA inhibition of flowering may be in part due to enhanced ethylene biosynthesis, that there is more than this to the IAA story. (2) Although exogenous application of either ACC or IAA stimulates ethylene evolution, each substance yields a very different maximum rate. (3) IAA has a profound effect on seedling morphology, whereas ACC does not.

A similar conclusion (e.g. IAA inhibition of flowering is not completely ethylene-mediated) was reached for Chenopodium rubrum (SDP) (Machácková et al., 1988; Machácková et al., 1989), tobacco (Zárský et al., 1990), and Pharbitis (Halevy et al., 1991). Thus another approach was taken to attempt to identify the mechanism of IAA-mediated inhibition of floral evocation. As stated above, flowering in both Chenopodium rubrum and tobacco is inhibited by exogenously applied IAA. Endogenous IAA levels in these systems during induction were found decrease (Machácková et al., 1988; Lozhnikova et al., 1988), as determined by fluorimetric detection (a less definitive method) of IAA level from partially purified methanolic extracts (the values may not be accurate). The basis for this decrease in endogenous IAA in both tobacco and Chenopodium rubrum was concluded to be due to the presence of increased levels of IAA-oxidases (Zárský et al., 1990), a class of enzymes which degrades IAA by oxidation.

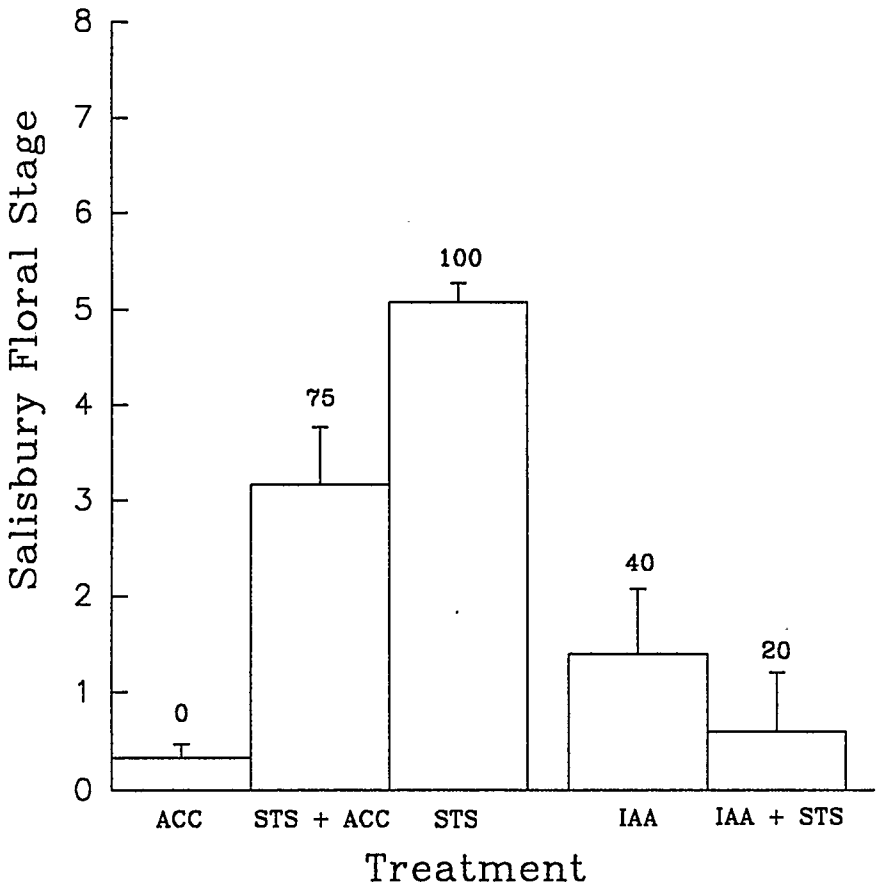


Figure 4.8 Effect of STS on IAA- or ACC-induced inhibition of floral evocation in Xanthium seedlings. Seedlings were dipped into freshly prepared STS (2.5 mM $AgNO_3$/10 mM $Na_2S_2O_3$ in 0.1% Activator™) 10 min prior to application of IAA or ACC at 100 µg/seedling. The IAA and ACC were applied to the primary leaf bases. Data represent the mean of five seedlings. Error bars represent 95% confidence intervals.

Based on their reasoning and on Japanese literature showing promotion of floral evocation in Pharbitis under non-inductive LD by certain phenolics and flavenoids (Umemoto, 1971; Shonozaki et al., 1987; Shonozaki et al., 1988), an approach using exogenous application of a competitive inhibitor (chlorogenic acid; CGA), for the cofactor site of this enzyme, as well as a cofactor of IAA oxidases (*p*-coumaric acid) was attempted (see figure 4.3). In essence it was postulated that the putative inhibitor of IAA oxidases (CGA) might reduce the flowering response in marginally-induced plants, possibly by increasing the in vivo IAA level. By comparison, exogenous application of the cofactor (*p*-coumaric acid) might "build" the flowering response by reducing the endogenous IAA level.

Neither of CGA nor *p*-coumaric acid influenced epicotyl or petiole elongation, but CGA did affect flowering in an almost opposite (from expected) manner (compare figures 4.9 and 4.10). For plants given a marginally-inductive long night, CGA strongly promoted floral evocation (up to 4 stages, but on average 3 stages; see figure 4.9), whereas *p*-coumaric acid had no effect on flowering (figure 4.10). In fact, the CGA promotion of flowering was linear in response to a log dose up to 3.3 µg/seedling. Above the 3.3 µg/seedling dose, floral stage was only marginally promoted, but 100% of the plants flowered (figure 4.9). In all three repeat trials CGA continued to show a strong promotion of floral evocation which was generally in excess of that exhibited by the $GA_{4/7/9}$ mixture (compare figure 4.9 and 3.14).

Given this unexpected result, an explanation is in order. A link between flowering and secondary metabolism has been reported by Zucker et al. (1965), Umemoto (1971), Taylor (1965) and Shonozaki et al. (1988). In Pharbitis, benzoic acid, L-phenylalanine and trans-cinnamic acid (the latter two being precursors of CGA) can replace a "high light followed by cold induction of flowering," photoperiod being non-inductive LD (Shonozaki

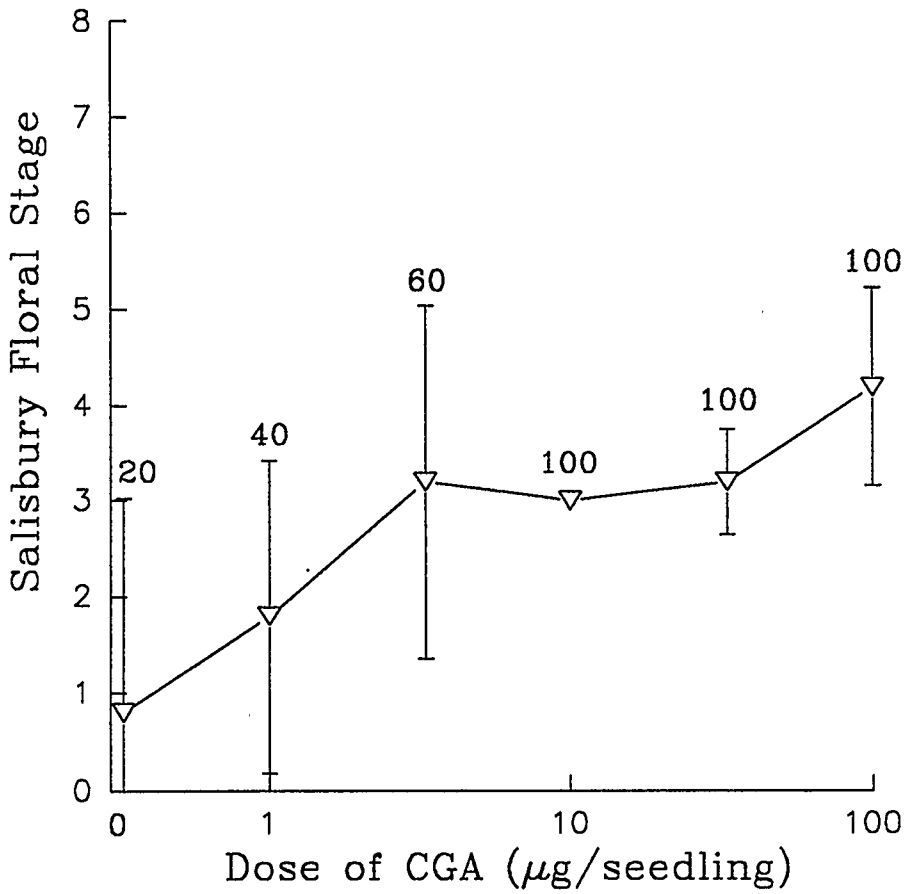


Figure 4.9 Effect of CGA on floral evocation in the Xanthium seedling bioassay. Doses of CGA were applied to the primary leaf bases at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings (Salisbury floral stages) dissected 9 days post-induction. Error bars represent 95% confidence intervals. Numbers above the bars indicate flowering quotients.

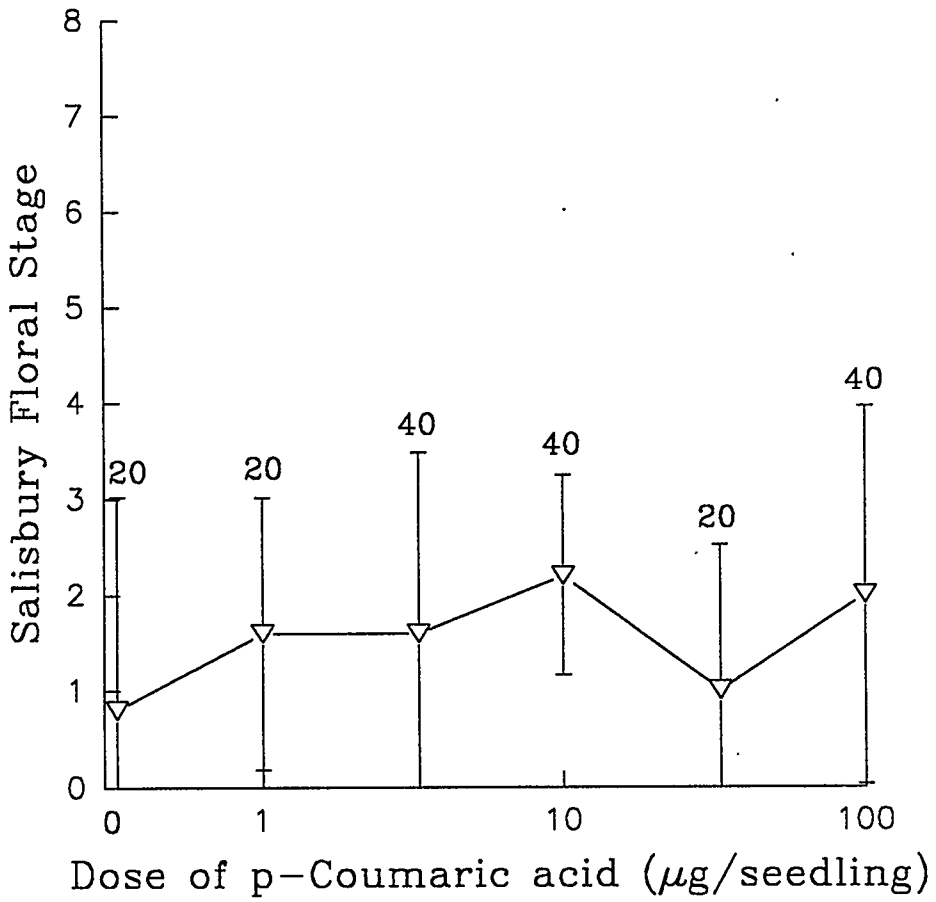


Figure 4.10 Effect of *p*-coumaric acid on floral evocation in the Xanthium seedling bioassay. Doses of *p*-coumaric acid were applied to the primary leaf bases at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings (Salisbury floral stages) dissected 9 days post-induction. Error bars represent 95% confidence intervals. Numbers above the bars indicate flowering quotients.

et al., 1987). It is known that when long nights are used to induce SDP, a high light period following induction will stabilize the floral stimulus (Salisbury, 1982). In Pharbitis, aminooxyacetic acid (an inhibitor of the enzyme phenylalanine lyase) suppressed flowering (apparently) by depressing CGA synthesis during induction (Shonozaki et al., 1987). Elevated levels of CGA are also associated with inhibition of flowering in tobacco (Zucker et al., 1965). In Xanthium, CGA was found to be the major phenolic present in leaves (Taylor and Zucker, 1968). However, based on my review of the literature, CGA does not appear to have been tested for florigenicity in the Xanthium system. An additional piece of circumstantial evidence suggesting a role for CGA in floral evocation is the fact that CGA content in the young Xanthium leaves is 3 to 5-fold higher than in older tissue (Taylor, 1968). As mentioned in the general introduction, it is the most rapidly expanding leaf which seems to be most sensitive to induction (and/or is the most effective leaf at synthesizing flowering stimulus).

Another possible link between florigenicity and phenolics or flavenoids may be suggested for the LD Lolium system where the class of ring A didehydro polyhydroxylated GAs are highly active (e.g. GA_{32}) (Pharis et al., 1990; Pharis et al. 1987; Evans et al., 1990) (see figure 4.3). The florigenicity of these polyhydroxylated GAs may be due to some structural motifs which are similar to those of CGA (i.e., multiple hydroxylations and presence of a double bond) (compare A (ii) and B on figure 4.3).

Now, to come back to IAA-inhibition of floral evocation, it is apparent that further experiments must be conducted to better determine the mechanism of IAA inhibition of flowering. These might include the co-application of IAA and a known inhibitor of auxin action/transport such as 2,5-dichloroanisole or TIBA. Such inhibitors would be expected

to offset the inhibiting effects of applied auxin on floral initiation (Bonner, 1949; Cooke, 1954 and refs. cited in Lang, 1965). In addition the effect of applied IAA on ACC synthesis and also of leaf-applied GAs and CGA on leaf and apex IAA synthesis (e.g. $GA_{4/7/9}$ mixture versus $GA_{1)}$ (and vice-versa) should be further studied. To this end tissue for the analysis of IAA and ACC following application of $[^2H_2]$-ACC and $[^{13}C_6]$-IAA has been collected. Such applications and the study of endogenous hormone levels following exogenous applications of these growth substances may shed light on the nature of the auxin inhibition of flowering and how IAA inhibition of flowering relates to floral inhibition induced by ACC.

Ethylene production by intact Chenopodium rubrum plants was found to be under photocontrol (Machácková et al, 1988; Machácková et al, 1989), although its role in photoinduction of flowering remains obscure. In several systems, including tobacco (Kasperbauer and Hamilton, 1978), Chenopodium rubrum and Chenopodium murale (SDP) (Machácková et al., 1989), ethylene is known to have an inhibitory effect on flowering. However, its role was not (until now) examined in Xanthium. In the SDP Chenopodium rubrum, and SD and LD strains of tobacco, endogenous ethylene levels decrease during photoperiodic induction (Machácková, 1988; Lozhnikova et al., 1988). In addition, the his-1 mutant of Arabidopsis, which is deficient in ethylene synthesis, bolts (elongates the flower stem) earlier than its wildtype counterpart (Guzmán and Ecker, 1990). By comparison, the ein1-1 and ein2-1 mutants of Arabidopsis, which are ethylene insensitive are slightly delayed in their bolting responses (Guzmán and Ecker, 1990). Lastly, in Chenopodium rubrum, flowering is inhibited by the application of IAA or ethylene (applied in the form of ethephon) (Khatoon et al., 1973). All these results point to the possibility of ethylene levels playing a role in the regulation of photoperiod induction of flowering. Although flowering was not studied by Woodrow et al. (1989),

one possible mechanism for the inhibition of flowering in Xanthium by IAA and ACC (possibly mediated by ethylene) may be the fact that treatment with ethephon (ethylene), as shown by Woodrow et al. (1989), can cause an alteration in partitioning of current photosynthate away from shoot and toward root sinks.

Further experiments with the Xanthium system could include treatment of plants during long night induction with ethylene to determine exactly when the plants are most sensitive, as well as determining the threshold concentration of ethylene at which the floral evocation becomes inhibited. Additionally, the mechanism whereby ethylene inhibits floral evocation requires further study. Several avenues may be pursued in this regard. Pearce et al. (1991) showed that ethylene inhibits the metabolism of $[^2H]GA_{20} \rightarrow [^2H]GA_1$ (e.g. inhibits 3ß-hydroxylation). An increased endogenous level of ethylene caused by long night induction may explain the decreased elongation growth of petiole and epicotyls for such plants. It is also possible that the 3ß-hydroxylation of $GA_9 \rightarrow GA_4$ is also inhibited at the apex in Xanthium. Hence, exogenous ethylene might lower the endogenous GA_4 level (by reducing its synthesis), thereby reducing the level of floral evocation under inductive long night. One could speculate that under a marginally long night the effect of endogenous ethylene on the 3ß-hydroxylase of GA_{20} may be greater than its effect on the 3ß-hydroxylase of GA_9. This assumes GA_1 might be floral inhibitory. These speculative hypotheses should be tested as time permits.

4.2 Inhibition by ABA

Application of s-ABA was significantly inhibitory to floral evocation (See figure 4.11), though the inhibition was only partial compared to that of IAA or ACC (compare figure 4.11 to figures 4.1 and 4.4). A maximum inhibition of 3 floral stages (and reduction

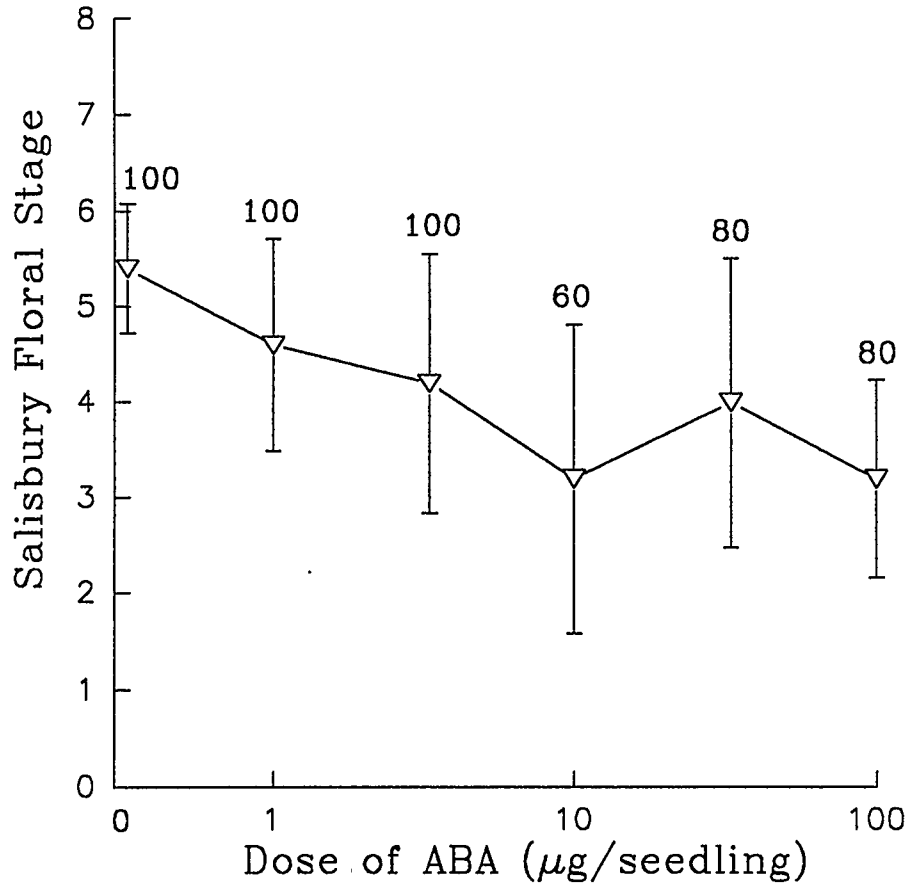


Figure 4.11 An example of s-ABA inhibition of floral evocation in the Xanthium seedling bioassay. Log doses of s-ABA were applied at 1200 h, 4 h before the beginning of the single inductive long night (16 h dark). Values represent the mean of 5 seedlings (Salisbury floral stages) dissected 9 days post-induction. Error bars represent 95% confidence intervals. Numbers above the bars indicate flowering quotients.

to 70% flowering) was obtained at either 10 or 100 µg/seedling. At the intermediate 33 µg/seedling dosage a slight promotion (NS) of flowering was observed compared to either the 10 or 100 µg/plant dosages. In all repeat experiments a similar trend was observed both in average Salisbury stage of floral development as well as in floral quotient. This result is consistent with the inhibitory effect of exogenously applied ABA noted for Lolium, Kalanchöe and Pharbitis (Evans and King, 1985; El-Antably and Wareing, 1966; Schwabe, 1972; Kamuro et al., 1990). The degree of inhibition in the Pharbitis system by s-ABA was absolute. However, its effect on Xanthium was only partial. Characteristic differences in the response to exogenously applied hormones between these species (as detailed throughout chapters 3 and 4), may also account for this partial inhibition in Xanthium, relative to total inhibition in Pharbitis.

Conclusions

All of ABA, ACC and IAA significantly inhibited floral evocation in Xanthium, although the inhibition by s-ABA was only partial compared to that by ACC or IAA at the highest dose tested. Exogenously applied ACC inhibition of flowering appears to be mediated by ethylene, since exogenous application of ACC raised the rate of ethylene production 100-fold over that of the control plants. In addition, STS prevented (or reversed) the ACC-induced inhibition of floral evocation. The inhibition of flowering by IAA, however, appears to be distinct from that of ACC, since STS did not prevent (or reverse) this inhibition. Even so IAA inhibition may be mediated, in part, by ethylene by way of IAA-stimulated ACC synthesis (Yoshi and Imaseki, 1981; Yu et al., 1979).

As demonstrated in the previous chapter, GAs promote floral evocation in marginally-induced Xanthium. In carnation flowers it has been recently demonstrated that

GA_3 treatment can reduce endogenous ACC content (Saks and Van Staden, 1993). While this is the only system which, to date, has been shown to behave in this way, one hypothesis of how applied GA "builds" a floral apex might be through an inhibition of ACC synthase. This assumes that high endogenous ethylene is a negative floral evocation factor in marginally-induced Xanthium.

A few investigators have tested auxin and several weak acids, which are known to inhibit photoperiodic response, and to also permeate cells thus acidifying the cytosol (Halevy et al., 1991; Friedman et al., 1990). This was done to make the general point that acidification of the cytosol might be the means by which auxin evokes its inhibition. Although a cytoplasmic effect on cytosol acidity by IAA and ACC cannot be ruled out in this study, it is unlikely that this is the mechanism whereby both elicit their effect. First, throughout the course of this thesis research, ABA, ACC, CGA, *p*-coumaric acid, IAA, and GAs (all weak acids) were exogenously applied and found to have substantially differing effects. Some were significantly promotory of floral evocation under marginally-inductive long nights, whilst others were significantly inhibitory under 16 h fully-inductive long nights. From these results it seems likely that these exogenously applied substances must gain their effect by merit of their structure, rather than simply by means of acidifying the cytosol. It must also be noted that the dose of weak acids (GAs, IAA, etc.) tested in the present study was 10 to 100-fold lower than those used by Halevy et al., (1991) and Friedman et al. (1990). Additionally, Halevy et al. (1991) determined the maximum inhibition by the non-hormonal weak acids, when compared with auxin, was at best 30% of the auxin inhibition. In conclusion, inhibition of floral evocation by IAA, ACC and ABA are likely not by way of acidification of the cytoplasmic pH, though this can not be ruled out.

CHAPTER 5

QUANTITATION OF ENDOGENOUS GIBBERELLINS, INDOLE-3-ACETIC ACID, AND ABSCISIC ACID IN PHOTOPERIODICALLY-INDUCED LEAF AND APICAL TISSUE OF XANTHIUM STRUMARIUM AND RELATION OF THESE LEVELS TO THE EFFECT OF EXOGENOUSLY APPLIED GIBBERELLINS, INDOLE-3-ACETIC ACID AND ABSCISIC ACID

Introduction

Hormonal physiology can be researched by three distinct, yet overlapping methods: (1) studies comparing biological activities of exogenously applied compounds, (2) quantitative analyses of endogenous hormones, and (3) hormone metabolism studies. Through the exogenous application of ABA, ACC (an immediate precursor of ethylene), several GAs and IAA, (cf. chapters 3 and 4) I obtained results indicating that hormone metabolism may change during the long night inductive period and subsequent to it. For this reason, and also since the endogenous GAs in Xanthium remain uncharacterized, the profile of endogenous GAs was studied at selected times for plants given two consecutive inductive 16 h long nights, and also for parallel LD control plants. Additionally, the endogenous IAA and ABA levels were analyzed at these same selected times. The GA profiles were obtained for both leaf and apical region tissue since (a) the leaf is the unit of perception of the long night inductive stimulus, and (b) the apex is the unit of morphological change (Mukherjee, 1974). Harvest times for tissue included, (i) immediately prior to the first long night at 1600 h, (ii) at dawn after the first long night (0800 h), (iii) just prior to the second long night (1600 h) and (iv) at dawn after the second long night (0800 h) (see figure 5.1).

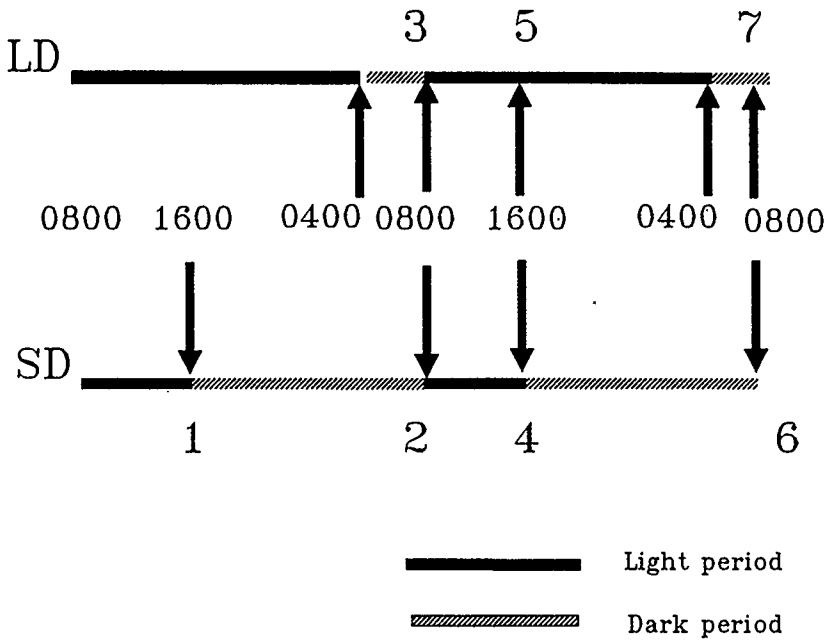


Figure 5.1 Harvest times of tissue (1-7) for analysis of endogenous GAs in leaf and apical tissue of Xanthium.

It has been previously reported, by several investigators, that the application of extracts from continuously induced leaves of Xanthium could cause flowering under completely non-inductive conditions (Hodson and Hamner, 1970; Lincoln et al., 1964; Roberts, 1951), although most attempts to repeat this work have ended in total or partial failure (Salisbury, 1982). In the present study, several experiments were conducted in attempt to repeat this work.

Methods

5.1 Plant material for extraction

Tissue for quantitative analysis by GC-MS was germinated as above (chapter 2, methods), however, after 9 days the seedlings were transplanted into 4" pots to eliminate all possibility of nutrient deficiency during the experiment. Prior to photoperiodic treatment the plants were grown for 6 weeks . Treatments were of seven types:

(1) LD (20 h light/ 4 h dark period) time 0 control; plants harvested prior to long night (SD) treatment.

(2) long night (SD) treatment with tissue harvested at dawn immediately following the first single long night (e.g., 16 h dark→harvest).

(3) LD control as in (1), but with tissue harvested parallel to above treatment (2).

(4) long night (SD) treatment with a single long night and a subsequent 8 h photoperiod; tissue harvested at dusk immediately following the 8 h photoperiod (16h dark→8 h light→harvest).

(5) LD treatment, this is a 3rd control harvest, parallel to treatment (4).

(6) Two inductive long nights (SD), tissue harvested at dawn immediately following the second long night (16 h dark→8 h light→16 h dark→harvest).

(7) LD treatment, this is a 4th control harvest parallel to treatment (6) above.

Figure 5.1 is a schematic diagram illustrating the harvest times. These harvest times were chosen to enable quantitation of changes in GA levels and metabolism following photoperiodic induction. While a single long night is known to be sufficient for absolute induction, a second long night will "firm-up" the inductive signal (Salisbury, 1963). All treatments (e.g. harvested tissue) consisted of 12 plants each, replicated three times. Prior to treatment, the plants were defoliated to the single most rapidly expanding leaf (generally the third visible leaf from the apex) the day prior to inductive treatment. The inductive dark period was provided by moving plants for induction to a dark room maintained at 23°C at 1600 h, and then transferring them back to the LD chamber at 0800 h the next day. At the specified treatment time (figure 5.1) the shoot tip (apical tissue), petioles and leaf blades were harvested and quickly frozen in liquid N_2. Shoot tips had all visible leaf primordia removed during harvest. Tissue was stored at -70°C prior to freeze-drying. Thereafter, lyophilized tissue was stored at -20°C.

5.2 Extraction of shoot tips

Apical region tissue (termed apex, hereafter) for hormone analysis was extracted utilizing the methods of Koshioka et al. (1983). The lyophilized shoot tips (2-3 mg dry weight) were ground with liquid N_2 in a glass homogenizer and extracted in 5 mL of cold 80% aqueous methanol (MeOH) at 3 to 5°C. This solution was filtered under vacuum through Whatman No. 1 paper and the tissue residue re-extracted with another 5 mL of 80% aqueous MeOH two more times. The pooled extracts (30 mL) were then adjusted to pH 6.5. At this point, 5 ng of $[^2H_2]GA_{1,3,5,8,20}$, 20 ng of $[^2H_2]GA_{4,7,9}$, 50 ng of $[^2H_2]GA_{19}$, 100 ng of $[^2H_2]$ kaurenoic acid (KA) and $[^2H_2]$ kaurene (K), and 20 ng of each of $[^{13}C_6]$IAA and $[^2H_6]$ABA were added as quantitative internal standards for gas chromatography-mass spectrometry-single ion monitoring (GC-MS-SIM). In addition, 100,000 dpm (1670 Bq) of $[^3H]GA_1$ and $[^3H]GA_4$ and 20,000 dpm (333 Bq) of $[^3H]$IAA and ABA, respectively were added for determination of recovery efficiency during the purification process and to help locate various fractions after HPLC.

5.3 Extraction of leaves

Lyophilized leaf tissue (0.5 g per sample, single leaf) was ground with liquid N_2 in a mortar with a pestle and extracted in 10 mL of 80% aqueous MeOH at 3 to 5°C. As above, the solution was filtered and the tissue residue re-extracted two more times with another 10 mL of 80% MeOH. The pooled extracts (30 mL) were then adjusted to pH 6.5. At this point, 100 ng of $[^2H_2]GA_{1,3,4,5,7,8,9,19 \text{ and } 20}$, 100 ng of $[^2H_2]$KA and $[^2H_2]$K, and 100 ng of each of $[^{13}C_6]$IAA and $[^2H_6]$ABA were added as quantitative internal standards for GC-MS-SIM. In addition, 100,000 dpm (1670 Bq) of $[^3H]GA_1$,

[^{3}H]GA_4, [^{3}H]IAA and [^{3}H]ABA were added for determination of recovery efficiency during the purification process.

5.4 Purification of shoot tip extract

The methanolic hormone extract was passed through a syringe barrel column (1.5 cm i.d. X 10 cm) packed with 1.0 g of preparative grade C_{18} matrix (55-105 μm particle size, Prepak 500/C_{18}™, Waters Scientific). This column was termed 'C_{18}-PC' (Koshioka et al., 1983). It was first equilibrated by elution with 3 X 20 mL of 100% MeOH, then equilibrated by elution with 3 X 20 mL of pH 6.5 80% aqueous MeOH. The MeOH extract for GA analysis was then passed through the C_{18}-PC and the eluate diluted to 20 mL with an aqueous MeOH rinse. Plant pigments and non-polar substances (i.e., K and KA) are retained on this column and are eluted subsequently with a 100% MeOH wash. This MeOH wash was stored at -70°C for subsequent "GA precursor" analysis. These were, however, not examined in this study. Duplicate aliquots of 0.25 mL were taken from the total eluate as well as from the MeOH wash for liquid scintillation counting to determine the recovery of the radioactive internal standards. Then the C_{18}-PC eluate was taken to dryness in vacuo on a rotary flash evaporator at 35°C.

5.5 Purification of leaf extract

The leaf extract was also purified using a C_{18}-PC. However, the syringe barrel column (3 cm i.d. X 11 cm) was packed with 2.0 g of preparative grade matrix. This column was equilibrated with 3 X 25 mL of 100% MeOH, followed by 3 X 25 mL of pH 6.5 80% aqueous MeOH. Thereafter, the MeOH extract for GA analysis was passed

through the C_{18}-PC and the eluate diluted to 40 mL with an aqueous MeOH rinse. Non-polar substances, retained by the column, were eluted with a 100 mL 100% MeOH wash. Duplicate aliquots of 0.5 mL were taken from the total eluate as well as for the MeOH wash for liquid scintillation counting to determine the recovery of the radioactive internal standards. Both the 80% aqueous MeOH eluate and the 100% MeOH wash were taken to dryness in vacuo on a rotary flash evaporator at 35°C.

The dried 80% aqueous MeOH eluates for the leaf extracts were subjected to a further purification step prior to HPLC chromatography. A short SiO_2 partition column (5 g silica gel deactivated with water) was prepared as a slurry in 95:5 (v/v) hexane:ethyl acetate (EtOAc) (both 0.5 M formate saturated). Then the leaf sample, which had been dried on 1.0 g Celite, was layered over the silica gel. Elution with 80 mL of 95:5 (v/v) EtOAc:0.5 M formate-saturated hexane yielded free GAs and some of the less polar GA-glucosyl esters. Putative GA glucosyl ether conjugates and polyhydroxylated GAs (i.e., GA_{32}) were eluted from the column with a subsequent 100 mL of a 100% MeOH wash. Again duplicate 1 mL aliquots were taken from all fractions to establish radioactive internal standard recovery. Both the free GA fraction and the fraction which contained most GA glucosyl conjugates were taken to dryness in vacuo on a rotary flash evaporator at 35°C prior to reversed phase C_{18}-high performance liquid chromatography (HPLC).

A single reversed-phase C_{18}-HPLC run was then used to separate free GAs and GA glucosyl conjugates (Koshioka et al., 1983). Separation of these two classes will not be absolute. The dried eluate from the silica gel partition column was then chromatographed on a 10-73% MeOH gradient-eluted Waters Associates (Mississauga, Ontario) reversed-phase μ-Bondapak C_{18} column (3.9 mm i.d. X 300 mm) at a flow rate of 2 mL per min (Table 5-1). The separation of GAs using this column was generally on the basis of polarity (degree of hydroxylation and location of the hydroxyl(s)). Two min

Table 5-1 HPLC columns and gradients for separation of GAs, ABA and IAA.

Column	Solvents	Time (min)	Flow rate (mL/min)	Volume (mL/fraction)
C_{18}-μ-Bondapak	10% MeOH→	10	2	4
	10% MeOH→73% MeOH	30	2	4
	73% MeOH→	10	2	4
	100% MeOH→	10	2	4
Nucleosil	99.9% MeOH	50	1	2
	0.1% HOAc	(Isocratic)		

(4 mL) fractions were collected and aliquots withdrawn to locate the radioactive internal standards. A separate C_{18}-HPLC run was also performed using 20,000 dpm (333 Bq) of each of [^{3}H]$GA_{8,1,4,5,9}$ standards (termed external standard retention time (R_t)).

The HPLC fractions from the shoot tip extract were bulked into four groups based on internal and external [^{3}H]GA R_t (see figure 5.2):

i) GA_8 (fr. 11-13)

ii) IAA (fr. 18-21)

iii) $GA_{1/3}$ (fr. 22-26)

iv) $GA_{5/20}$, ABA and JA (fr. 29-33)

v) $GA_{4/7/9}$ (fr. 34-41)

The leaf HPLC fractions were bulked into four groups based on internal and external [^{3}H]GA retention times (R_t) (figure 5.2):

i) GA_8 (fr. 11-13)

ii) IAA/$GA_{1/3}$ (fr. 18-26)

iii) $GA_{5/20}$, ABA and JA (fr. 29-33)

iv) $GA_{4/7/9}$ (fr. 34-41)

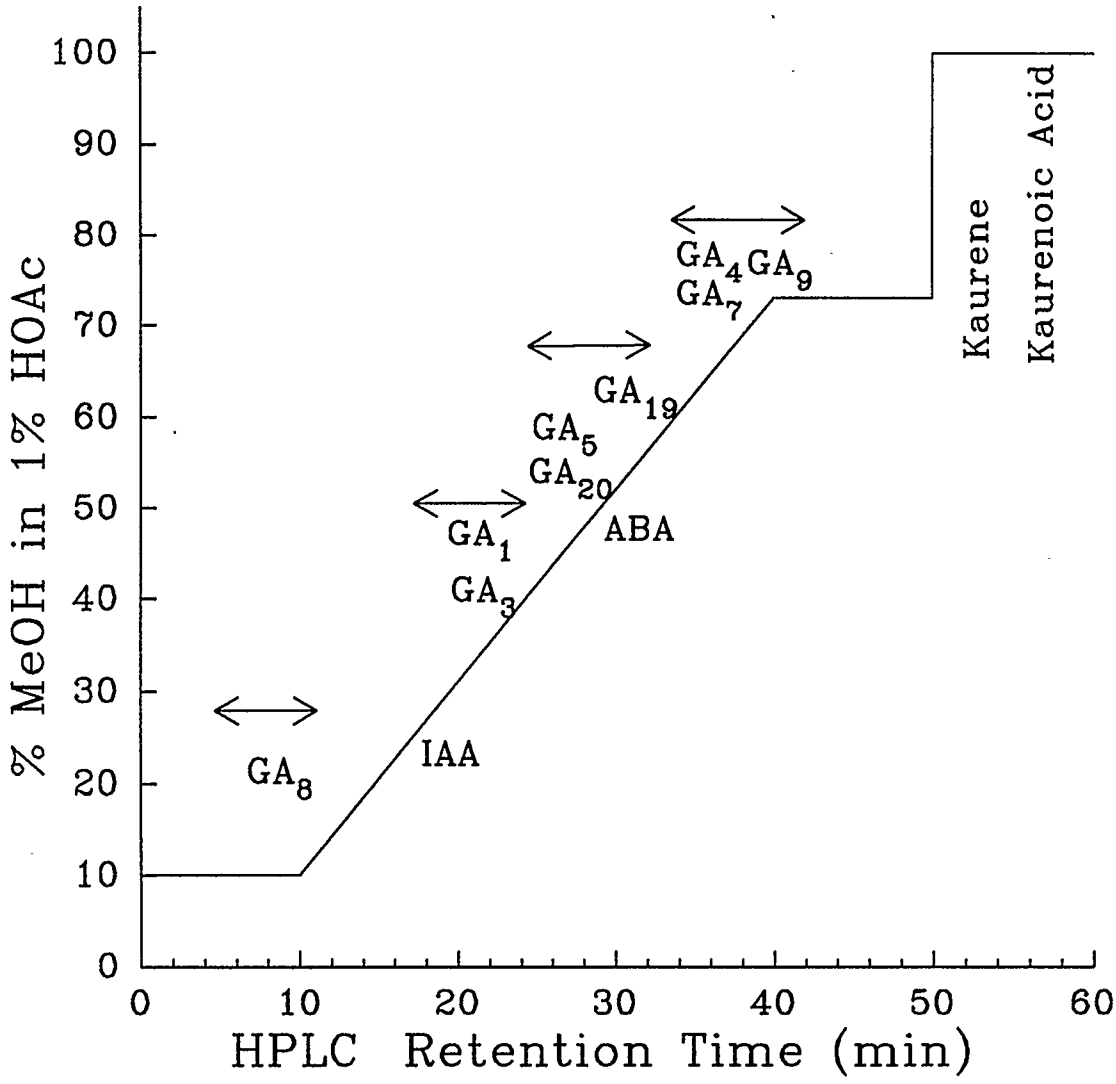


Figure 5.2 Chromatographic separation of free (acidic) plant hormones following 10-73% MeOH gradient-eluted reversed phase C_{18}-HPLC. The R_ts were determined by combined use of [^{3}H] external and internal standards, Tan-ginbozu dwarf rice bioassay (GA) and detection by GC-MS-SIM. Horizontal bars represent regions that were grouped for further purification on an isocratic-eluted Nucleosil HPLC.

Each of the four HPLC groupings of leaf samples and shoot tip groupings ii, iii and iv were then chromatographed on a Nucleosil [$N(CH_3)_2$] HPLC column (4.6 mm i.d. X 150 mm) packed with 5 μm particles (Alltech Associates Inc., Deerfield, IL) using an isocratic gradient of 99.9% MeOH and 0.1% acetic acid at a flow rate of 1 mL per min (Takahashi et al., 1986) (Table 5-1). One min fractions were collected and aliquots taken to locate the radioactive internal standards by liquid scintillation spectrometry. Due to the substantial drift of R_ts on Nucleosil HPLC, separate runs were also performed prior to and subsequent to each group of natural samples using 20,000 dpm (333 Bq) of [^{3}H]GA_8 or [^{3}H]$GA_{1,3}$ or [^{3}H]$GA_{5,20}$ or [^{3}H]$GA_{4,7 \text{ and } 9}$ as external standards respectively. The Nucleosil HPLC purification step separated GAs in relation to functional groups (methyl, delta lactone, aldehyde, carboxyl and hydroxyl(s)) and was used immediately before GC-MS-SIM analysis.

5.6 Bioassay

After each round of chromatography the residues of all HPLC fractions were analyzed using a modified Tan-ginbozu dwarf rice microdrop bioassay to localize bioactive GAs (Murakami, 1972). Oryza sativa L., cv. Tan-ginbozu seeds were soaked for 24 h in a 1 ppm solution of technical grade Uniconazole (S-3307) (Nishijima and Katsura, 1989) at room temperature. A further 24 h soaking of the seed was carried out in running water at 32°C. Germinated seeds were planted in 1% water agar media and incubated for 48 h at 32°C, at nearly 100% RH under continuous light. Aliquots of post-C_{18} and post-Nucleosil HPLC samples were applied to the growing leaf of the rice seedlings in 0.5 μL or 1.0 μL of 95% EtOH. The seedlings were incubated for a further 48 h at 32°C under high humidity. Following this incubation the elongation of the second leaf sheath was

measured. The elongation of the second leaf sheath is a semi-quantitative measure for bioactive GAs present in a sample. Although relative levels of such GA bioactivity may be determined by relating elongation from test fractions to GA_3 external standards, the bioassay in this case was used only to locate bioactive GA fractions (which were composed of endogenous GAs, or endogenous GAs plus the $[^2H_2]$GA internal standards). These fractions were then quantified by GC-MS-SIM (see below).

5.7 Quantitation of endogenous levels of gibberellins

Approximate groupings from the Nucleosil HPLC fractions containing the GAs for GC-MS-SIM analysis were made using a combination of external standard R_t, the rice bioassay data and relative R_t data for these GAs (Takahashi et al., 1986). Fractions for GC-MS were transferred into 1 mL Reacti™-vials and dried under a flow of N_2 at room temperature. Fractions were then dissolved in 10 µL of 100% MeOH and 100 µL of ethereal diazomethane (CH_2N_2), sealed and allowed to react for 30 min at room temperature. The methylated samples were subsequently dried with N_2 and silylated with 50:50 pyridine:BSTFA with 1% TCMS (Pierce Chemical Co.). Derivatization was carried out at 70°C for 30 min. Samples were concentrated to 10 µL with a gentle flow of N_2 and a 1 µL sample injected into a Hewlett-Packard model 5890 Series II GC fitted with a DB-1 15N capillary column (0.25 mm i.d., 0.25 µm film thickness; J and W Scientific) directly interfaced to a model 5970A Series MSD. The GC temperature was programmed from 60°C to 195°C at 15°C min, immediately followed by a 5°C ramp to 275°C. Each GA was analyzed by SIM of three characteristic ion pairs. Endogenous GA levels were quantitated using the ratio of these ion pairs, correcting for the contribution of

[^{2}H]ions to [^{1}H]ions and vice versa. Data analysis was via the integration functions of the Hewlett-Packard 5970 MS Chemstation.

The IAA- and ABA- containing fractions were collected separately, as determined from radioassay of Nucleosil fractions from groupings (ii) and (iv). These regions were methylated as described above, dried under a gentle flow of N_2 with hand warming (very important for IAA methyl ester which readily sublimes) and then dissolved in 10 µL of n-hexane. Of this, a 1 µL aliquot was injected into a DB-1701 15N column (0.25 mm i.d., 0.25 µm, film thickness; J and W Scientific) fitted on the same GC and MSD described above. The GC temperature was programmed from 60°C to 165°C at 20°C/min, immediately followed by a 5°C ramp to 230°C. Endogenous levels of IAA and ABA also were analyzed by comparison of three ion pairs, as described above for the GA samples, correcting for the contribution of heavy isotope to natural isotope and vice versa.

5.8 Xanthium leaf extract applications

A methanolic extract (80% MeOH in water) was prepared, as described above (5.3), from 0.5 g of 3 month old continuously induced, by 21 long nights, lyophilized Xanthium leaves (one leaf). This extract was dried under reduced pressure on a rotary flash evaporator. The residue was subsequently dissolved in an appropriate amount of EtOH to achieve extract doses of 1/100, 1/200 and 1/400 (e.g. fraction of leaf dry weight to be applied to the seedlings). Ten µL of each of these dilutions was applied to groups of 10 seedlings. Groups of 5 seedlings from each application were subjected to two different photoperiods: (1) non-inductive photoperiod (20 h light/ 4 h dark period), and (2) a single 12 h marginally-inductive dark period followed by growth under non-inductive photoperiod (20 h light/ 4 h dark period) until dissection. Nine days following marginal-

induction, apices were dissected and scored according to the Salisbury floral stage system (1955).

Since the application of this extract failed to induce flowering under non-inductive conditions several other methods were used in further attempts to induce flowering under non-inductive conditions. These included: (i) application of the extract in DMSO, (ii) injection of the appropriate induced leaf extract dilution and (iii) uptake of the extract as an aqueous solution by a stem-flap.

Results and Discussion

5.1 Endogenous GAs to Xanthium

Characterization of the endogenous GAs in Xanthium, was an important first step since these had not previously been assessed. Using the methods described above for the analysis of free GAs, GA_1, GA_4, GA_8, GA_9 and GA_{20}, by GC-MS-SIM were found to be endogenous to Xanthium apical tissue with GA_1, GA_3, GA_4, GA_8, GA_{19}, GA_{20} and GA_{29} being endogenous in leaves (see Table 5.2). Although analyses of the level of GA_3 and GA_9 were attempted, in most cases their levels were too low for quantitation. In all but one case, GA_5 was non-detectable as was GA_7 in all analyses. The fact that these GAs were found to be endogenous to the Xanthium system suggests that both the early-13-hydroxylation pathway and the early-non-hydroxylation pathways are present in leaf tissue (see figures 5.3 and 5.4), but since GA_{19} was absent from apices, only the early-non-hydroxylation pathway may be present in apical region tissue (figures 5.4).

Table 5-2 Relative intensities of characteristic m/z ions after GC-MS-SIM for MeTMSi derivatives of GAs from 21-day-induced Xanthium leaves.

Identified compound	Kovat's retention index (KRI)	Diagnostic ions (m/z) with % abundance in sample				
GA_1		Ion	357	376	448	491	506
	2674		4.2	15.0	16.8	9.7	100
GA_3		Ion	387	431	445	489	504
	2696		24.2	7.8	8.4	28.1	100
GA_8		Ion	379	448	504	579	594
	2819		18.5	26.0	2.0	6.4	100
GA_{19}		Ion	374	402	434	462	481
	2610		53.5	50.8	100	6.1	11.9
GA_{20}		Ion	343	359	375	403	418
	2461		1.3	15.2	74.1	7.8	100
GA_{29}		Ion	447	465	477	491	506
	2692		13.1	4.5	10.9	16.1	100

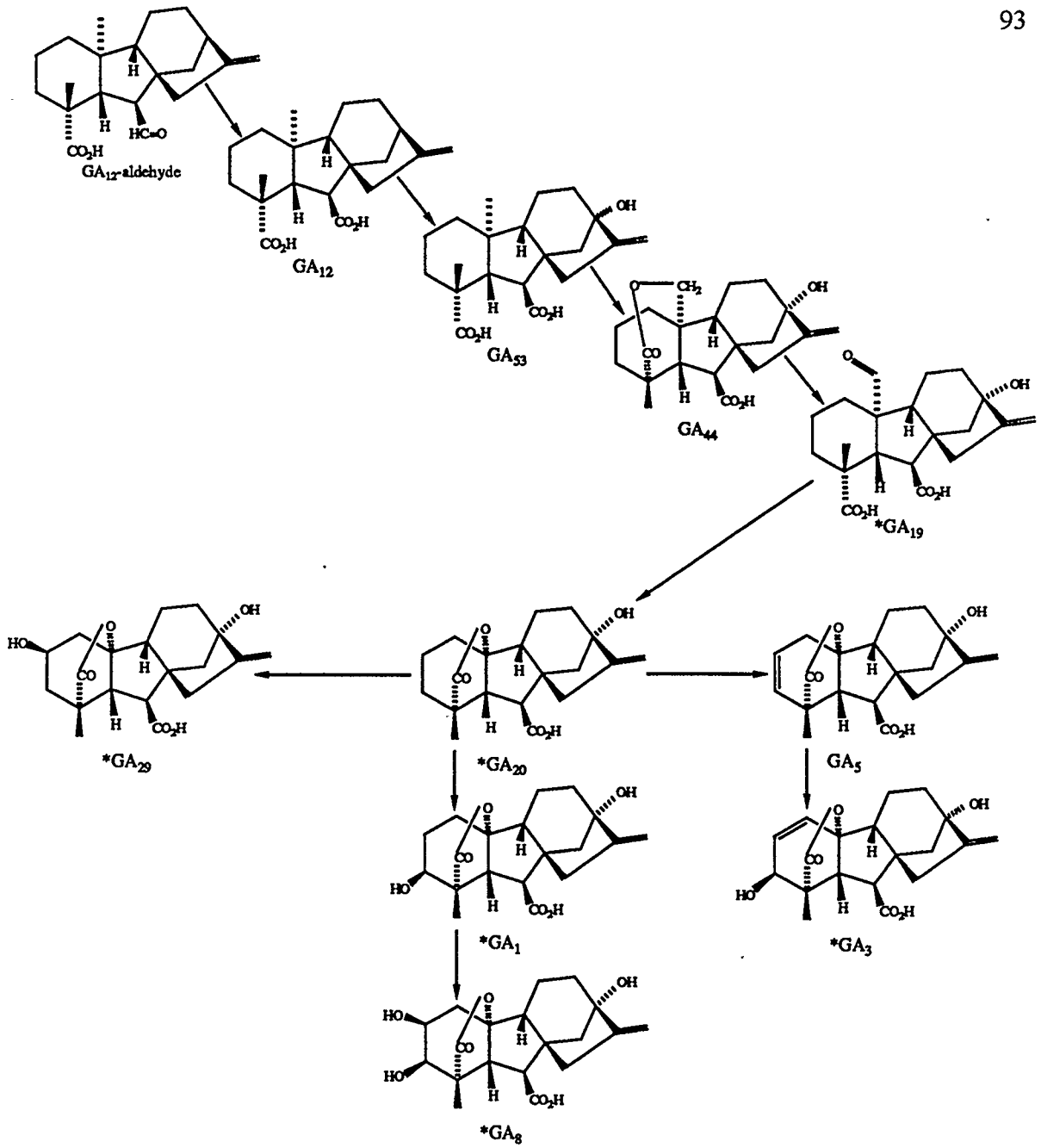


Figure 5.3 Structures and the early-13α-hydroxylation GA biosynthetic (metabolic) pathway from GA_{12}-7-aldehyde to the C_{19} GAs for Xanthium based on known endogenous GAs, feeding experiments and known pathways from other species. Endogenous GAs to the Xanthium system are denoted by a *.

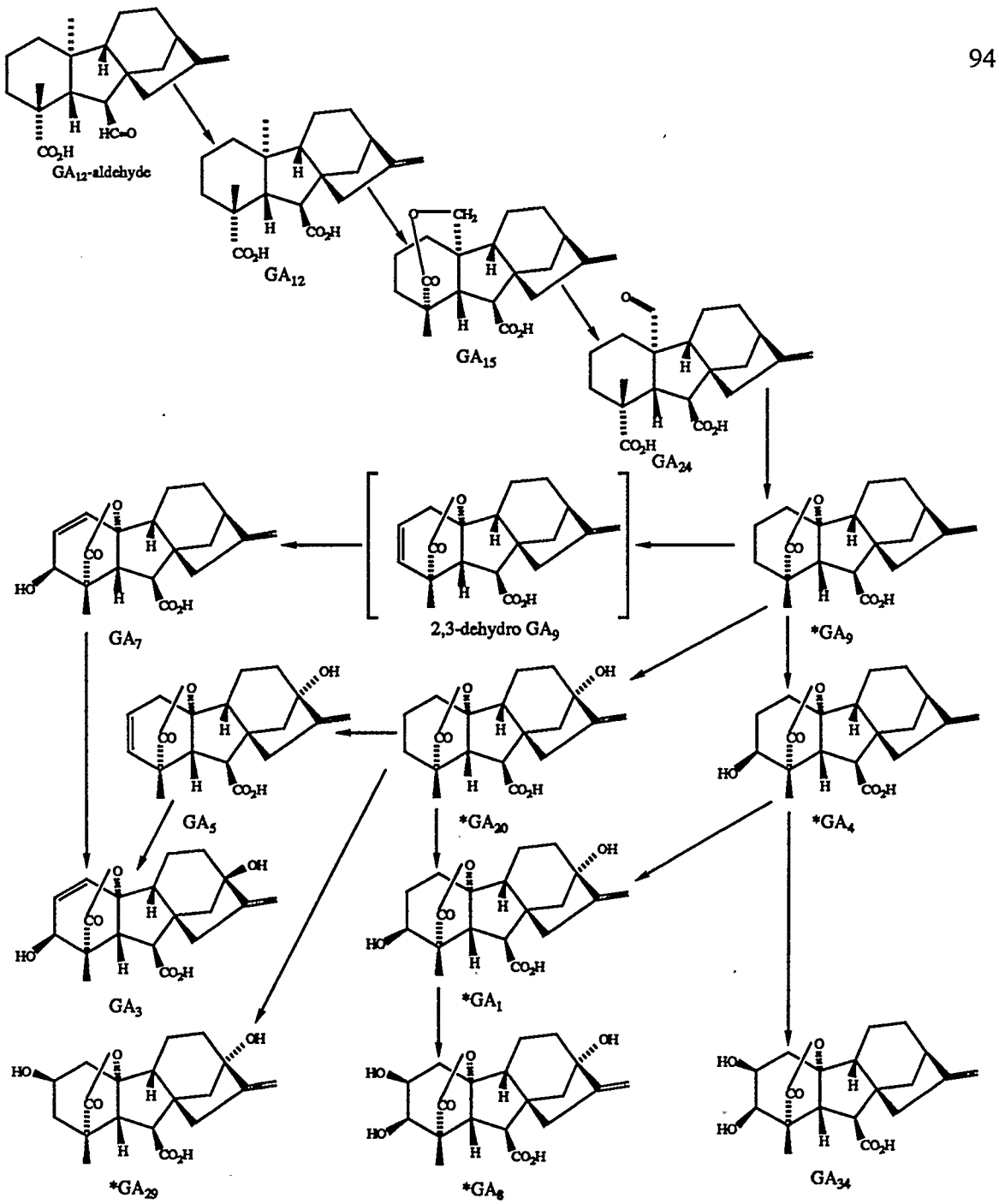


Figure 5.4 Structures and proposed early-non-hydroxylation biosynthetic (metabolic) pathway from GA_{12}-7-aldehyde to the C_{19} GAs for Xanthium based on known endogenous GAs, feeding experiments and known pathways from other species. Endogenous GAs to the Xanthium system are denoted by a *.

5.2 Endogenous levels of GAs in leaf tissue

In several systems induced to flower by either photoperiod or vernalization [e.g., spinach (Zeevaart, 1971b), Agrostemma (Jones and Zeevaart, 1980a; Jones and Zeevaart, 1980b), Lolium (Pharis et al., 1987) and Thlaspi (Metzger and Dusbabek, 1991), changes in endogenous GAs or GA-like substances have been found to occur. In the present study, the relative levels of endogenous GAs in leaves correlate roughly (e.g. GA_4 is highest) with the efficacy rank of exogenously applied GAs on floral evocation, as well as on epicotyl growth. Of the endogenous GAs to the Xanthium system, GA_1, GA_4 and GA_8 show the most appreciable difference between long night (SD-induced) and LD (control) leaves (see figure 5.5). Additionally, GA_1, GA_3 and GA_8 tend to follow a rhythm (which may have a period greater than the 48 h sampling period), in that their levels were lower at dawn (after the dark period) than at dusk (after the light period) (figure 5.5). Increased inactivation (2ß-hydroxylation and/or conjugation), or increased degradation and/or decreased GA synthesis during the dark period, compared to that in the light, may account for this rhythm. Or, the apparent rhythm may result from the events associated with leaf development (expansion) since the leaves analyzed were those which were most rapidly expanding.

Key changes in GA levels in leaves under various photoperiod treatments, are thus superimposed on changes in GA levels associated with leaf development. These include a GA_1 decline in long night (SD) induced leaves, compared the LD control leaves (figure 5.5). At dawn after the first long dark period, the level of GA_1 is approximately equal to that of the LD control (this dawn similarity is a general rule for all GAs which were analyzed). However, during the light period (8 h) before the second inductive long night, the level of GA_1 is even lower (significantly lower than that of the LD controls) and

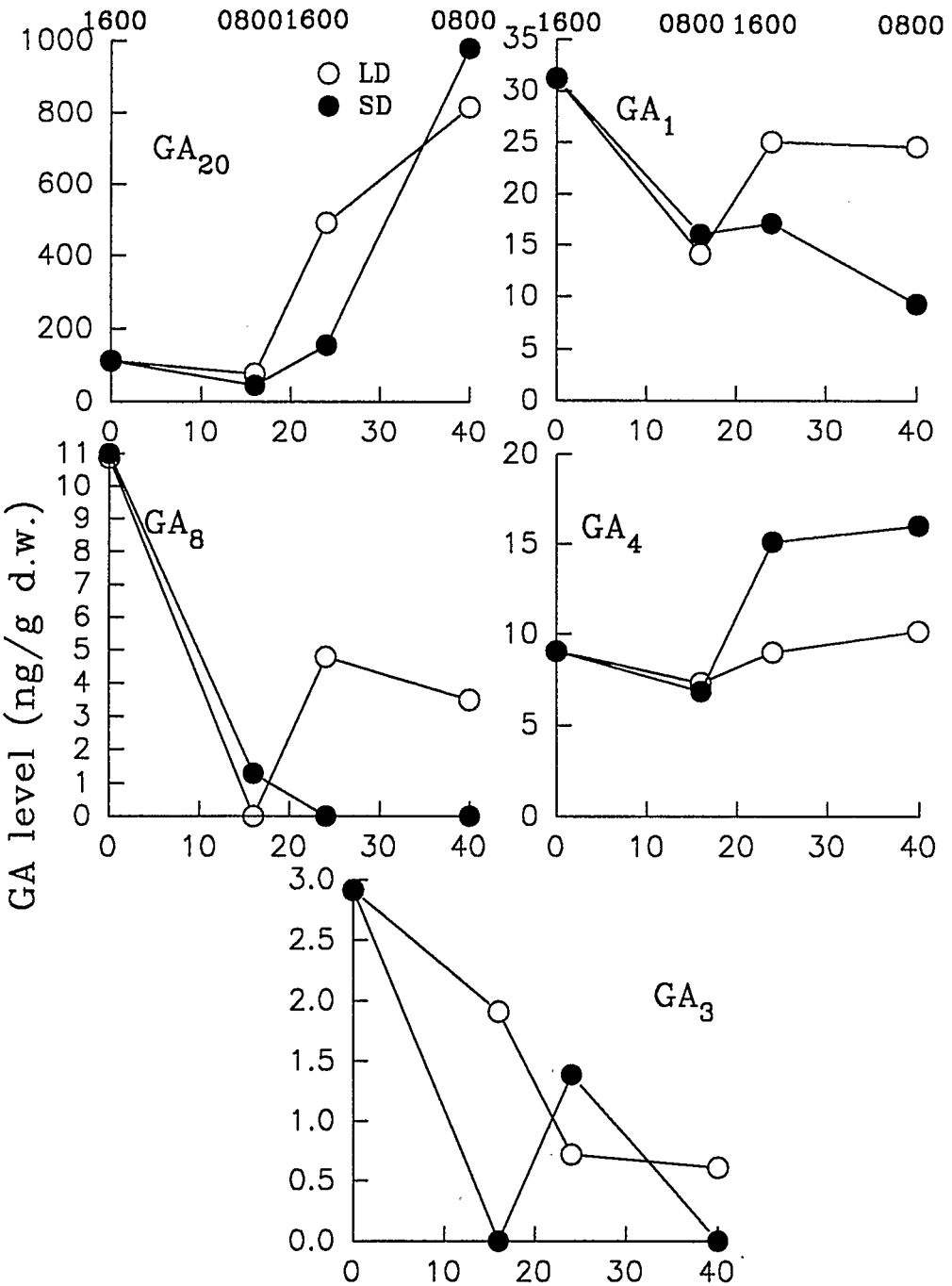


Figure 5.5 Gibberellin $A_{1, 3, 4, 8 \text{ and } 20}$ levels (ng/g d.w.) extracted from the most rapidly expanding leaf of induced (two 16 h dark periods within the 40 h) and non-induced (20 h LD) Xanthium plants (6 weeks old). Values represent results of a single experiment typifying these changes quantitated by GC-MS-SIM. Note the different y-axis scales for the GAs.

remains lower thereafter. The same general trend was observed for endogenous GA_8 (GA_8 is a 2ß-hydroxylation inactivation product of GA_1) levels in induced leaves (figure 5.5). However, during the light period (8 h) GA_8 levels in LD leaves increased, whereas GA_8 in SD leaves decreased. At dawn the second day, the level of GA_8 in the SD leaves remained much lower than that of the LD plants. Hence, a sequence of 2 long nights appreciably reduced the levels of both GA_1 and GA_8.

Since internode and petiole growth were also significantly reduced in SD (long night) plants compared to in the LD controls (see chapter 4 results), it might well be expected that the levels of GA_1 and/or GA_3 in leaves might be reduced after long nights (SD), compared to LD controls. Indeed, the GA_1 results fit well with such a thesis. The level of GA_3 (which could also be an "effector" of elongation growth) in young leaves is 10-fold lower than that of GA_1. Thus, the predominant effect on elongation of the epicotyl and petiole organs is likely to be mediated by GA_1 (assuming it to be the endogenous effector) (fig 5.5). However, the level of GA_3 decreases more rapidly than the level of GA_1 in the long night (SD)-induced leaves than in the LD leaves. At dawn on days 1 and 2, the level of GA_3 is much lower in the SD leaves (not detectable) than in the LD leaves.

From the early-13-hydroxylation pathway, GA_{20} (by 3ß-hydroxylation) is the precursor of GA_1, whereas for the non-hydroxylation pathway, GA_4 or GA_{20} via GA_9 its precursor (by 13α-hydroxylation) (see figures 5.3 and 5.4). Since GA_1 levels from SD leaves are lower than that from LD leaves, it might be expected that the levels of its possible precursors (GA_{20} and GA_4) might also change with photoperiod. This seems to be the case for both potential precursors of GA_1, GA_4 and GA_{20} (figure 5.5). In all repeat samples, the levels of GA_4 in SD and LD leaves is the same at dawn the first day.

Thereafter, the level of GA_4 extracted from the induced leaves increased by 50% (from 10 to 15 ng/g d.w.) and remained relatively higher level to the last sampling time. The level of GA_4 in the LD control samples was stable at 10 ng/g d.w. at all sample times.

The levels of leaf GA_4 may thus be under photoperiod regulation, and the increase in GA_4 in leaves after the long night could (speculatively) be involved in the promotion of floral evocation. Long night may thus be speculated to reduce 13α-hydroxylation of GA_4 (and/or 2ß-hydroxylation to GA_{34} and/or conjugation), and/or increase the synthesis of GA_4 from GA_9. This is an especially attractive hypothesis, since GA_4 applied to the leaf blade is very active in promoting floral evocation under a marginally-inductive long night. In addition, its precursor from the non-hydroxylation pathway (GA_9) and GA_7, a molecule structurally similar to GA_4 (both GA_7 and GA_4 are 3ß-hydroxylated) were also potent promoters of floral evocation in plants given a marginally-inductive long night (see figure 3.13).

In comparison to GA_4, GA_{20} was not stable under either LD or long night (SD) conditions throughout this study. In leaves from both SD and LD plants, the level of GA_{20} increased 5-fold, with the GA_{20} profile being similar under both photoperiods (figure 5.5), however, it must be noted that the GA_{20} level, at the same point when long night (SD) GA_1 and GA_8 levels diverge from those of the LD controls, is significantly lower in the SD leaves as compared to leaves of plants under continuous LD. Thus, GA_1, GA_3 and GA_8 levels are decreased under SD, whilst the levels of GA_4 and GA_{20} are increased. This suggests that there is photoperiodic control of GA metabolism in these Xanthium leaves, and that shortly after long night induction said changes may occur at both the 13α-hydroxylation step and the 3ß-hydroxylation step. Although GA_{19} levels were not quantitated in this study, the $GA_{19} \rightarrow GA_{20}$ step could also be a photoperiodically controlled step, as was determined to be the case for the LDP, spinach

(Zeevaart, 1971b) and Brassica spp. (Zanewich, 1993), and also for the LSDP Bryophyllum (ref. in Zeevaart, 1978). Hypotheses of the photoperiodic control of GA_{20} metabolism will be discussed in the chapter to follow. A study of GA_4 metabolism could also increase our understanding of how levels of this hormone are controlled by photoperiod.

As alluded to above, the exogenous application results, epicotyl growth response to long night, and trends for the endogenous GAs correspond fairly well. Gibberellin A_1 and GA_8 levels decrease after a single SD (16 h dark/8 h light), and GA_3 levels decrease even more rapidly under SD. Thus, there is a strong correlation between endogenous GA trends and the reduced epicotyl growth of the long night (SD)-induced plants. Concomitantly the levels of GA_4, a potent promoter of floral evocation in this system increase by 50% after a single or several long nights. However, given the lag period between the end of the long night and the occurrence of the detectable changes in hormone levels (dusk before the second long night), it would seem that the difference in leaf GA_4 will not be involved causally in the initial floral evocation events (which take place during or immediately after the inductive dark period) (Salisbury, 1982). The fact that the levels of GA_4 increase during the intervening light period following induction by a single long night suggests, however, that this GA may be part of a broad floral evocation stimulus. Evidence for this is two-fold: Firstly, Maksymowych and Erickson, (1977) and Erickson and Meichenheimer (1977) noted that the change in phyllotaxy induced by exogenous application of GA_3 was the same as that which occurred naturally in the transition from the vegetative to the reproductive state. The increase in GA_4 levels proximal to the end of the first SD cycle (1600 h) might explain the change in phyllotaxis which occurs under long night (SD), though GA_4 has not been tested in this regard. Additional evidence that the observed changes in GA_1 and GA_4 may be important in floral

evocation arises from defoliation experiments at times following induction in Xanthium. Essentially flowering is reduced (in Salisbury floral stage) if the induced leaf is defoliated up to 44 h following induction. However, maximal flowering occurs if the induced leaf is removed thereafter (Bernier, 1988; Salisbury and Ross, 1978; Vince-Prue, 1975; Salisbury, 1990). Changes in GA_1 and GA_4 levels may form the hormonal basis for this observation, since changes in their levels are apparent by 2 days after induction. Although GA_{20} could be detected at two harvest times in the apical tissue of LD plants it was not found in apical tissue after any long night (SD) treatment. Odén and Heide (1989) also determined that the level of GA_{20} was not strongly correlated with long night induction in Begonia another SDP.

In several LD plants, including Agrostemma, Spinacia, Thlaspi, and Lolium, GA levels and levels of GA-like substances are higher after LD treatments than under continuous SD (Zeevaart and Gage, 1993; Jones and Zeevaart, 1980a; Jones and Zeevaart, 1980b; Talon and Zeevaart, 1990; Zeevaart, 1971b; Metzger, 1985; Metzger and Zeevaart, 1982; Pharis et al., 1987). In LDP grown under inductive LD conditions there is usually a correlation between growth rate and rate of GA metabolism. However, this correlation has never been substantiated definitively by GC-MS-SIM for any SDP grown under LD conditions. Odén and Heide (1988) did determine, by use of the Tan-ginbozu dwarf rice bioassay, that the level of GA-like substances decrease in Begonia following induction (under SD and/or low temperature). In contrast to the results following induction for Begonia, where levels of GA_9 and GA_4 (determined by GC-MS-SIM) decrease significantly within 2 to 4 days of long night induction (GA_1 level was too low to quantitate accurately) (Odén and Heide, 1989), I observed an increase in GA_4 for Xanthium.

The relationship of endogenous GA levels in Xanthium relative to their efficacy of promoting floral evocation in marginally-induced Xanthium is similar to the changes noted in the Pinaceae family of conifers, where the less-polar GAs (GA_4 and GA_9) are potent inducers of flowering and cultural treatments known to promote flowering, increase less-polar endogenous GAs for several species (Pharis et al., 1989 and 1992). In Sitka spruce there is indirect evidence that metabolism of GA_4 to GA_1 may be blocked (e.g. in poor-flowering clones GA_1 and GA_3 are detectable, whereas, in good-flowering clones only GA_4 and GA_9 are found) (Moritz et al., 1990). Evans et al. (1993) also noted, using an acyclohexanedione (which blocks 3β-hydroxylation), a synergistic promotion of flowering by this inhibitor with 1 LD (a marginally inductive treatment) in Lolium. Both results provide evidence for inhibition of late stage hydroxylations being associated with promotion of flowering. The story in Xanthium may thus be similar.

5.3 Endogenous levels of GAs in apical tissue

In the apical region tissue the levels of GA_4 (e.g. 100 to 700 ng/g d.w.) were always far in excess of the levels of other GAs which were present (figure 5.6). Of the remaining GAs, GA_1 (a 3ß-hydroxylation product of GA_{20} and/or a 13α-hydroxylation product of GA_4) was present at about $^1/_{10}$ the level of GA_4. The level of GA_8 (an inactive 2ß-hydroxylation product of GA_1) was ca $^1/_{10}$ the level of GA_1. Finally, the level of GA_{20} was nil or trace in long night-induced (SD) apex tissue, but was detectable in LD apex tissue (only at 1600 h, days 1 and 2) (fig 5.6). Levels of endogenous GA_3, GA_5, GA_7 and GA_9 were either nil or too low for them to be quantified.

The changes in levels of GA_1 and GA_4 in apical tissue roughly parallel changes in these GAs extracted from the corresponding leaf samples, e.g. the level of GA_1 tends to be slightly lower in long night-induced (SD) apex tissue than in the LD control apex tissue (figure 5.6). The difference in GA_1 level between photoperiod treatments is more pronounced at dawn [long night-induced (SD) approximately 20% less than LD] than at dusk (long night-induced and LD are equal). For GA_4 the level declined steeply at all harvest times after time-zero (1600 h), even in the LD-treated apices. However, since the leaf tissue supplying these apices is undergoing rapid growth, this decline in GA_4 may reflect ontogenetic changes, and photoperiod effects are minor when superimposed on such a large decline. Nonetheless, the decline was less pronounced for long night-induced apices, and at 1600 h on day 1 GA_4 level was 4-fold higher than in the LD, non-induced apices (figure 5.6). Gibberellin A_4 was present at all harvest times in long night-induced apices (figure 5.6), but was undetectable in LD control apices after dawn of day 1. Gibberellin A_4 can promote flowering the next year when sprayed on apple spur leaves in heavy crop years (Looney et al., 1985). It is also abundant in various reproductive tissues including rice anthers, maize tassels, immature Brassica spp. siliques and induced Lolium apices (Kobayashi et al., 1988; Murofushi et al., 1991; Zanewich, 1993; Takagi et al., 1993), although no definitive role has yet to be assigned for GA_4 in these tissues. The levels of GA_8 in the LD and long night-induced apical samples are approximately equal with the exception of the harvest following the initial inductive long night (0800 h) where the LD GA_8 level is 3.5-fold higher than the GA_8 level in the long night-induced apices (figure 5.6). The level of a wide range of bioactive endogenous GA-like substances is also higher just after an inductive LD in Lolium apices (Pharis et al., 1987).

Apparent rhythms (dusk/dawn/dusk/dawn) of endogenous GAs are less pronounced in the apical samples than they are in the leaves. However, a fluctuation in

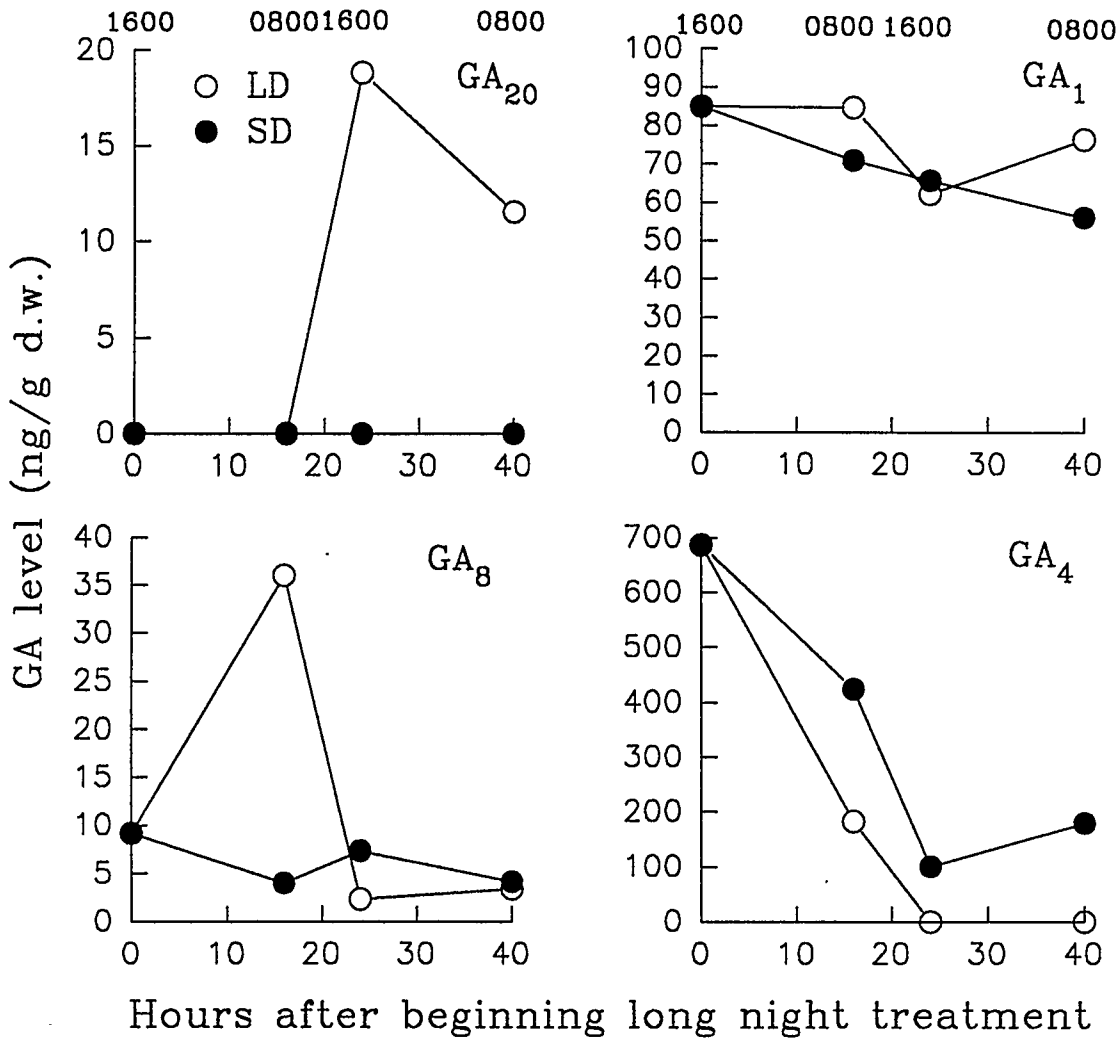


Figure 5.6 Gibberellin $A_{1, 4, 8 \text{ and } 20}$ levels (ng/g d.w.) in apical tissue (12 buds per sample) of induced (two 16 h long nights) and non-induced (20 h light) Xanthium plants (6 weeks old). Values represent results of a single experiment typifying these changes quantitated by GC-MS-SIM. Note the different y-axis scales for the GAs.

GA_1, and to a lesser extent GA_8, is detectable, levels of these GAs being higher at dawn than at dusk (figure 5.6). Furthermore this rhythm is stronger in the LD apices than in the long night-induced apices, possibly because the rhythm is stabilized in the LD plants, but had not yet had time to stabilize in plants induced with two long nights. The fact that these rhythms are not diurnal may indicate that the period is longer than 48 h, or conversely the rhythm may be affected by developmental (rapidly growing primary leaves) fluctuations of hormone levels.

5.4 Endogenous levels of IAA and ABA

In agreement with Zeevaart (1978) there was no clear trend relating endogenous levels of ABA or IAA in leaves to flowering. In all analyses leaves of the long night-induced plants demonstrated a noticeable diurnal fluctuation, with peaks of IAA occurring at dawn after both inductive long nights (see figure 5.7). At dawn the first day (after one long night) the level of IAA in leaves is 3-fold higher than in leaves from the LD plants, but by dusk the situation is reversed (figure 5.7). Surprisingly, the endogenous IAA of leaves from LD plants shows a sigmoidal increase over all sampling points. Again, this may relate to ontogenetic developmental changes in leaf growth. If there is a rhythmic cycle for IAA level, it is longer than 24 h. A peak in endogenous IAA is also observed in the apex samples at dusk after the first long night (SD) cycle. At this point the level of IAA in the induced apices is significantly greater (almost double) than that in the non-induced apices. It is interesting that these changes in endogenous IAA levels occur concomitantly with the significant changes in GA metabolism. Are these changes related to the events of floral evocation? It is also unclear whether the "valley" in endogenous

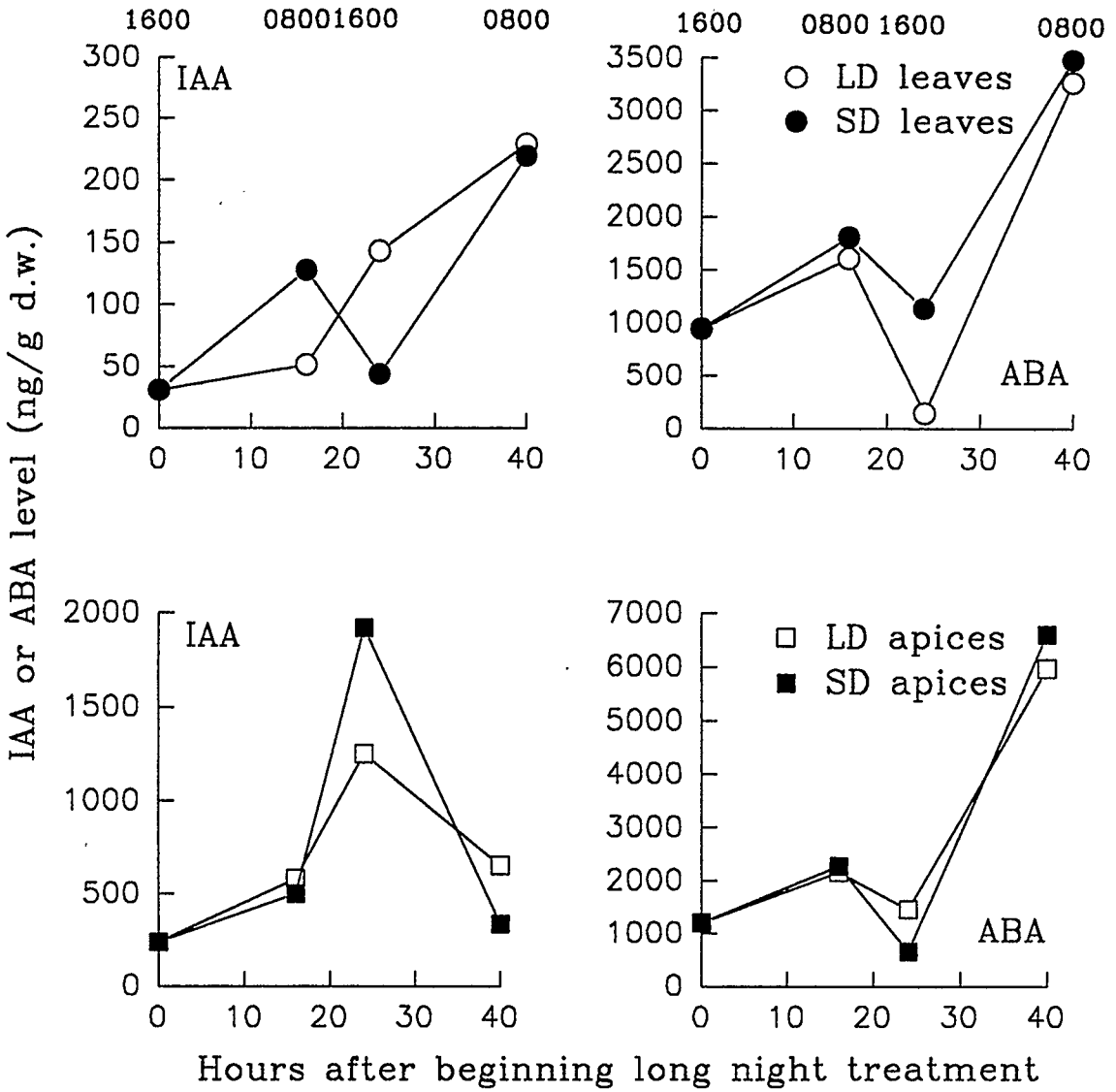


Figure 5.7 Levels of IAA and ABA (ng/g d.w.) in the leaf (most rapidly expanding) and apical tissue (12 buds per sample) of induced (two 16 h long nights) and non-induced (20 h light) Xanthium plants (6 weeks old). Values represent results of a single experiment typifying these changes quantitated by GC-MS-SIM. Note the different y-axis scales for the GAs.

IAA in induced leaves at 1600 h of the first day is related to the peak which occurs at this time later in the apices (it does follow an 0800 h peak in induced leaves).

Although exogenously applied IAA was quite inhibitory to floral evocation, this effect may be pharmacological. That is, if the endogenous levels of this hormone are causal to the inhibition of flowering under LD (as was speculated based on results from exogenous applications of IAA), the IAA level should have fallen in leaves, and especially in apices of the induced plants. This was obviously not the case. Keeping this in mind, it is possible that there is an optimum range of IAA required for induction, with inhibition of floral evocation resulting at levels of IAA above or below this optimum. Such a result was obtained in Sinapis, where low doses of exogenous IAA were correlated with successful induction (even promotion of flowering) and high doses of IAA were also correlated with the absence of flowering (Bodson, 1985).

The curve shapes are similar for endogenous ABA levels from both apices and leaves under either 20 h LD or after long night induction (SD) (figure 5.7). Peaks in ABA level are observed at dawn after both long nights. This similarity in curve shape and peak times indicates that ABA levels are mainly responding to a factor(s) other than photoperiod, perhaps to a developmental factor such as rapid leaf growth (see above re IAA and GA_4). The endogenous level of ABA shows a diurnal fluctuation for the first 24 h, then (perhaps) the "developmental influence" takes over and ABA level skyrockets in both the leaves and apices. In Spinacia, no causal relationship was observed between endogenous ABA in leaf tissue and flowering (Zeevaart, 1971a).

The apparent hormonal rhythm is (roughly) reversed for leaf ABA and leaf IAA compared to that seen for the endogenous GAs. This could indicate that IAA and ABA in the leaves are: (i) photo-oxidized., (ii) undergo increased conjugation in the light, or (iii)

undergo decreased synthesis in the light. No significant difference in ABA level with photoperiod treatment is observed at any sample point in both leaves and apices. Thus, ABA seems unlikely to play a significant role in floral evocation.

5.4 Effect of an extract from continually induced leaves on flowering

The partially purified extract from continually induced leaves was **not** florigenic at any dose tested under completely non-inductive 20 h LD conditions, regardless of the method of application used (See Table 5-3). There was, however, significant promotion of floral evocation, both in stage and in flowering quotient (compared to the ethanol controls) when extract-treated plants were given a single marginally-inductive long night (12 h dark). This may indicate either that the extract does not contain the primary inductive signal, or that the highest dose of extract applied did not contain enough primary inductive signal to initiate the vegetative to floral state transition. The latter hypothesis was tested by applying more concentrated doses of the extract to another batch of Xanthium plants. However, this resulted in toxicity and the apical buds were killed. It might also be hypothesized that the florigenicity of the extract may result from endogenous GAs, potentially GA_4, since this GA (when applied exogenously to marginally-induced plants) had the highest florigenicity of the all the known endogenous GAs. Unfortunately, an extract of non-induced LD leaves was not prepared to act as a parallel control treatment.

In addition to the fact that the leaf extract promoted floral evocation, it is interesting that the extract's effect on floral stage and flowering quotient weakened with dilution. Such a dilution of the stimulus indicates that even if the extract does not contain

Table 5-3. Effect of several dilutions of a crude extract made from continually-induced leaves (given 21 long nights of 16 h) on flowering of Xanthium seedlings maintained under non-inductive 20 h LD photoperiod (-) or induced with a single marginally-inductive long night (12 h dark) (+). The extract was applied 8 h prior to the beginning of the 12 h dark period.

Extract dilution⊗	Single Long Night (+/-)	Average Floral Stage	Flowering Quotient (# plants flowering out of 5 or 10)
EtOH	-	0	0/10
	+	1.3	3/10
1/100	-	0	0/5
	+	3.00	4/5
1/200	-	0	0/5
	+	2.60	3/5
1/400	-	0	0/5
	+	0.75	1/5

⊗ to convert dilution of extract applied to portion of an extracted leaf applied, multiply by ten (e.g. 1/100 represents 1/10 of a leaf).

the primary stimulus, it certainly contains a stimulus important in the vegetative to floral state transition pathway.

Conclusions

To summarize, the levels of GA_{20} (at dusk the first day), GA_1 and GA_8 decreased in extracts of leaves from plants given 2 inductive long nights, compared to the LD controls. This trend began after the first complete (24 h) SD cycle. The level of GA_1 also decreased in SD apices compared to the non-induced apices. Further, in LD apices, GA_8 (inactive 2β-hydroxylation product of GA_1) and GA_{20} (precursor to GA_1) levels were higher than those of the induced apices. Concomitant with the decrease in GA_1, the level of GA_4 in leaves increased after a single inductive long night (SD) cycle. The GA_4 level also decreased less rapidly in induced apex tissue than in non-induced LD apices. The decreased level of GA_1 in leaves of the SD plants is well correlated with the shorter petioles and epicotyls seen for these plants. It is speculated that reduced GA_1 and increased GA_4 may form a part of the floral evocation stimulus, since these levels change at the same time as translocation of the floral stimulus is occurring. Long night induction did not affect the IAA or ABA levels and curve shapes of ABA were roughly similar under both SD and LD conditions. However, the level of IAA in the leaves was lower at dusk the first day, while the level in the apices increased at this sampling time.

CHAPTER 6

METABOLISM OF [^{3}H] AND [$^2H_2$]GA_{20} BY INDUCED AND NON-INDUCED LEAVES OF XANTHIUM STRUMARIUM

Introduction

The results of chapter 3 show that seedlings induced with a single long night had significantly shorter epicotyls and petioles than those organs in the LD control seedlings. In part this altered phenotype may be explained by reduced levels of GA_1 (and GA_3) following the long night (see chapter 5). The spectrum of GAs found in Xanthium suggests that both the early C-13 hydroxylation and non-hydroxylation pathways may be present in leaves of this plant.

In the early C-13 hydroxylation pathway GA_{20} (likely not in itself bioactive in either elongation or flowering) is the immediate precursor of GA_1 (the presumed elongation effector). Another metabolite of GA_{20} is its inactive 2ß-hydroxylation product GA_{29}. Further, GA_1 can be metabolized via 2ß-hydroxylation to GA_8, which is relatively inactive (at least as an effector of elongation in most plants). The present study restricts the analysis of GA_{20} metabolism to these latter two steps. As alluded to above, the mechanism underlying reduced epicotyl growth in the long night (SD)-induced Xanthium plants possibly involves a decline in the levels of GA_1 (putative elongation effector). The purpose of performing this metabolism study was to determine whether the decline in GA_1 was due to decreased rate of metabolism of $GA_{20} \rightarrow GA_1$, or whether the change was due to conjugation and/or inactivation (by 2ß-hydroxylation to GA_{29}) of the GA_{20} precursor. Investigations of GA metabolism can be an important tool in the study of hormonal

physiology, since by its very nature it measures fluxes rather than static levels of hormones. Thus, it was through the analysis of GA_{20} metabolism that I attempted to assess whether there was a correlation between reduced epicotyl growth in the SD-induced plants and endogenous GA physiology.

Preliminary hormone analyses had determined that GA_{20} was endogenous in Xanthium leaves (see Table 5-1). Therefore, it seemed logical to study its metabolism using exogenously applied labeled GA_{20}. An additional reason for conducting this study was to investigate the possibility that GA_{20} might be converted to GA_5 (Phinney et al., 1991), since GA_5 was a "fairly florigenic" GA in Pharbitis (King et al., 1987), and somewhat florigenic in the Xanthium system. Gibberellin A_5 is also a precursor to GA_3.

Methods

6.1 Effect of Exogenously Applied [^{2}H]GA_{20} on Induction

Since [^{2}H]GA_{20}, or for that matter GA_{20} had never been applied previously it was important to assess the florigenicity of [^{2}H]GA_{20}, so as to be absolutely certain that the LD controls were not induced by GA_{20} application (e.g. that they remained vegetative and application of this GA did not interfere with SD induction). Parallel with the above applications, 10 µg of [^{2}H]GA_{20} was applied alone to each of 6 LD control plants and 6 long night-induced plants ([^{2}H]GA_{20} was applied 5 min prior to the beginning of a 16 h long night at 1600 h). At 0800 h the following morning the long-night plants were transferred from the darkroom back to the LD chamber where they were allowed to resume their growth until dissection 9 days later to assess their floral response.

6.2 Isotope dilution analysis of metabolism

For metabolism study exogenous application of 10 μg of [^{2}H]GA_{20} and 1,000,000 dpm (16 700 Bq) of [^{3}H]GA_{20} per plant was made in 10 μL of 95% EtOH to the base of the most rapidly expanding leaf blade of 6-week-old physiologically mature plants grown in 4" pots and maintained under LD photoperiod prior to treatment. These plants were defoliated to the single most rapidly expanding leaf (generally the third visible leaf from the apex) the day prior to GA application. Immediately following the application (1600 h; within 5 min), half the treated plants were induced by transferring them to the darkroom to receive a fully-inductive 16 h long night. The remaining plants (controls) were returned to the LD chamber immediately following treatment. Both treatments consisted of 5 plants each. Immediately following photoperiodic treatment (at 0800 h), the single most rapidly expanding leaf was rinsed with methanol and shoot tips, petioles and leaf blades of induced plants were harvested into liquid N_2. Both LD and SD photoperiods co-terminated, so that the LD control plants and long night plants were harvested simultaneously. Shoot tips had all visible leaf primordia removed during harvest. Tissue was subsequently stored at -70°C prior to freeze-drying. Thereafter, lyophilized tissue was stored at -20°C. Through these harvest times perturbation of [^{2}H]GA_{20} metabolism due to the inductive long night was studied in the harvested leaf tissue. Shoot tips were not analyzed for this thesis.

The lyophilized leaf tissue was extracted (5.3), purified (5.5) and [$^2H_2$]GA metabolites quantified (5.6 and 5.7) as above (Chapter 5 Methods). The experiment was repeated twice, with results exhibiting similar trends.

Results and Discussion

6.1 Effect of exogenously applied [^{2}H]GA$_{20}$ on induction

Exogenously applied [$^2H_2$]GA$_{20}$ did not interfere with SD induction, nor did it induce the LD plants to flower. This was not unexpected, and is yet another example of an exogenously applied GA which was unable to induce a flowering response in Xanthium plants maintained under non-inductive LD.

6.2 Metabolism of [^{3}H]/[^{2}H]GA$_{20}$ in Xanthium leaves

Analyses of leaf tissue were made for the three main radioactive products of GA$_{20}$ metabolism, specifically GA$_1$ (3ß-hydroxylated GA$_{20}$), GA$_8$ (2ß, 3ß-dihydroxylated GA$_{20}$) and GA$_{29}$ (2ß-hydroxylated GA$_{20}$). Gibberellin A$_3$ and GA$_5$ were not found as major metabolite peaks. Although other possible GA$_{20}$ metabolites could exist in this system, mainly GA$_6$, epi-GA$_1$ (3α-hydroxy GA$_1$)(Graebe, 1987; MacMillan, 1990), various catabolites and glucosyl conjugates, these were not investigated in this study since they were not observed as major metabolite peaks in the [^{3}H] HPLC profiles (figures 6.1 and 6.2)

Between 30 and 40% of the applied radioactivity was extracted. Less than 1% of the extracted radioactivity eluted in the non-polar (precursor) fraction (this non-polar fraction was retained by the C$_{18}$-PC and subsequently eluted with 100% MeOH) for both induced and non-induced plants. This was known since all the expected hydroxylated metabolites of GA$_{20}$ are more polar in structure than GA$_{20}$ [C/D rearranged GA$_{20}$

excepted] (see figure 5.3 for structures of GA_{20} metabolites). By normal-phase chromatography using a short SiO_2 partition column, putative conjugates (EtOAc insolubles) were separated from free GAs. Glucosyl esters or glucosyl ethers are common metabolites of exogenously applied GAs (Graebe, 1987; MacMillan, 1990). This step revealed that leaves of induced plants conjugated (on average) less $[^3H]GA_{20}$ (and its metabolites) than their LD counterparts (4% and 6% respectively). In cotyledons of Pharbitis nil, Takeno and Cleland (1990), also determined that the levels of GA conjugates were approximately equal in induced and non-induced plants.

Following C_{18}-HPLC, radioassay and bioassay of these fractions, [results presented in figures 6.1 (LD control) and 6.2 (SD induced)], by comparison with the R_ts of external standards the major identified metabolites of $[^3H]GA_{20}$ were putative $[^3H]GA_1$, $[^3H]GA_8$, and $[^3H]GA_{29}$.

Of the free GA radioactivity recovered from C_{18}-HPLC for the long night (SD) extract, 87,600 dpm (27%) was putative $[^3H]GA_{20}$, 2,848 dpm (1%) was putative $[^3H]GA_1$, 3,080 dpm (1%) was putative $[^3H]GA_{29}$ and 2,760 dpm (1%) was putative $[^3H]GA_8$. Of the free GA radioactivity recovered from C_{18}-HPLC for the LD extract, 166,760 dpm (23%) was putative $[^3H]GA_{20}$, 35,320 dpm (5%) was putative $[^3H]GA_1$, 4,480 dpm (1%) was putative $[^3H]GA_{29}$ and 30,960 dpm (4%) was putative $[^3H]GA_8$. The above results (Table 6-1) probably represent an underestimate of the actual metabolism occurring due to the positioning of the tritium atoms in ring A. The $[^3H]GA_{20}$ used in this study was labeled at the C-2 and C-3 positions, which are sites of substitution in the GA pathway (Murofushi et al., 1977). However, if one assumes (correctly or not) that there were proportional losses of labile $[^3H]$ in each of the LD and SD treatments, differences in radioactivity recovered may be compared. The results indicate that metabolism of GA_{20} to GA_1 is significantly faster in the plants maintained

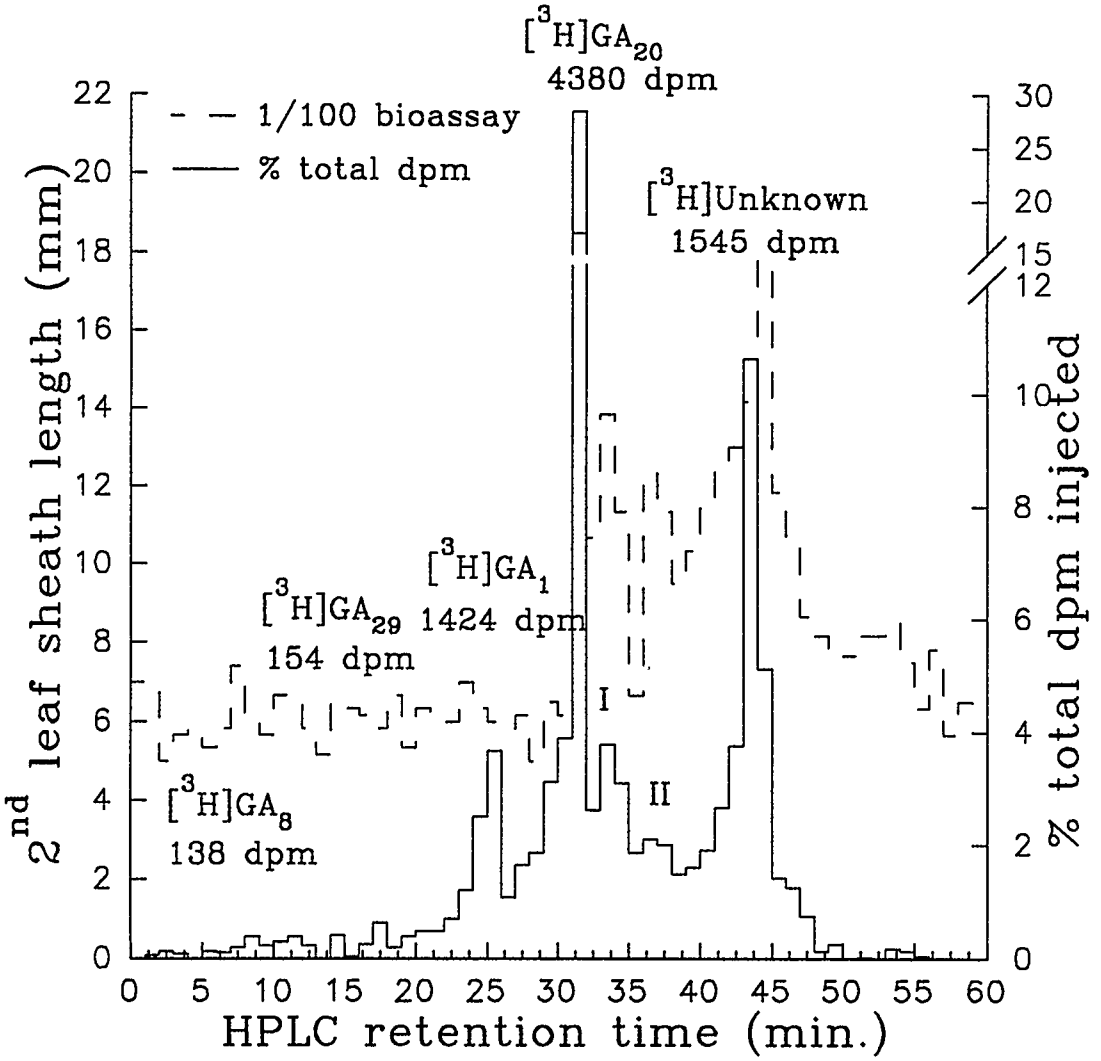


Figure 6.1 Metabolism of [^{3}H]GA_{20} in the most rapidly expanding leaf from a long night (SD)-induced Xanthium plant. Chromatogram of radioactivity (solid line) and bioactivity (dashed line) in the Tan-ginbozu dwarf rice microdrop bioassay (e.g. elongation growth in rice of a 1/100 fraction aliquot) for fractions from a reversed-phase C_{18}-HPLC column loaded with the EtOAc-soluble fraction following a feed of [^{3}H]GA_{20}. Peak I may be putative C/D rearranged GA_{20}, peak II may be a putative GA_{29} catabolite and the unknown peak may represent a GA_{20} catabolite.

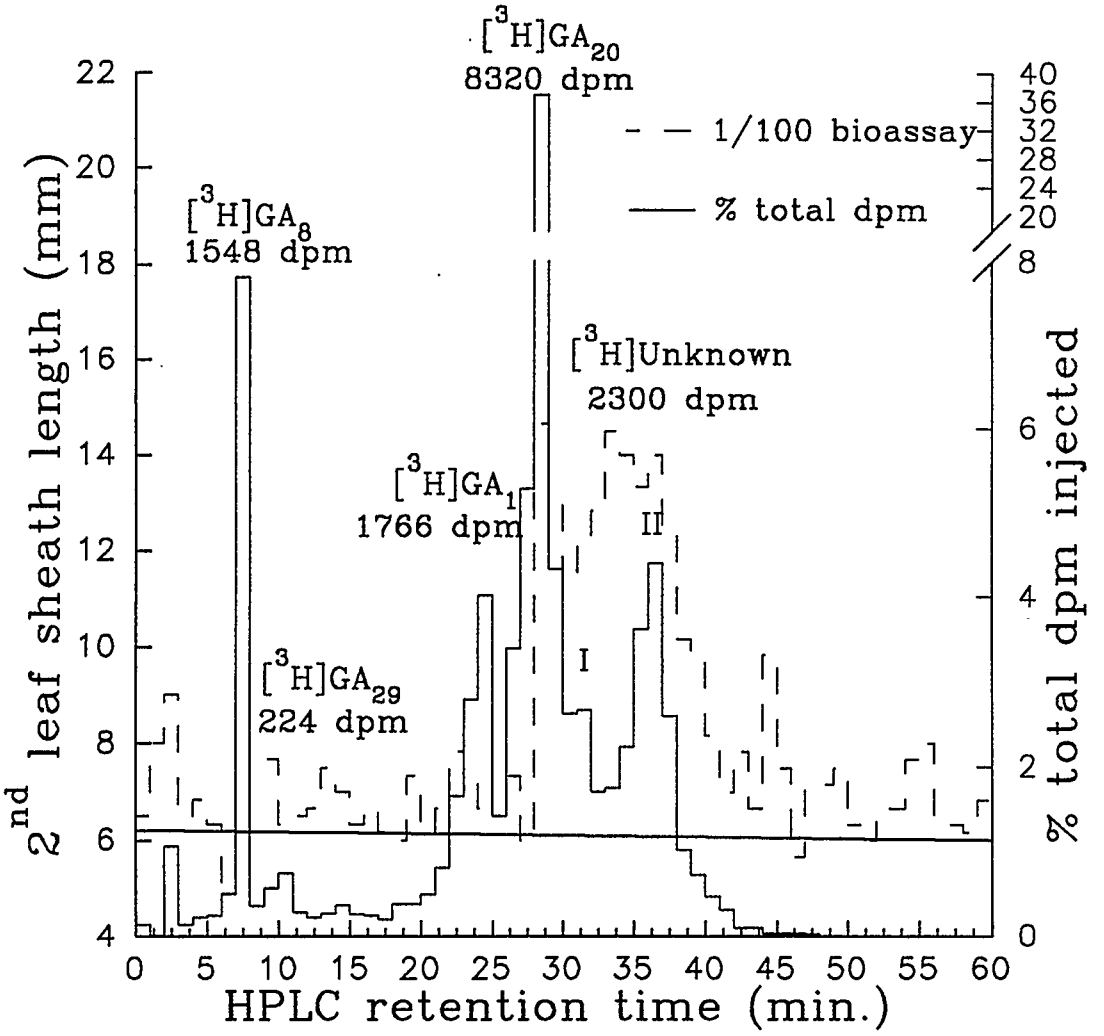


Figure 6.2 Metabolism of [^{3}H]GA_{20} in the most rapidly expanding leaf from a non-induced Xanthium plant. Chromatogram of radioactivity (solid line) and bioactivity (dashed line) in the Tan-ginbozu dwarf rice microdrop bioassay (e.g. elongation growth in rice of a 1/100 fraction aliquot) for fractions from a reversed-phase C_{18}-HPLC column loaded with the EtOAc-soluble fraction following a feed of [^{3}H]GA_{20}. Peak I may be putative C/D rearranged GA_{20} and peak II may be a putative GA_{29} catabolite.

Table 6-1 The effect of a single inductive 16 h long night (SD) on the metabolism of $[^3H]GA_{20}$ (applied at 1600 h) to putative $[^3H]GA_1$, $[^3H]GA_{29}$ and $[^3H]GA_8$. Extractions were made from long night-induced (8 h light/ 16 h dark) and non-induced (20 h light/ 4 h dark) leaves harvested at 0800 h the next day following induction. Values are the means of two samples.

Treatment	Radioactivity extracted as a % of $[^3H]GA_{20}$ applied*	Radioactivity as a percentage of total acidic eluting from C_{18}-HPLC			
		at GA_{20} R_t†	at GA_1 R_t†	at GA_8 R_t†	at GA_{29} R_t†
SD	44.6±0.1	26.9±0.1	0.84±0.52	0.82±0.41	0.66±0.31
LD	37.4±0.6	23.3±0.4	4.91±1.28	4.33±0.69	0.91±0.56

* for this column, the sum of all extractable, acidic, EtOAc soluble radioactivity was taken as a % total of the $[^3H]GA_{20}$ exogenously applied to the most rapidly expanding leaf.

† for these columns, the value is a % of the total radioactivity eluting from the C_{18}-HPLC. Leaves were washed with MeOH prior to harvest.

under LD, relative to those plants receiving the inductive long night (SD). Furthermore, the increased rate of putative [^{3}H]GA_1 synthesis from [^{3}H]GA_{20} in plants maintained under LD is accompanied by an increased rate of [^{3}H]GA_8 synthesis (e.g. GA_{20}→GA_1→GA_8). The metabolism of GA_{20} to GA_{29} was not affected by the inductive long night, being only 1% of the total radioactivity eluted from C_{18}-HPLC for both photoperiod treatments.

Taken together, the increased level of conjugation, the increased biosynthesis of GA_1 (and of GA_8 from GA_{20} via GA_1) confirms the precedent that LD results in an overall increase in GA biosynthesis (Odén and Heide, 1988; Pharis et al., 1990; Metzger and Zeevaart, 1982; Zeevaart, 1971b), thus increasing the levels of elongation-active GAs (GA_1 in this case). It is also quite interesting that even a single long night could result in a decreased rate of synthesis of GA_1, as well as decreased conjugation of GA_{20} (and potentially GA_1 as well). Also, the reduced synthesis of GA_1 is presumably maintained even after induced plants are returned to LD conditions since, as detailed in chapter 3, induced plants have significantly shorter internodes and petioles than those maintained under LD from germination. In spinach, more rapid growth is also seen in petioles maintained under LD (inductive) than under SD conditions (Zeevaart, 1971b).

In spinach (LDP), a 2-fold increase in the rate of metabolism of [^{3}H]GA_{20} was recorded following induction under continuous LD, with the majority of the exogenous [^{3}H]GA_{20} being directed towards [^{3}H]GA_{29} (Metzger and Zeevaart, 1982). In the present experiment, long night induction in Xanthium resulted in GA_1 as the only [^{3}H] metabolite of [^{3}H]GA_{20} (figure 6.1), whereas in LD leaves putative [^{3}H]GA_1 and putative [^{3}H]GA_8 are present (figure 6.2). Based on radioactivity profiles, there was a 5-fold lower rate of synthesis in leaves from long night (SD)-induced plants than in the LD

controls. A lower rate of synthesis of GA_8 (via GA_1) in the long night (SD) leaves compared to the LD controls was also seen in Xanthium (compare figure 6.1 with 6.2, R_t 5-10 min). In spinach GA_{29} was also found to accumulate to a higher level under LD than under SD. This is in contrast to the result in Xanthium, where no appreciable change in $[^3H]GA_{29}$ synthesis was noted.

In addition, fractions from the radioactive regions were grouped (by R_ts based on tritiated-external standards) and run on GC-MS-SIM to confirm the presence of these metabolites in deutero form (Table 6.2a and 6.2b; LD and SD respectively). The GA_{20} region was chromatographed by Nucleosil $[N(CH_3)_2]$ HPLC in order to separate GA_{20} from GA_5 (another potential metabolite of GA_{20}). Fractions determined to be in the GA_5 region were subsequently analyzed for $[^2H_2]GA_5$ on GC-MS-SIM. However, no $[^2H_2]GA_5$ was detected. This confirms the fact that GA_5 is not likely an endogenous GA in Xanthium leaves. Likewise, $[^2H_2]GA_3$ was also not detected by GC-MS-SIM following analysis of appropriate fractions purified by Nucleosil $[N(CH_3)_2]$ HPLC of the $[^3H]GA_1/GA_3$ region. The conversion of $[^2H_2]GA_{20}$ to $[^2H_2]GA_1$ and $[^2H_2]GA_8$, and of $[^2H_2]GA_{20}$ to $[^2H_2]GA_{29}$ was thus confirmed (Tables 6.2a and 6.2b).

Large differences in M^+ peak area occurred for $[^2H_2]GA_1$ (3-fold higher), $[^2H_2]GA_8$ (2-fold higher) and $[^2H_2]GA_{29}$ (3-fold higher) between the non-induced leaves and the long night-induced leaves respectively (Tables 6.2a and 6.2b). Approximately equal aliquots of each fraction were injected and assuming equal work-up losses, these results corroborate the $[^3H]$ metabolism trends.

In each of the SD and LD samples, a large peak of radioactivity was found to the right of the $[^3H]GA_{20}$ peak (less polar than GA_{20}) (see figures 6.1 and 6.2). However, in both cases its identity remains unknown by GC-MS. This peak may represent alkylated

Table 6-2a The relative intensities of characteristic diagnostic ions for $[^2H_2]GA_1$, $[^2H_2]GA_8$ and $[^2H_2]GA_{29}$ from non-induced (LD) Xanthium leaves fed $[^2H_2]GA_{20}$ compared with those of authentic GA standards. The latter are normalized to the peak are counts at m/z 508 of the putative $[^2H_2]GA_1$ metabolite, at m/z 596 of the putative $[^2H_2]GA_8$ and at m/z 508 $[^2H_2]GA_{29}$. The relative intensity, expressed as a percentage of the M^+ ion is shown in ( ).

Treatment		Ratio at m/z ion	
	508 (M^+)	493	449
Putative Metabolite, $[^2H_2]GA_1$	6,519,825 (100)	680,551 (10.4)	717,731 (11.0)
Authentic $[^2H_2]GA_1$	6,519,825 (100)	697,445 (10.7)	687,806 (10.5)
	596 (M^+)	450	381
Putative Metabolite, $[^2H_2]GA_8$	797,187 (100)	159,179 (20.0)	134,602 (16.8)
Authentic $[^2H_2]GA_8$	797,187 (100)	163,398 (20.4)	104,702 (13.1)
	508 (M^+)	493	377
Putative Metabolite, $[^2H_2]GA_{29}$	4,662,423 (100)	555,908 (11.9)	987,289 (21.2)
Authentic $[^2H_2]GA_{29}$	4,662,423 (100)	581,793 (12.4)	762,551 (16.4)

Table 6-2b The relative intensities of characteristic diagnostic ions for $[^2H_2]GA_1$, $[^2H_2]GA_8$ and $[^2H_2]GA_{29}$ from 16 h long night-induced (SD) Xanthium leaves fed $[^2H_2]GA_{20}$ compared with those of authentic GA standards. The latter are normalized to the peak are counts at m/z 508 of the putative $[^2H_2]GA_1$ metabolite, at m/z 596 of the putative $[^2H_2]GA_8$ and at m/z 508 $[^2H_2]GA_{29}$. The relative intensity, expressed as a percentage of the M^+ ion is shown in ( ).

Treatment	Ratio at m/z ion		
	508 (M^+)	493	449
Putative Metabolite, $[^2H_2]GA_1$	1,834,972 (100)	191,290 (10.4)	210,213 (11.5)
Authentic $[^2H_2]GA_1$	1,834,972 (100)	196,292 (10.7)	193,579 (10.5)
	596 (M^+)	450	381
Putative Metabolite, $[^2H_2]GA_8$	469,086 (100)	90,889 (19.4)	69,007 (14.7)
Authentic $[^2H_2]GA_8$	469,086 (100)	96,148 (20.4)	61,609 (13.1)
	508 (M^+)	493	377
Putative Metabolite, $[^2H_2]GA_{29}$	1,810,763 (100)	208,096 (11.5)	226,772 (12.5)
Authentic $[^2H_2]GA_{29}$	1,810,763 (100)	225,953 (12.4)	296,155 (16.4)

[^{3}H]GA_{20} (via the C-7 carboxyl) or conversely [^{3}H]GA_{20} adducted or covalently linked (via the C-7 carboxyl or the 13α-OH) to an unknown less-polar compound. It is interesting that this unknown peak represents 1/3 of the [^{3}H]GA_{20} for the SD samples, while it only represents 1/4 of the [^{3}H]GA_{20} for the LD samples. Therefore it is possible that this peak is associated with the events of floral evocation. Further characterization of this peak will be attempted.

As King et al. (1987) suggested, pulse changes in GA metabolism (or applied GA) may be one factor leading to floral initiation. The photoperiod-induced changes in GA_{20} metabolism of the Xanthium system support this idea.

CHAPTER 7

CONCLUSIONS

Many plant processes occur as a result of interactions between positive and negative stimuli. Organ formation and male:female sex determination are examples and floral evocation also seems to fall into this category. The results of several earlier investigators have been extended by extensive experimentation in this research, thus indicating that analytical technology has advanced sufficiently to allow additional progress to be made toward the characterization of the florigenic stimulus, florigen. Earlier failures in the characterization of florigen may not only have resulted from inadequate analytical technology, but possibly also from the lack of a rapid, reliable and sensitive bioassay for flowering. Establishing such an assay was one objective which the present study accomplished.

Flowering seems to be controlled by a complex series of switches. If enough of these are flipped (i.e., more promoters and/or less inhibitors) then flowering results. However, if the critical number of switches are not triggered, then the apical meristem remains vegetative. Even with advances in technology, a general understanding of the partial processes which control floral evocation is essential to understanding the process as a whole. That is, the most effective manipulations of flowering can best occur when research has led to a thorough understanding of the flowering process. My thesis research has sought to come to grips with several of these partial processes, including those that may be influenced or controlled by ABA, ACC, ethylene, GAs and IAA, and by substances of the flavenoid/phenolic class.

Through flowering bioassay trials I examined three substances which could inhibit a 16 h long night-induced floral evocation. These were ABA, ACC and IAA. While ABA was only partially inhibitory, both IAA and ACC caused total inhibition of floral evocation at a high dose (100 μg/plant). Inhibition by ACC appeared to result from elevated levels of ethylene (as supported by STS prevention [or reversal] of ACC inhibition). Inhibition by IAA did not appear to be completely due to an IAA-enhanced ethylene production.

Gibberellins as a general rule were promotive of floral evocation under marginally-inductive long nights (SD), with those GAs of the non-hydroxylation pathway (e.g., GA_4, GA_7, GA_9 and a derivative of GA_4, 2,2-dimethyl GA_4) being highly promotive. Chlorogenic acid, one of the phenolics tested in this experiment, was also highly florigenic under marginally-inductive long night conditions.

Analysis of endogenous hormones showed that GA_1 levels decreased in both leaves and apices after a single long night (SD) cycle. This decrease appeared to be, at least in part due to decreased GA_1 synthesis from GA_{20}. Additionally, the amount of GA_8, the 2ß-hydroxylated inactivation product of GA_1, decreased in leaves after long nights (SD). In apices levels of both GA_8 and GA_{20} were substantially lower under after long nights (SD) than under LD. Biosynthesis of $[^3H]GA_1$ from $[^3H]GA_{20}$ and $[^3H]GA_8$ through $[^3H]GA_1$ was also enhanced under LD in leaves. These results indicate that the reduced petiole and epicotyl growth under SD may be caused by a decreased level of GA_1. Additionally, the level of GA_4 in leaves and apices was increased substantially after a single long night (SD) cycle, relative to LD. Since applied GA_4 and related GAs are the most florigenic (under marginally inductive long nights) of this hormone class, it is possible that endogenous GA_4 may be a part of the floral evocation stimulus, although it is not a florigen per se under totally non-inductive LD.

Work with GA biosynthesis inhibitors resulted in either partial inhibition of floral evocation e.g. delayed floral development (2 g/L uniconazole and CCC at all concentrations), total inhibition of this process (3 g/L uniconazole) or no inhibition at all (BAS 111..W and ancymidol). These effects might suggest that GA levels must decrease below a critical level to completely inhibit floral initiation. This is supported by the fact that uniconazole-induced inhibition (3 g/L) was reversed by the application of $GA_{4/7/9}$ (62:30:8) mixture. The GA biosynthesis inhibitor results thus confirm the conclusion that GAs are important components in floral evocation, but shed no light on the question of whether GAs are florigen(s) per se for this SD plant.

Future research will be needed in order to optimize the interactions of these partial processes. In the author's opinion the final composition of a florigen for SDP such as Xanthium may include: (1) a component which causes a reduction in the levels of elongation-promoting GAs, (2) a component which is involved in initially activating (inducing) the floral evocation process, (3) a component which transiently reduces the level of IAA (and thus ethylene) in situ to reduce apical dominance in the induced plant, and finally (4) a component which is important in the continued development of the floral apex. Components 1 and 4 may be related by simple changes in GA metabolism. That is, a decrease in the level of GA_1 may simply result from the decreased activity of the GA_{20} C-3ß-hydroxylase, with a concomitant increase in the level of GA_4 (either through enhanced 3ß-hydroxylation of GA_9, reduced 2ß-hydroxylation to GA_{34}, reduced conjugation of GA_4 or a combination of all of these). The increased level of GA_4 may be important in the early development of the floral apex.

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APPENDIX I

EFFECT OF A LOG DOSE OF EXOGENOUSLY-APPLIED GAS ON PETIOLE AND EPICOTYL GROWTH OF XANTHIUM STRUMARIUM—Results of Three Replicate Experiments

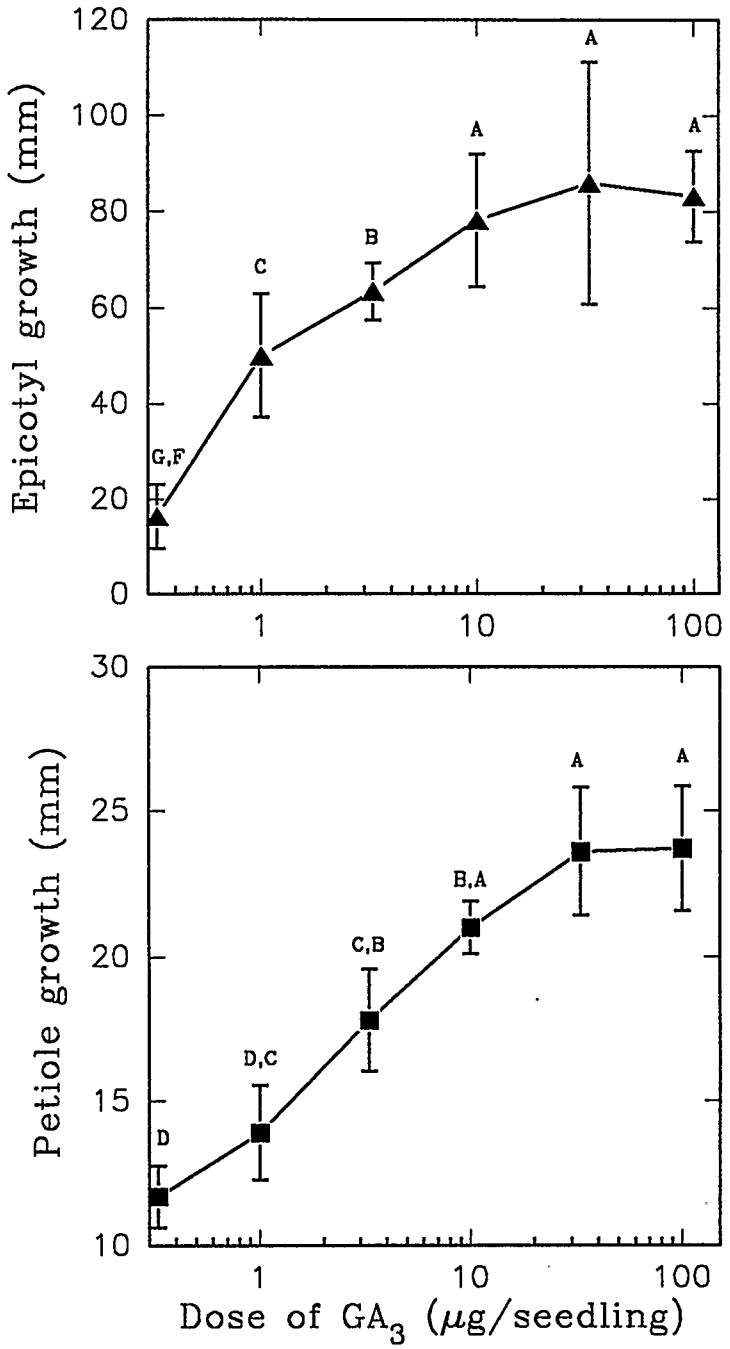

Figure A-1 Epicotyl and petiole growth in response to an increasing log dose of GA_3 applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

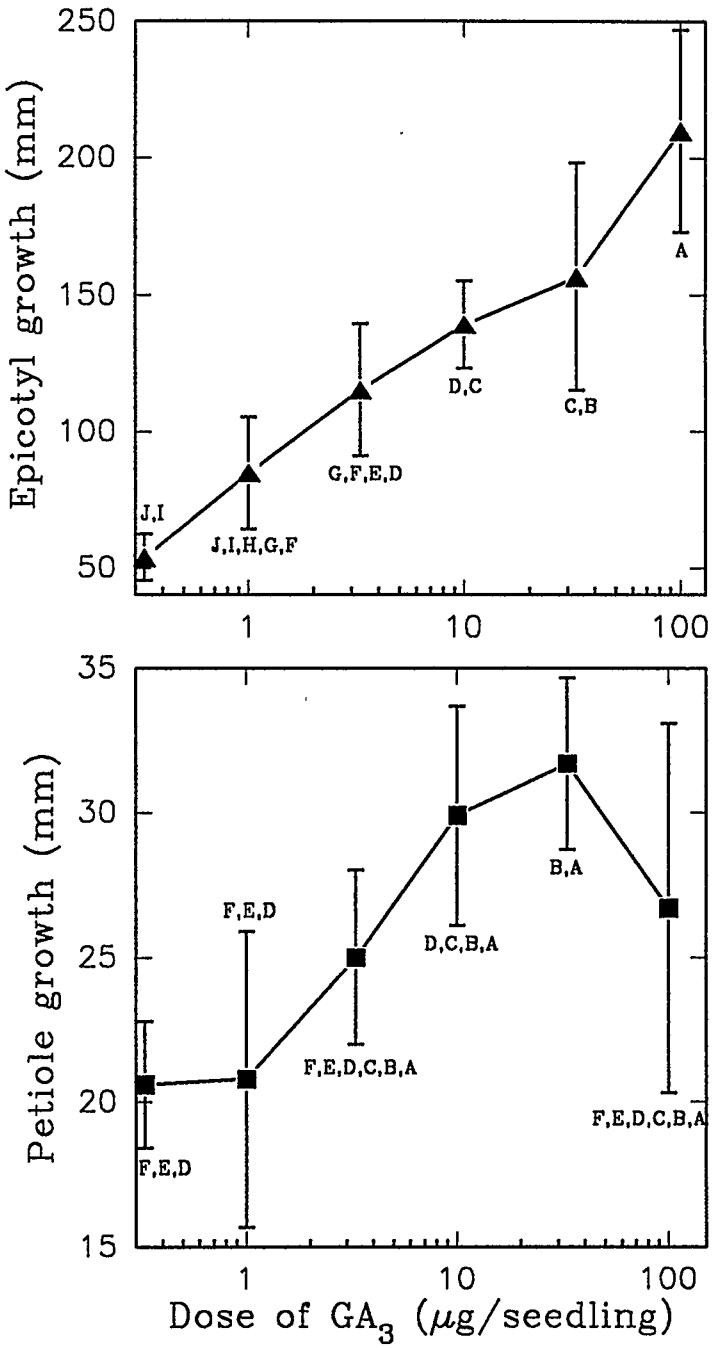

Figure A-2 Epicotyl and petiole growth in response to an increasing log dose of GA_3 applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

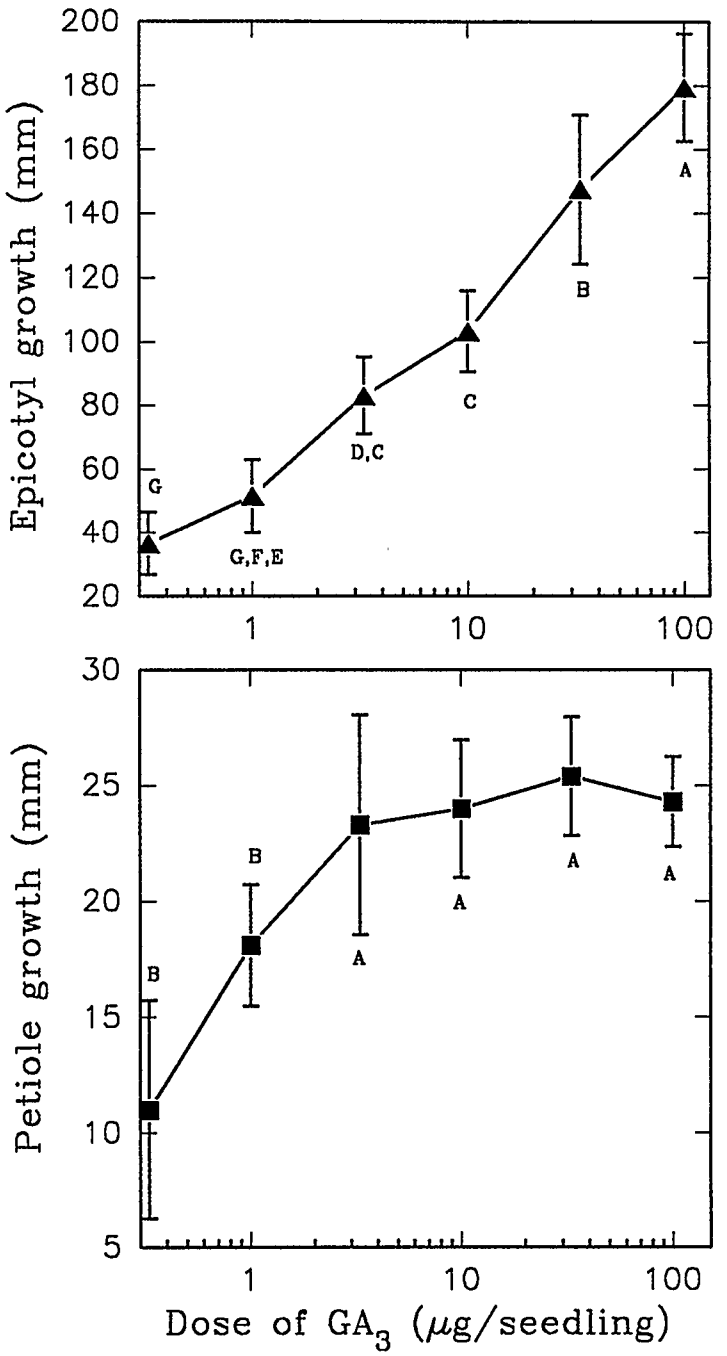

Figure A-3 Epicotyl and petiole growth in response to an increasing log dose of GA_3 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

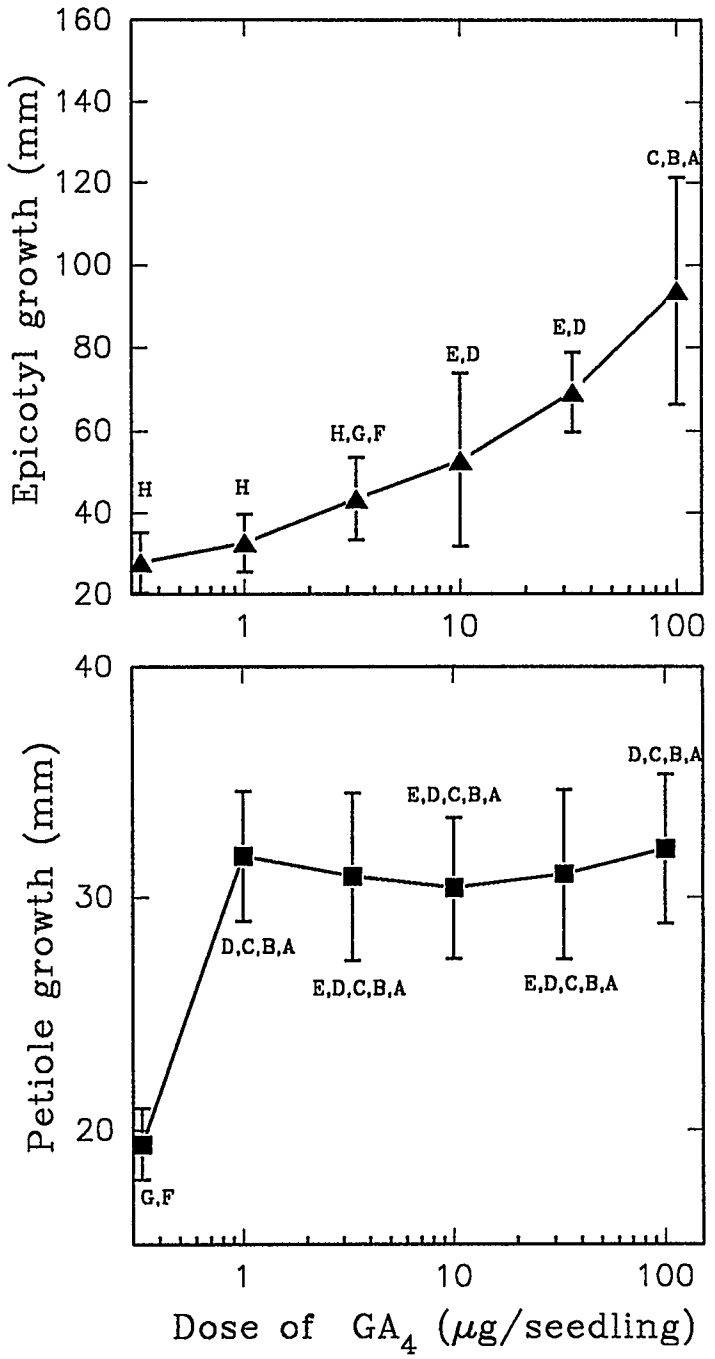

Figure A-4 Epicotyl and petiole growth in response to an increasing log dose of GA_4 applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

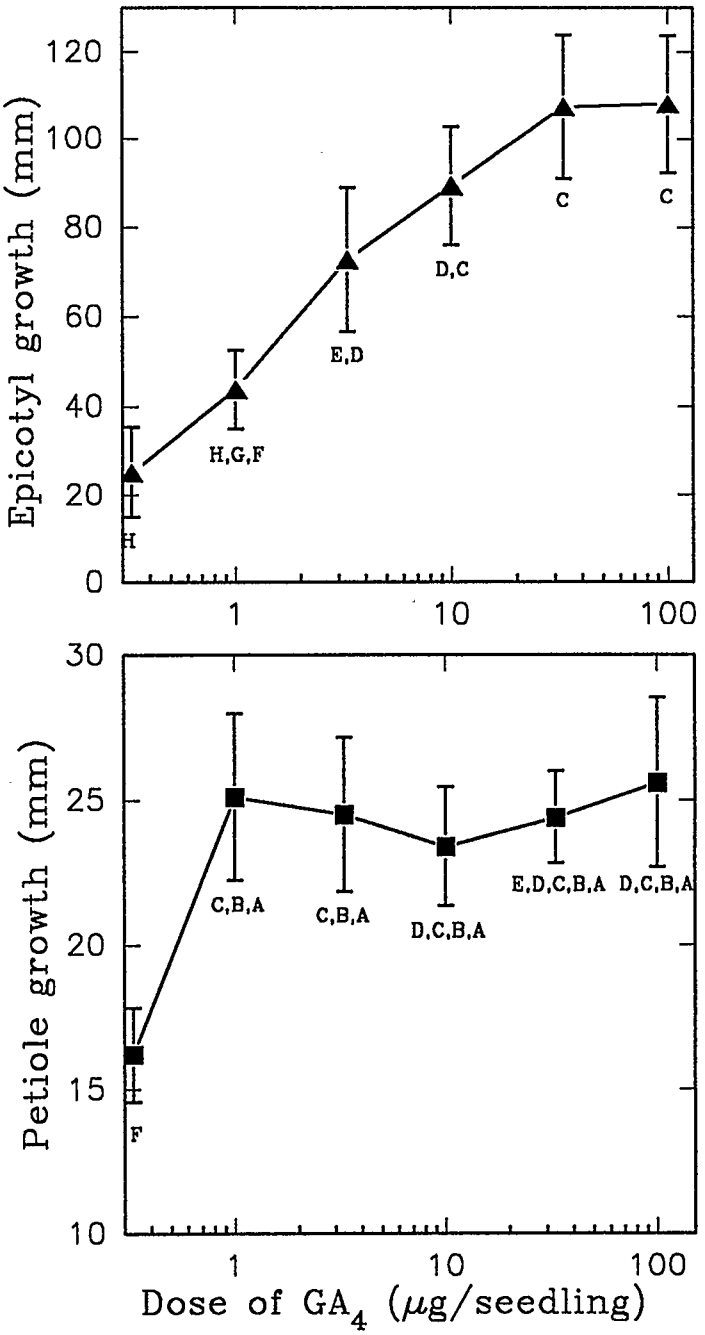

Figure A-5 Epicotyl and petiole growth in response to an increasing log dose of GA_4 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

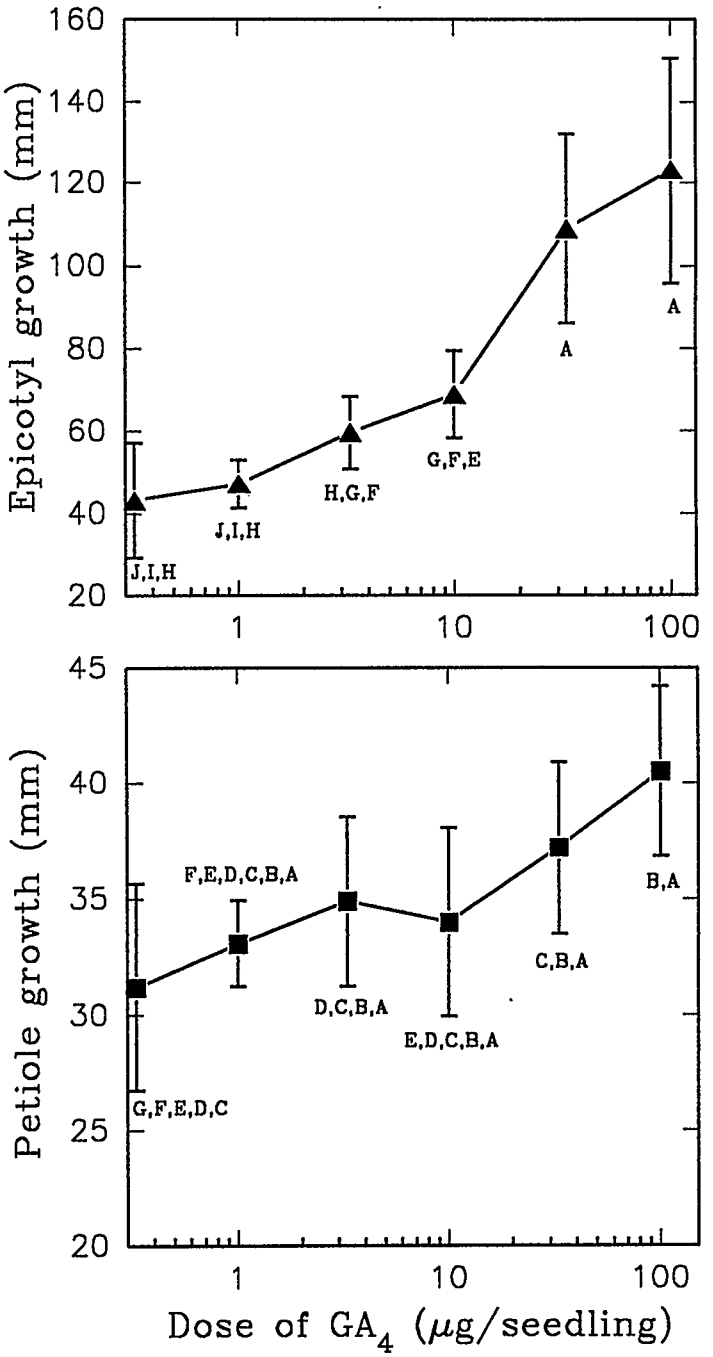

Figure A-6 Epicotyl and petiole growth in response to an increasing log dose of GA_4 applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

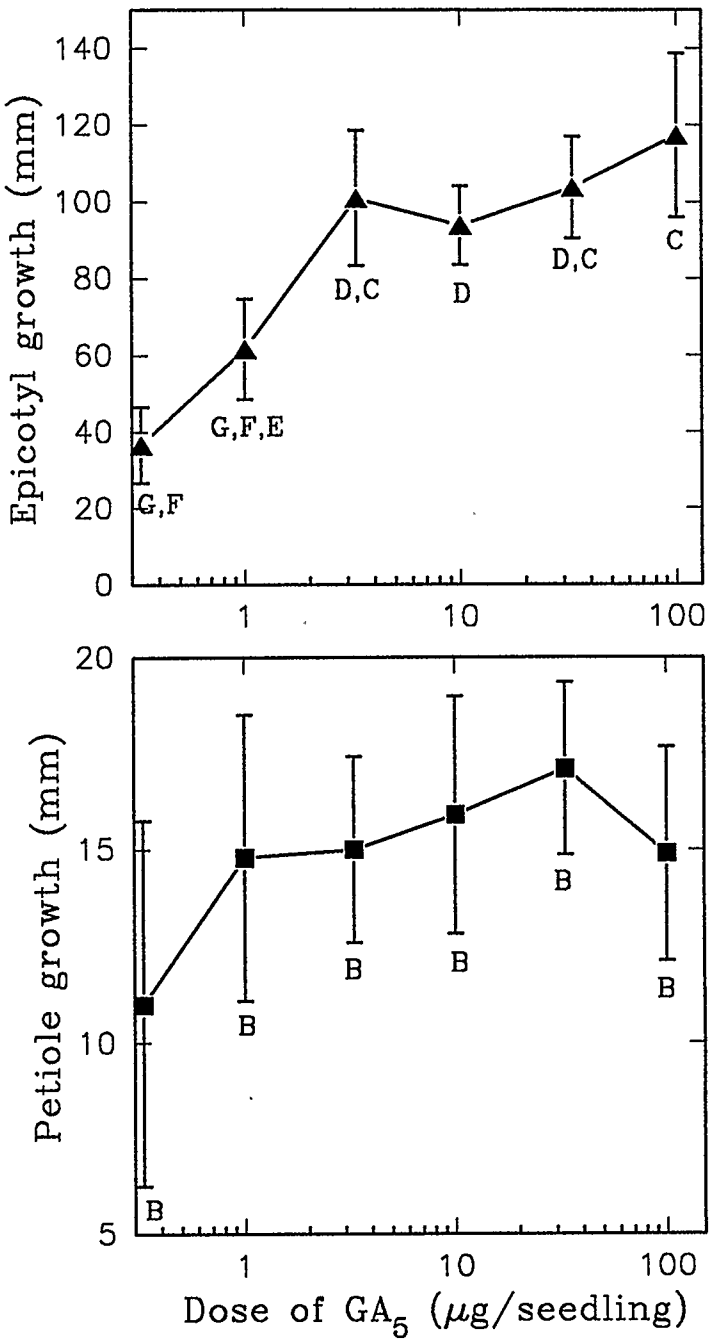

Figure A-7 Epicotyl and petiole growth in response to an increasing log dose of GA_5 applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

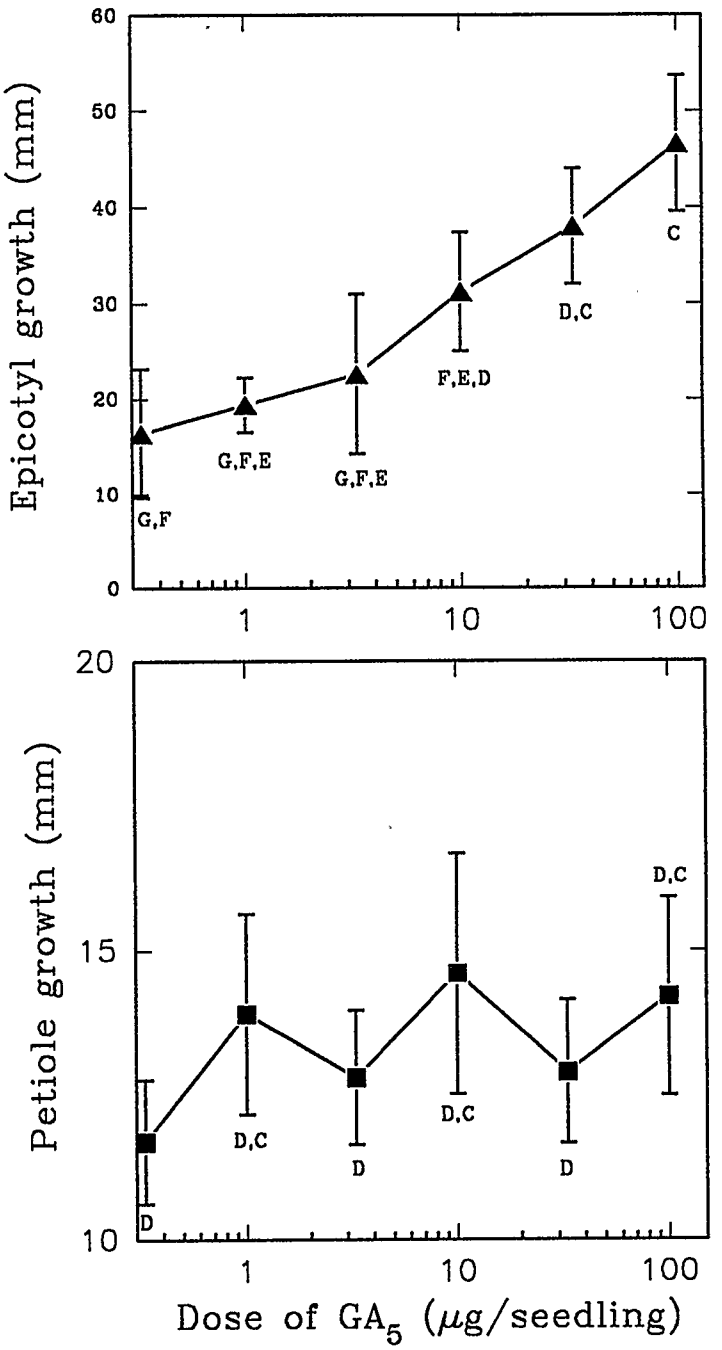

Figure A-8 Epicotyl and petiole growth in response to an increasing log dose of GA_5 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

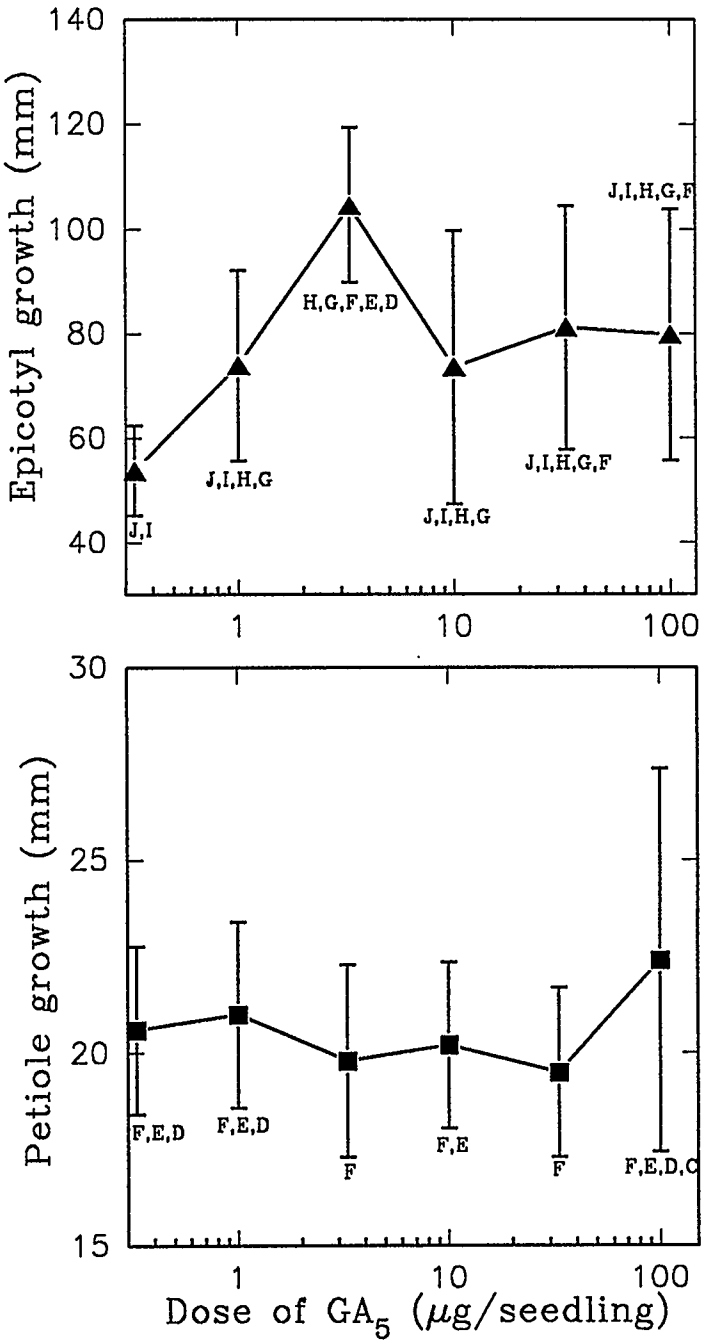

Figure A-9 Epicotyl and petiole growth in response to an increasing log dose of GA_5 applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

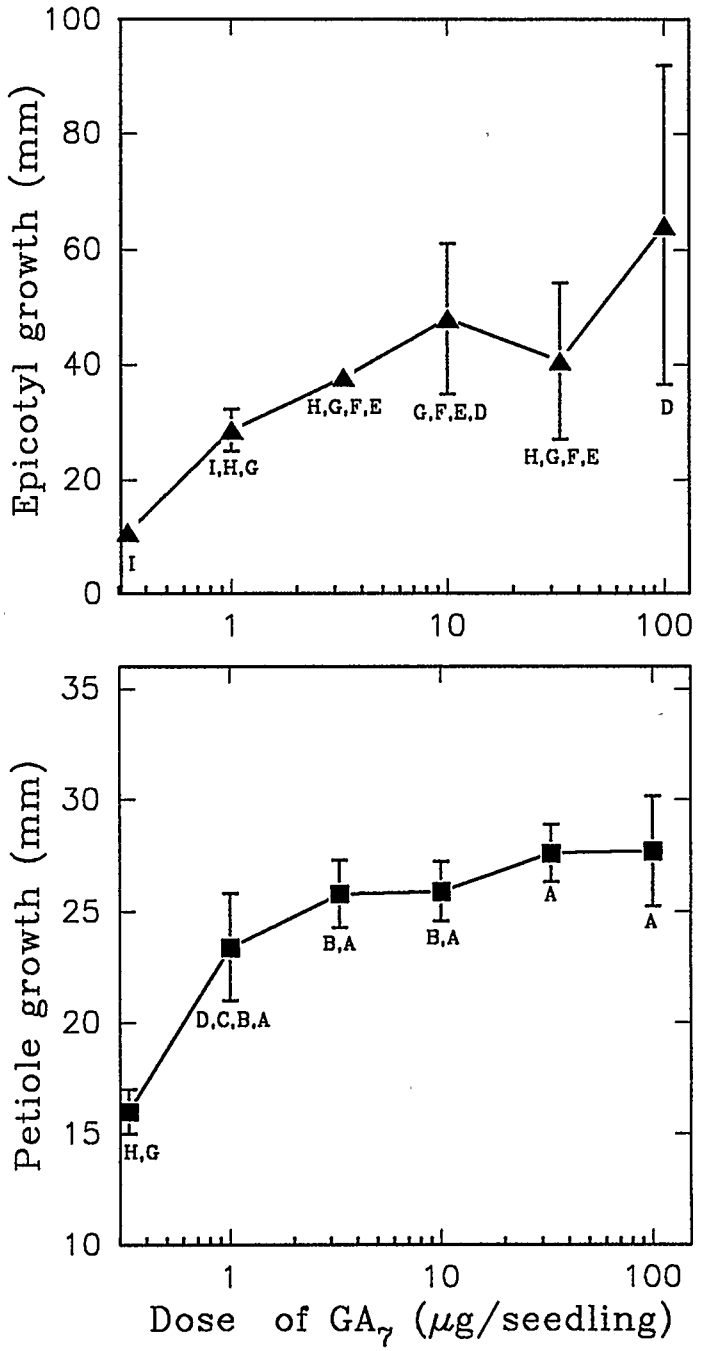

Figure A-10 Epicotyl and petiole growth in response to an increasing log dose of GA_7 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

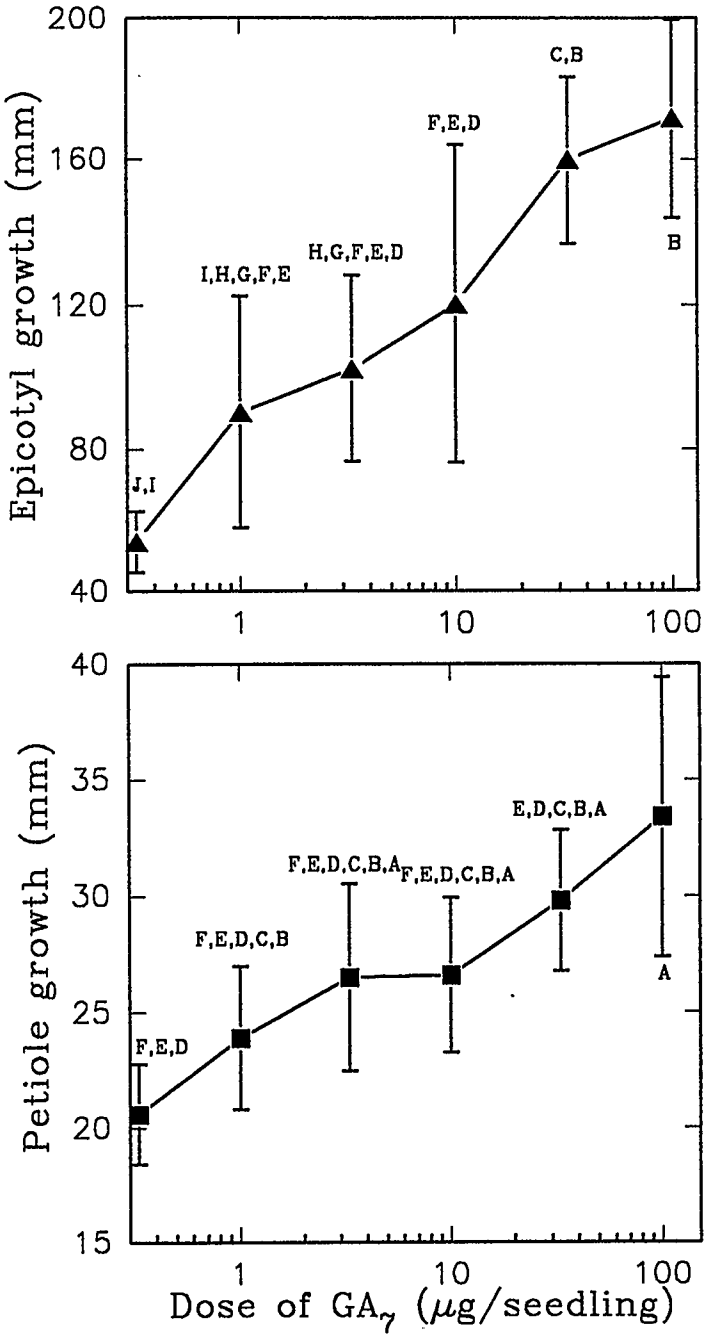

Figure A-11 Epicotyl and petiole growth in response to an increasing log dose of GA_7 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

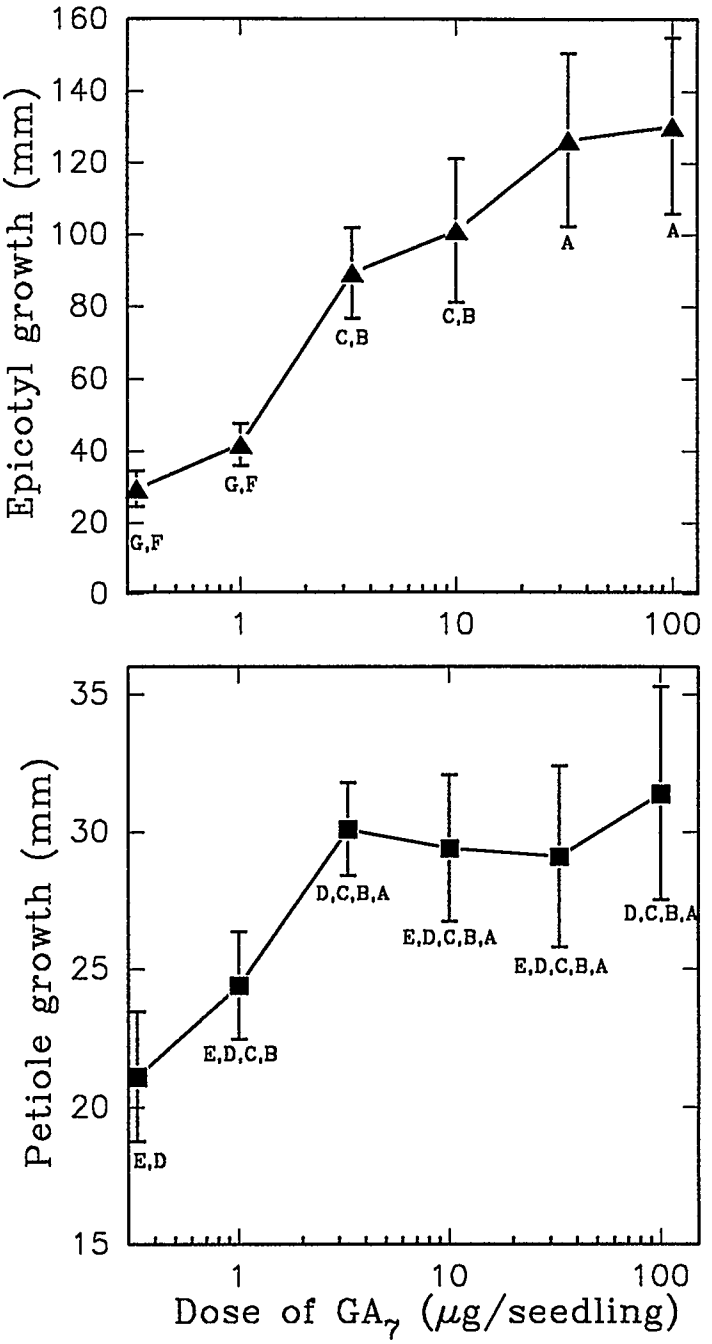

Figure A-12 Epicotyl and petiole growth in response to an increasing log dose of GA_7 applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

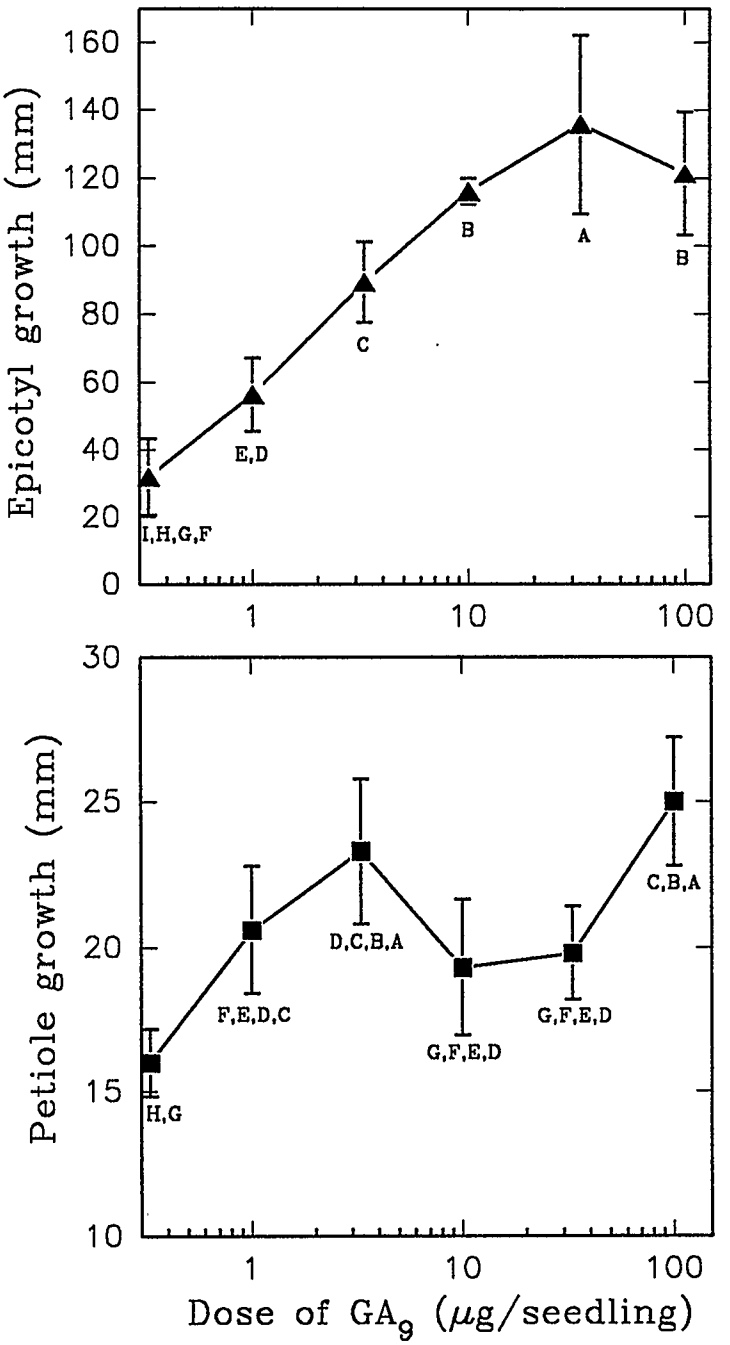

Figure A-13 Epicotyl and petiole growth in response to an increasing log dose of GA_9 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

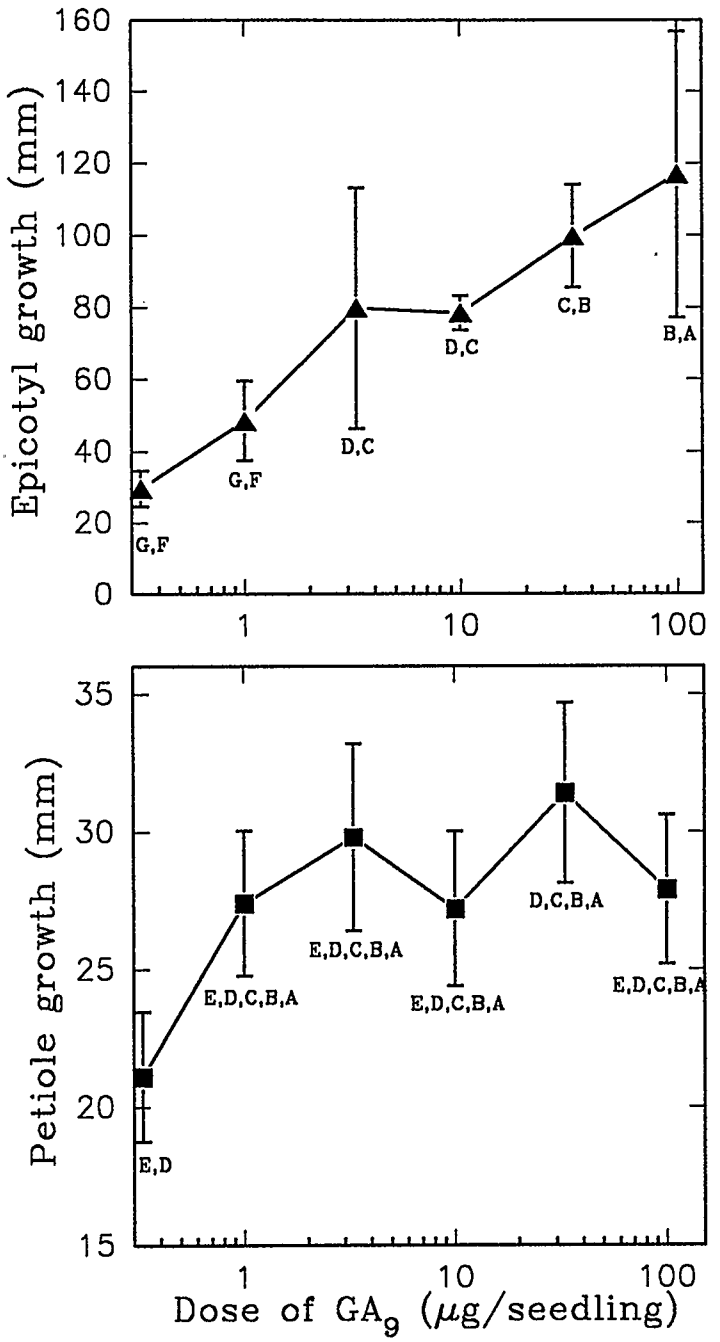

Figure A-14 Epicotyl and petiole growth in response to an increasing log dose of GA_9 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

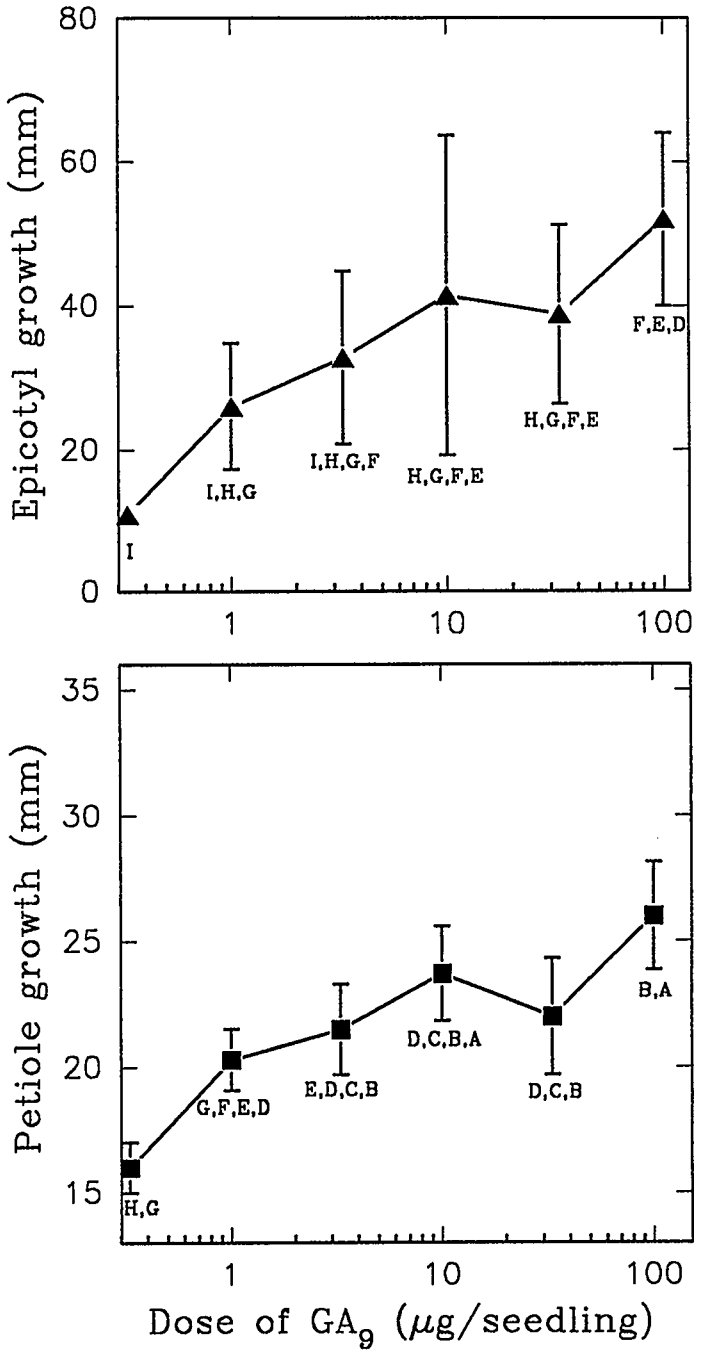

Figure A-15 Epicotyl and petiole growth in response to an increasing log dose of GA_9 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

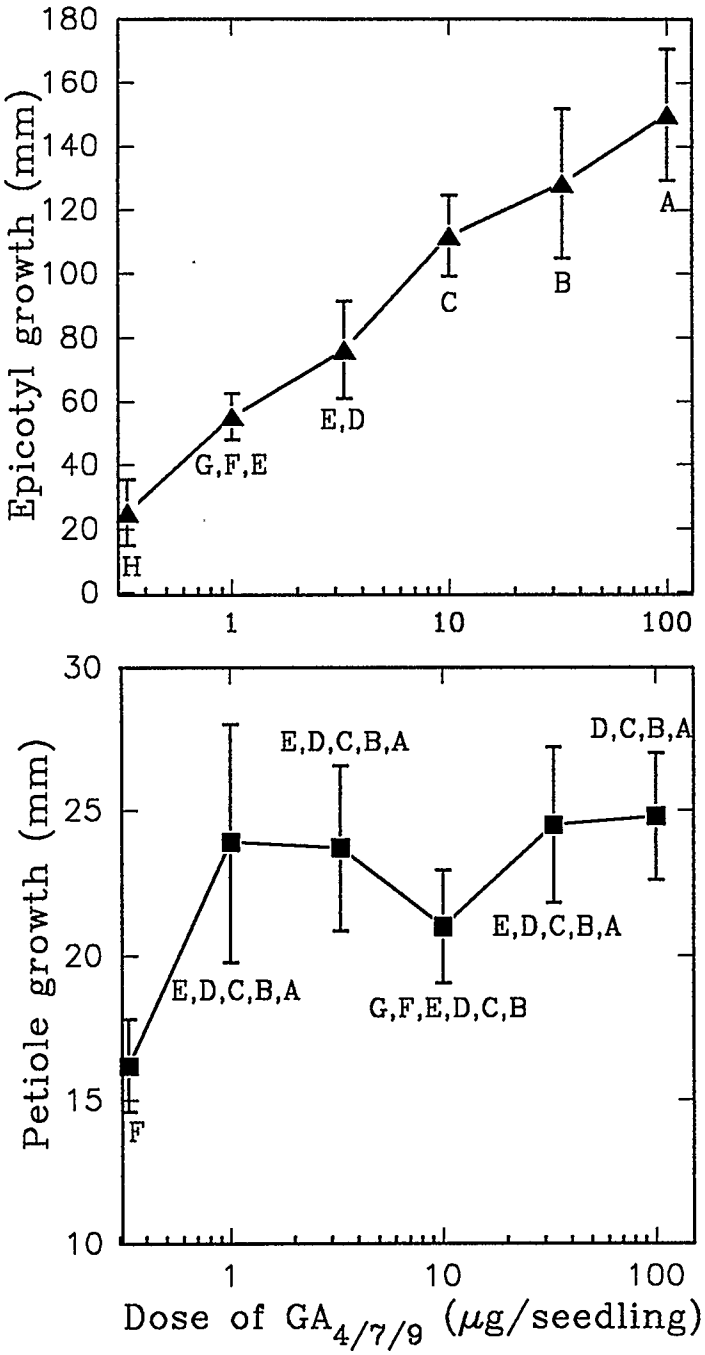

Figure A-16 Epicotyl and petiole growth in response to an increasing log dose of $GA_{4/7/9}$ (62:30:8) applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

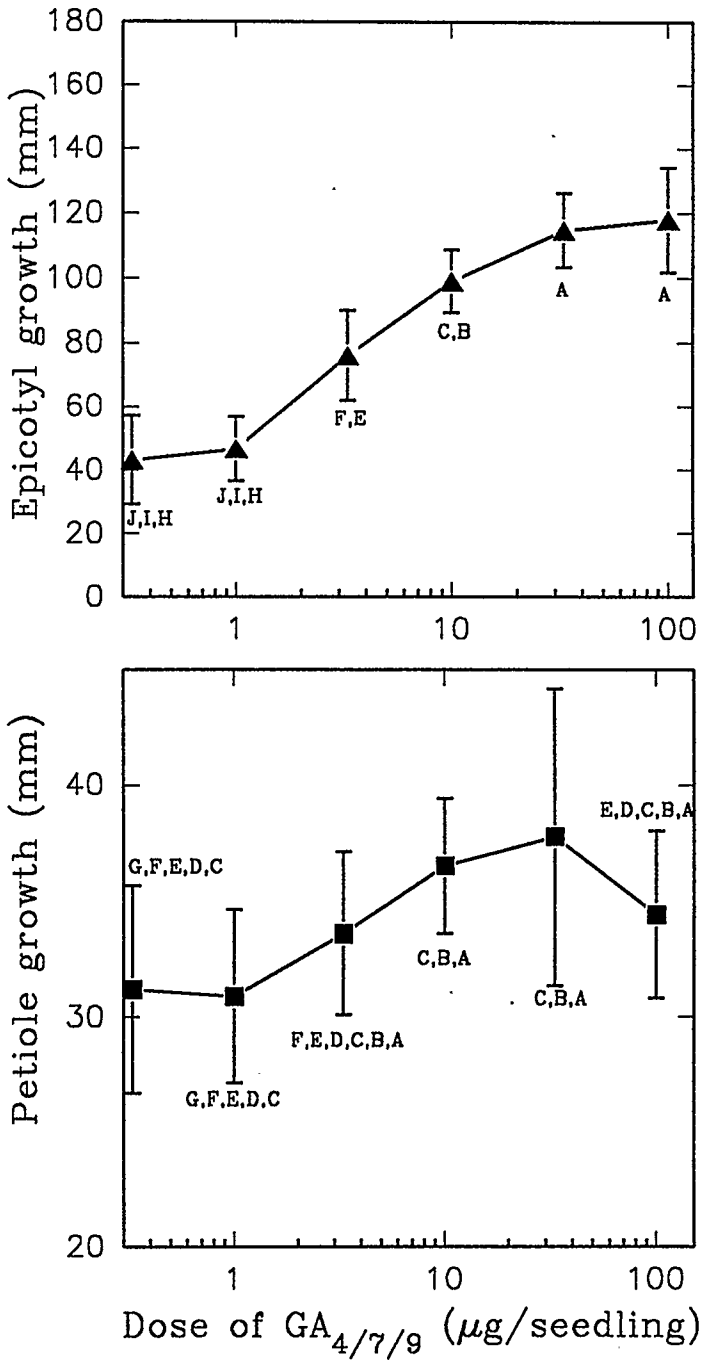

Figure A-17 Epicotyl and petiole growth in response to an increasing log dose of $GA_{4/7/9}$ (62:30:8) applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

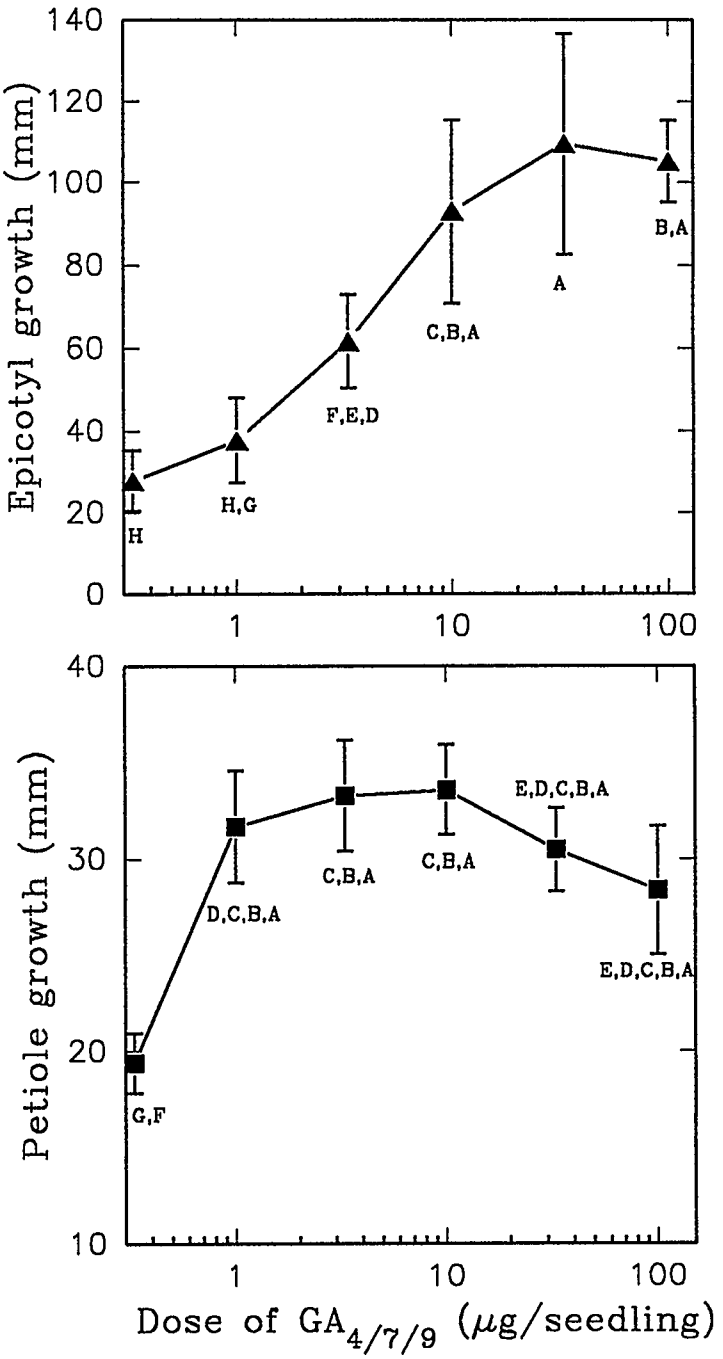

Figure A-18 Epicotyl and petiole growth in response to an increasing log dose of $GA_{4/7/9}$ (62:30:8) applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

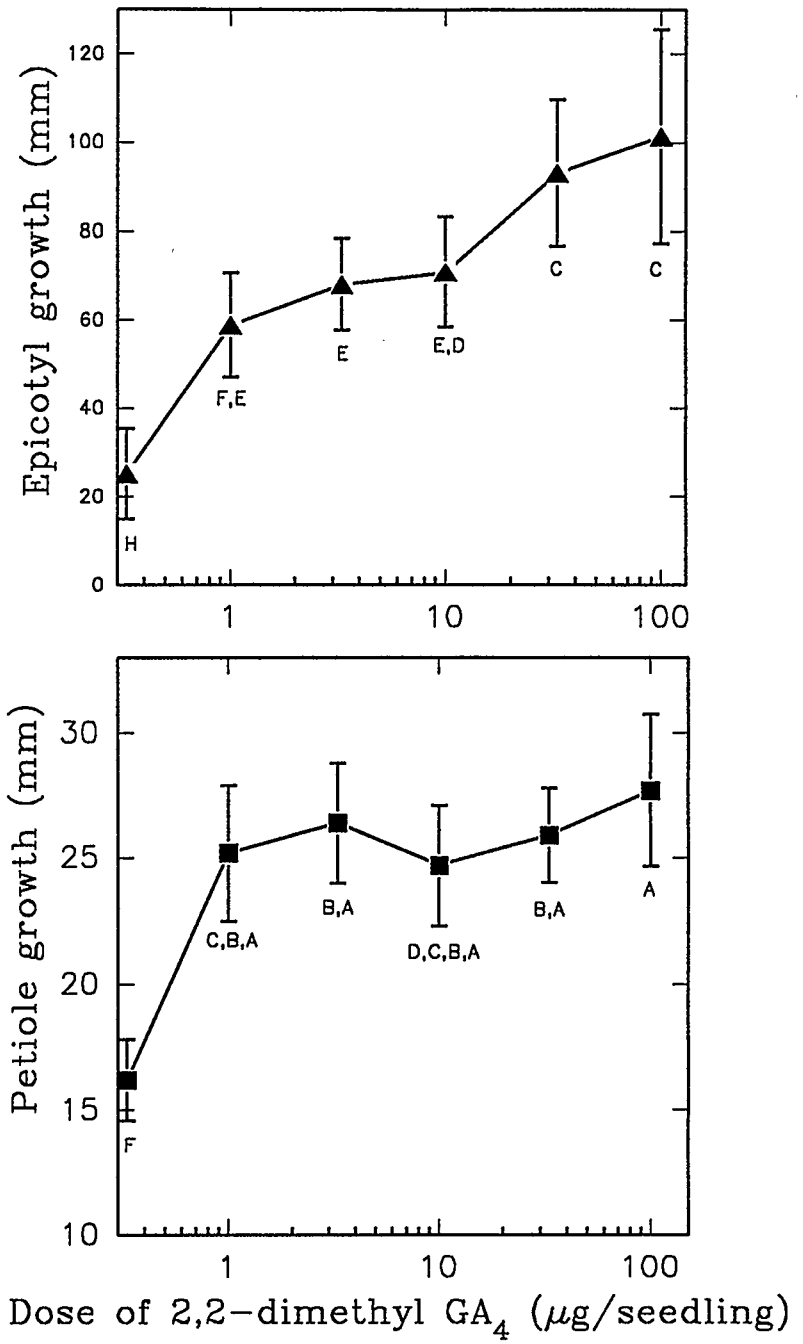

Figure A-19 Epicotyl and petiole growth in response to an increasing log dose of 2,2-dimethyl GA_4 applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

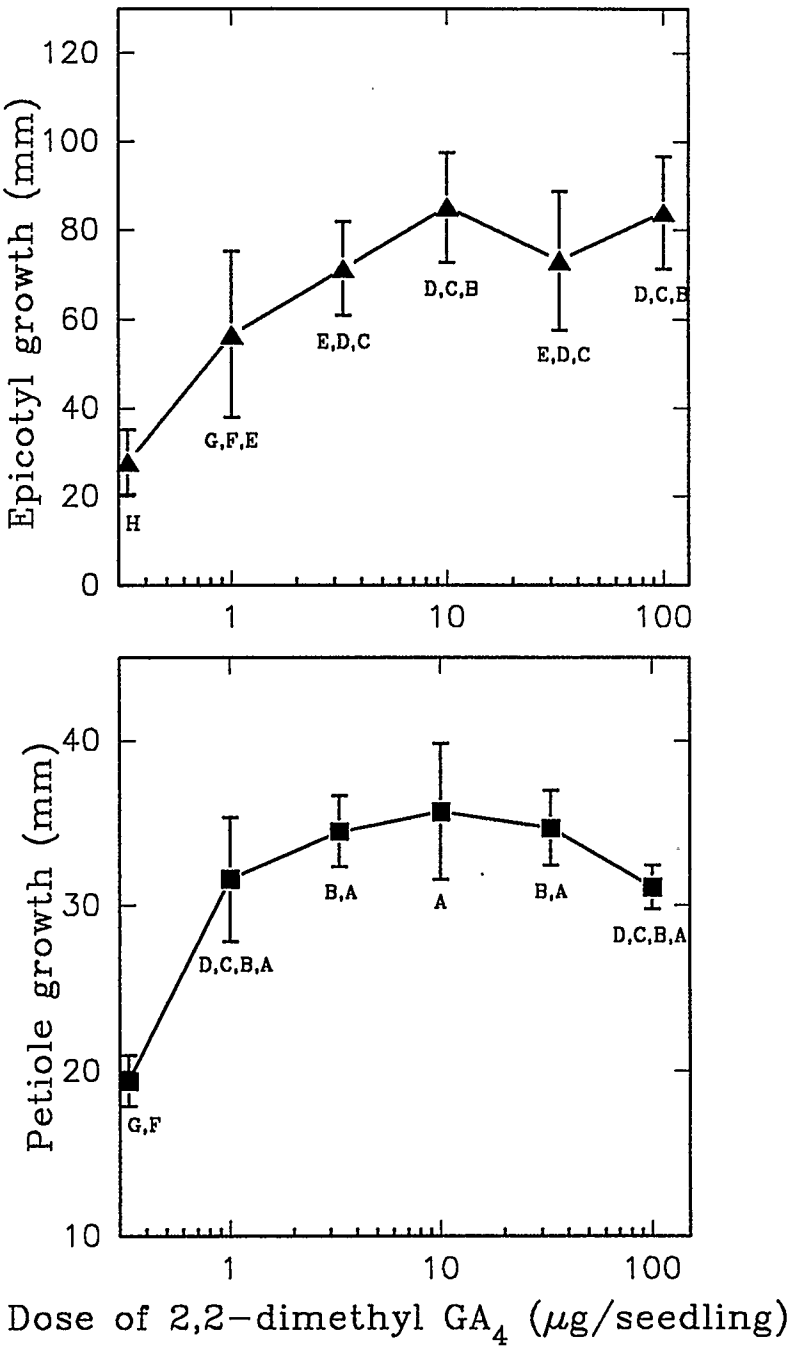

Figure A-20 Epicotyl and petiole growth in response to an increasing log dose of 2,2-dimethyl GA_4 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

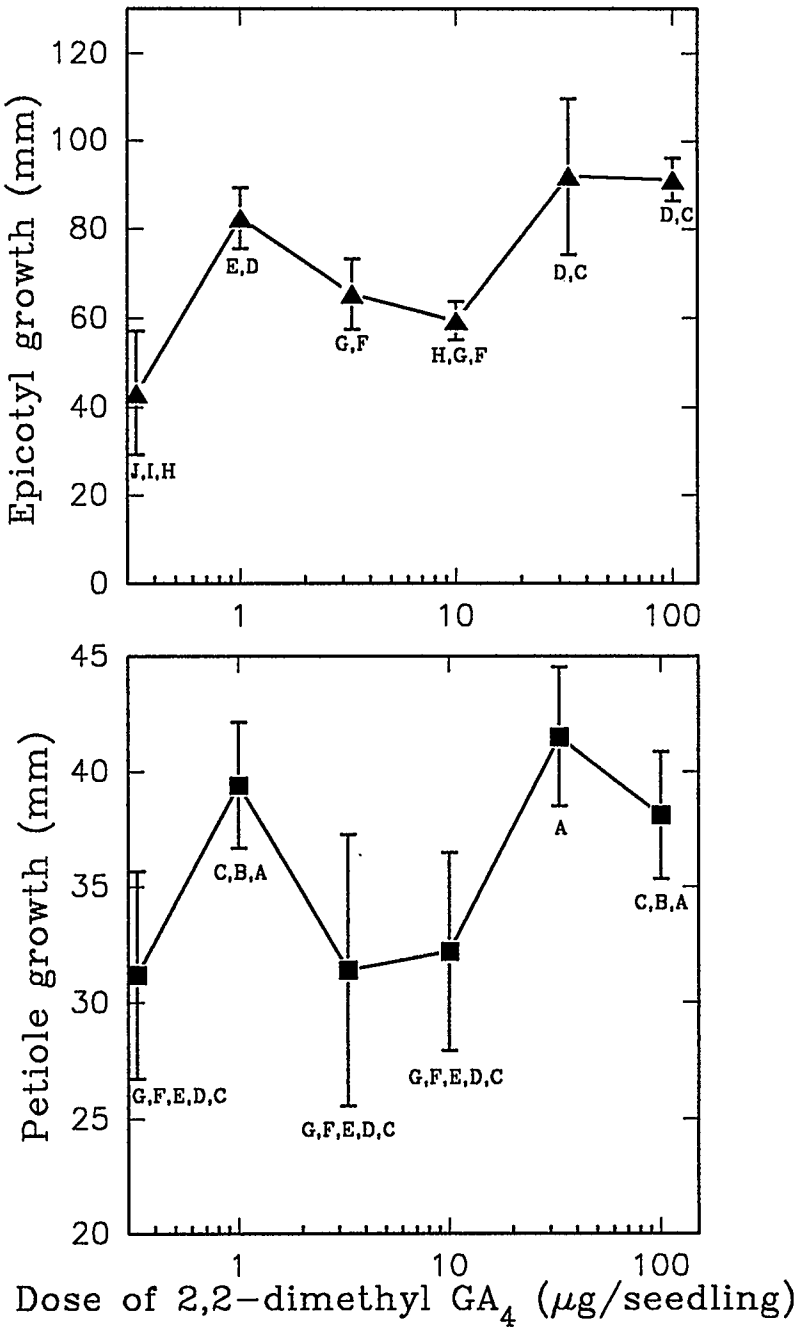

Figure A-21 Epicotyl and petiole growth in response to an increasing log dose of 2,2-dimethyl GA_4 applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

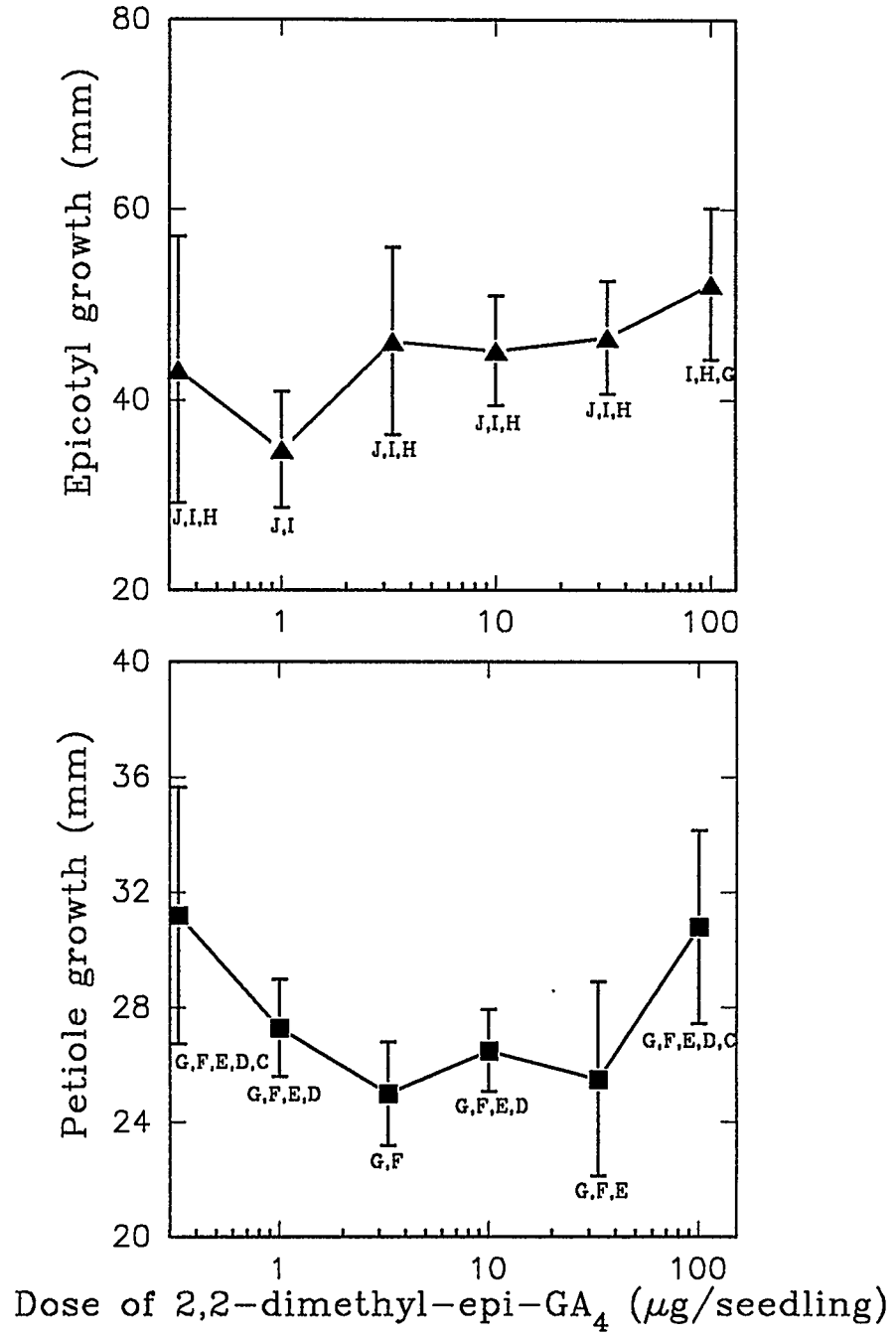

Figure A-22 Epicotyl and petiole growth in response to an increasing log dose of 2,2-dimethyl-epi-GA_4 applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

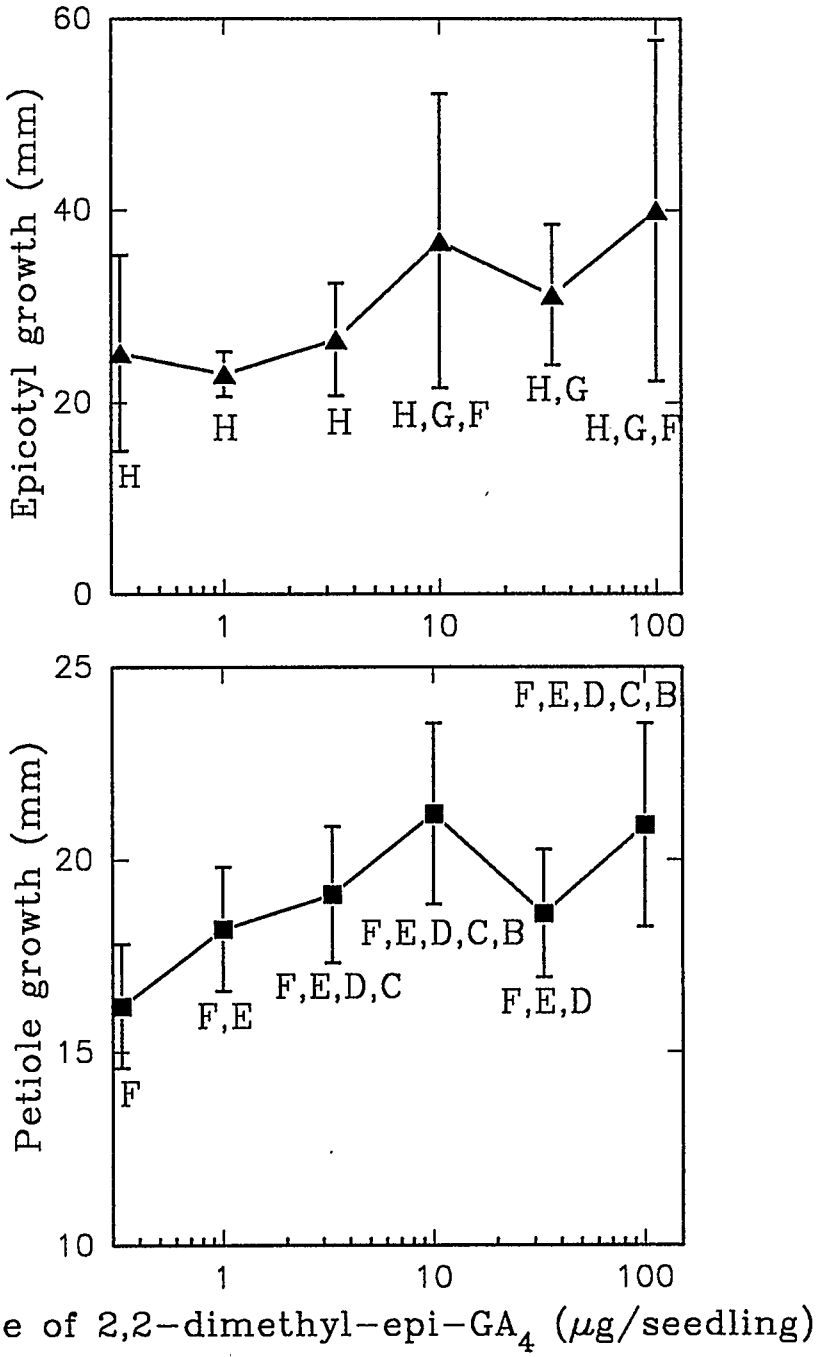

Figure A-23 Epicotyl and petiole growth in response to an increasing log dose of 2,2-dimethyl-epi-GA_4 applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

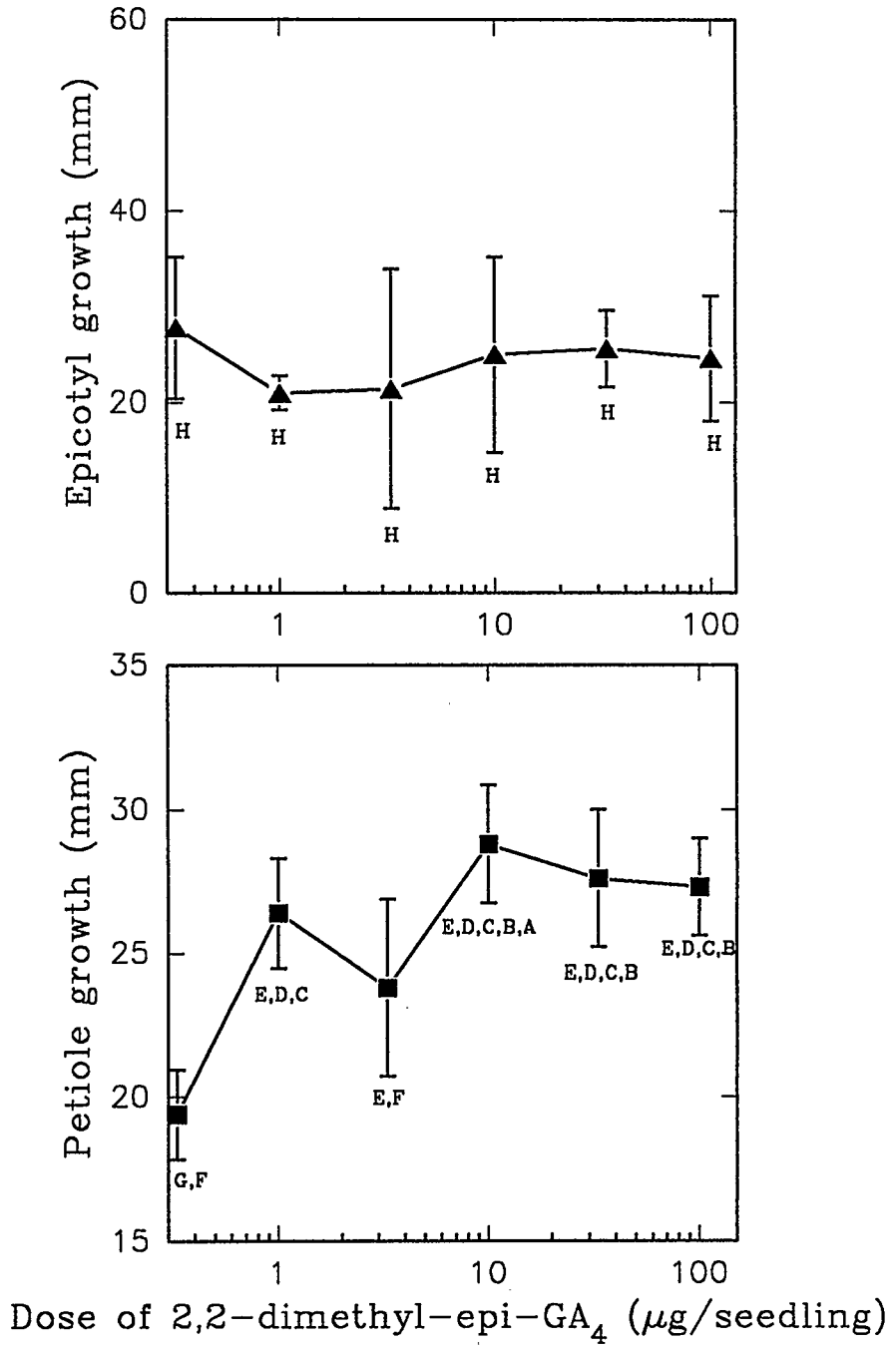

Figure A-24 Epicotyl and petiole growth in response to an increasing log dose of 2,2-dimethyl-epi-GA_4 applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings measured 9 days post-induction. Error bars represent 95% confidence intervals. Letters above the bars indicate significance groupings ($P \leq 0.05$) via the Student Newman-Keuls test.

APPENDIX II

EFFECT OF A LOG DOSE OF EXOGENOUSLY-APPLIED GAS ON FLORAL EVOCATION IN XANTHIUM STRUMARIUM—RESULTS OF THREE REPLICATE EXPERIMENTS

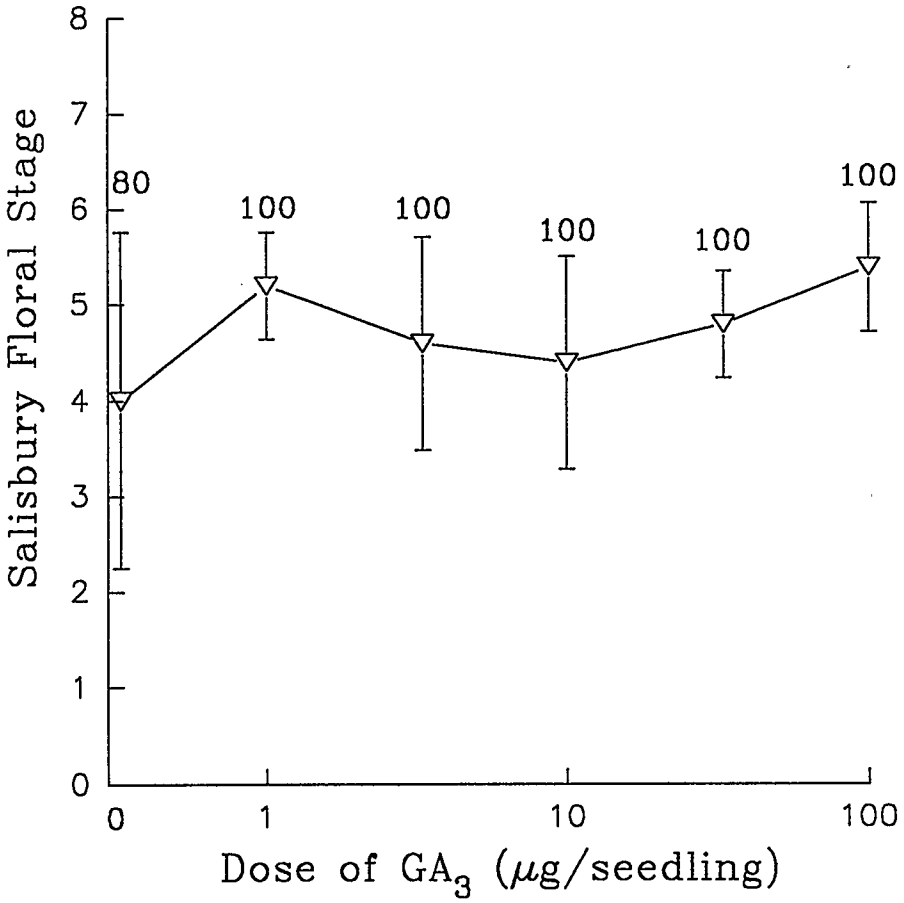

Figure B-1 Efficacy of GA_3 on floral evocation in the Xanthium seedling bioassay. Doses of GA_3 were applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

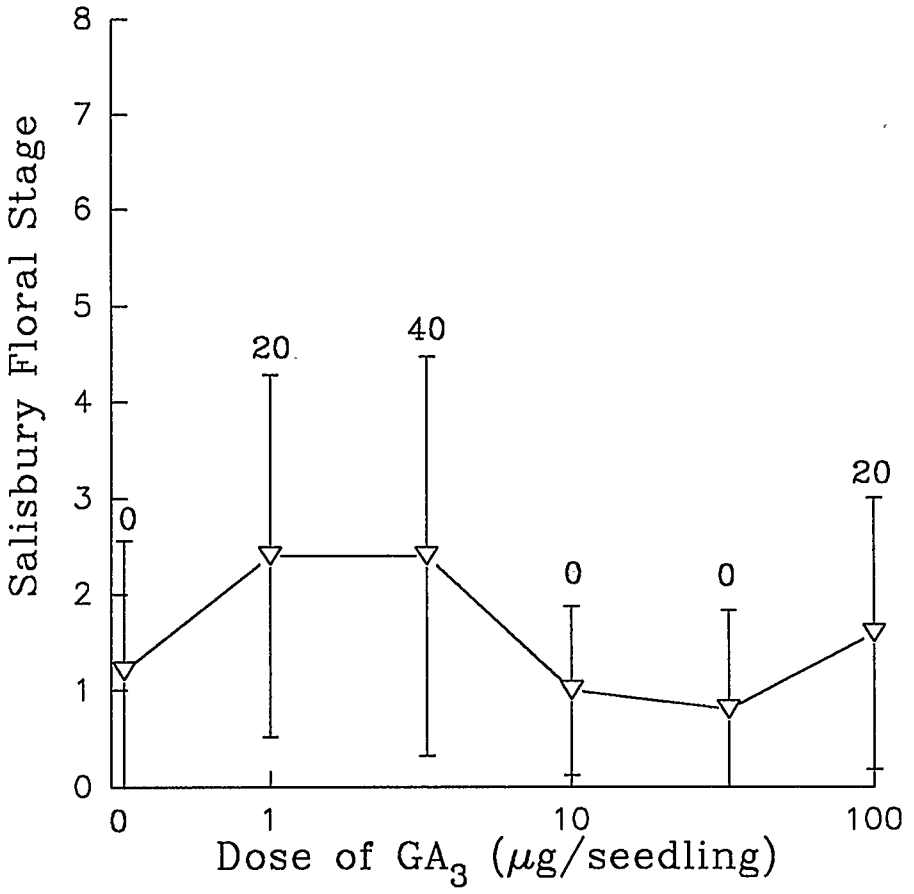

Figure B-2 Efficacy of GA_3 on floral evocation in the Xanthium seedling bioassay. Doses of GA_3 were applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

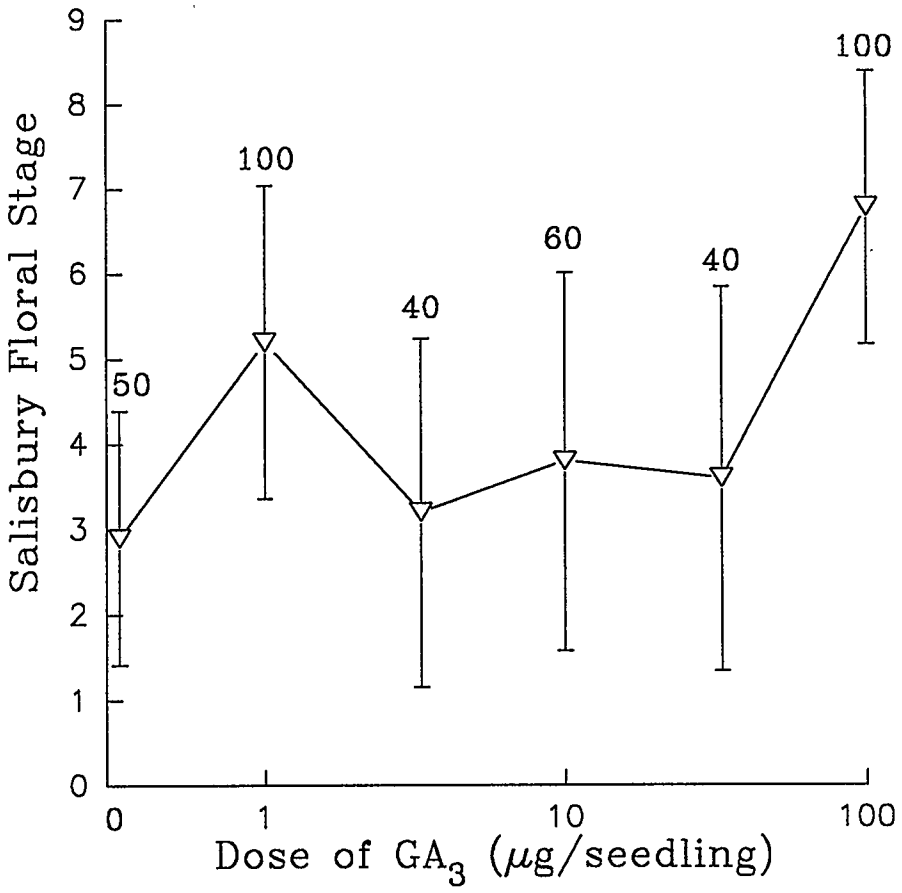

Figure B-3 Efficacy of GA_3 on floral evocation in the Xanthium seedling bioassay. Doses of GA_3 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

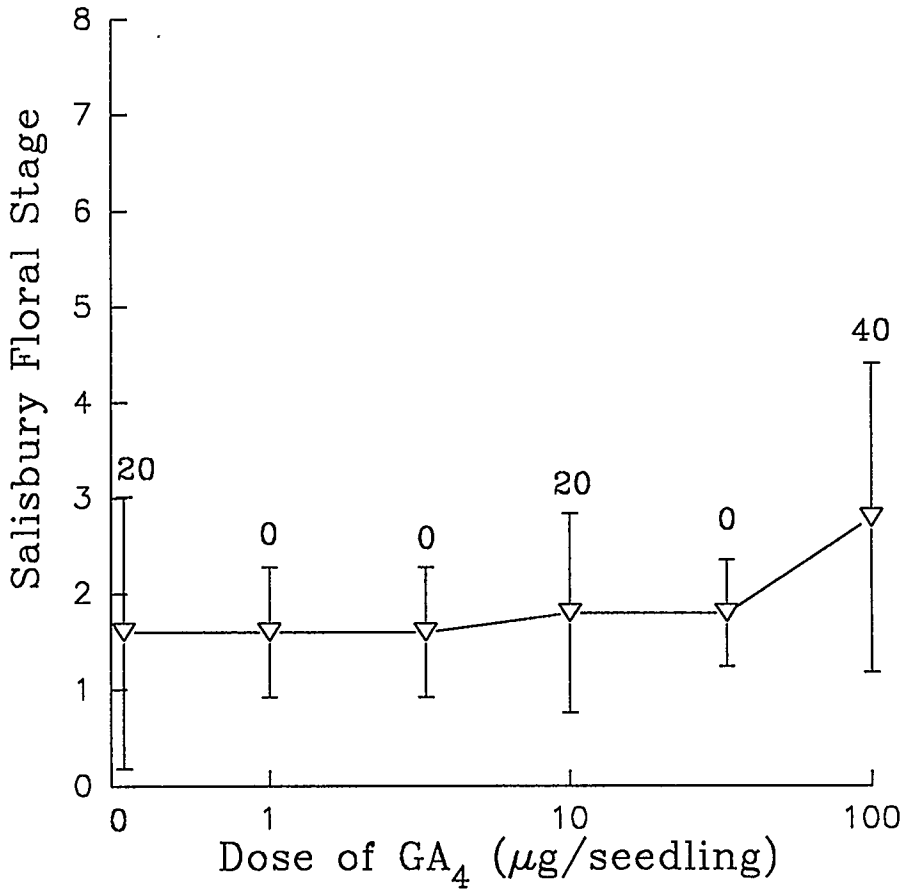

Figure B-4 Efficacy of GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of GA_4 were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

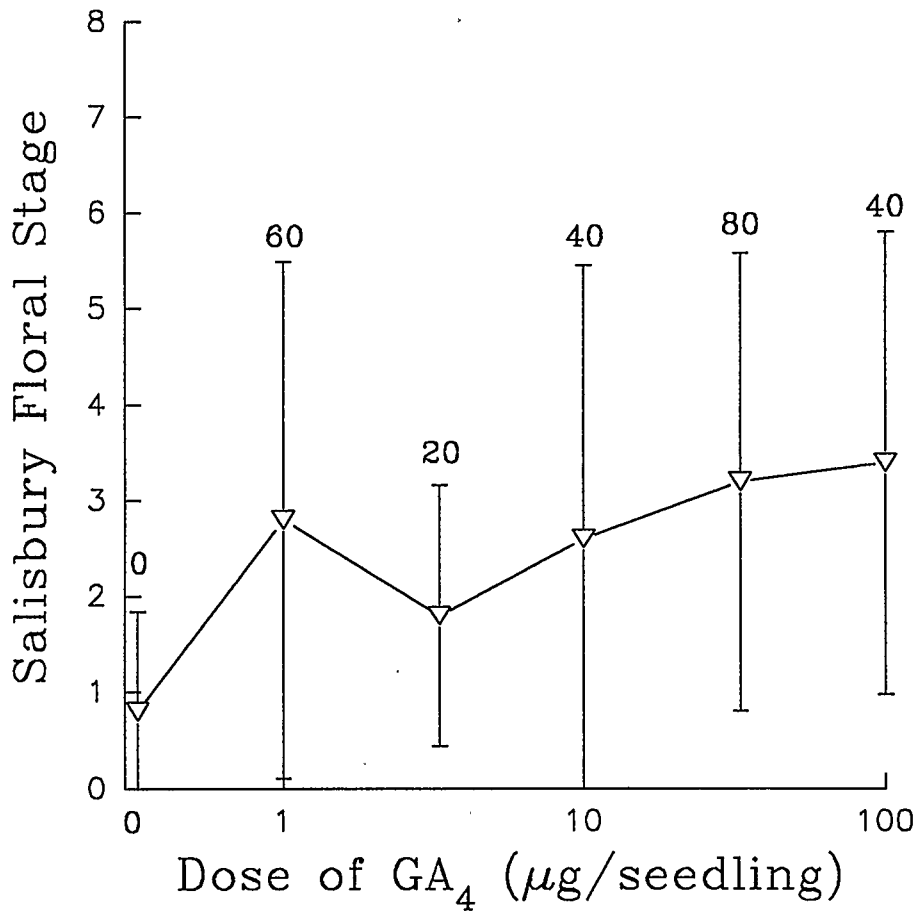

Figure B-5 Efficacy of GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of GA_4 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

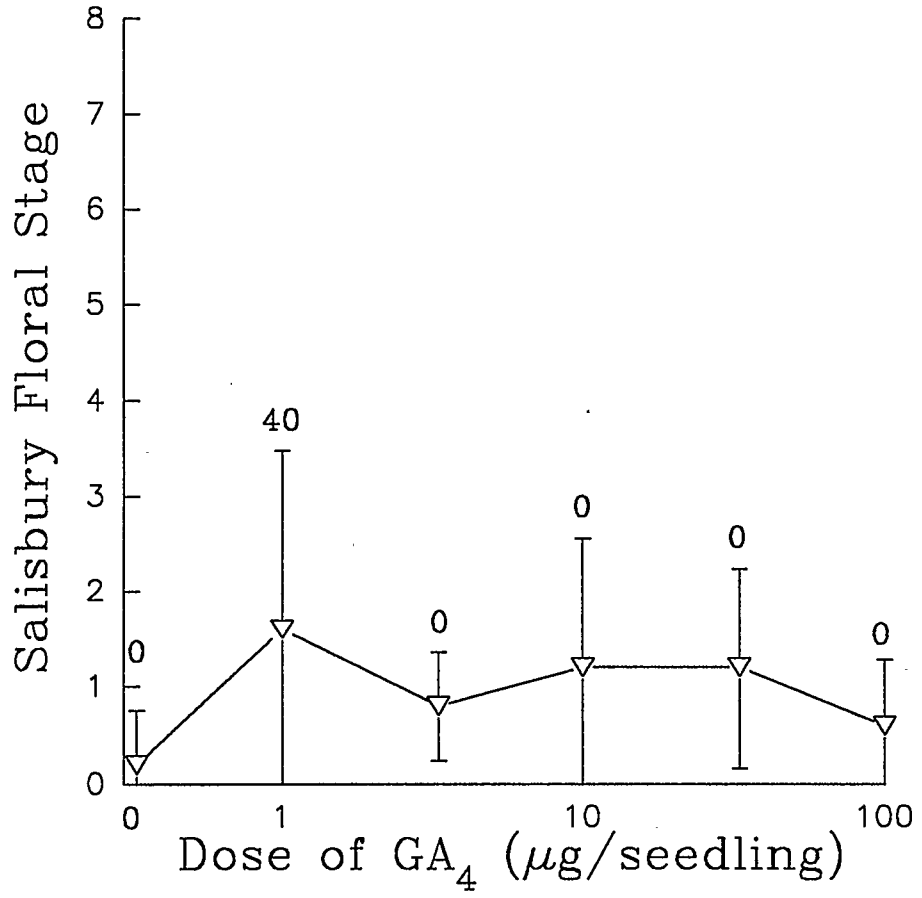

Figure B-6 Efficacy of GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of GA_4 were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

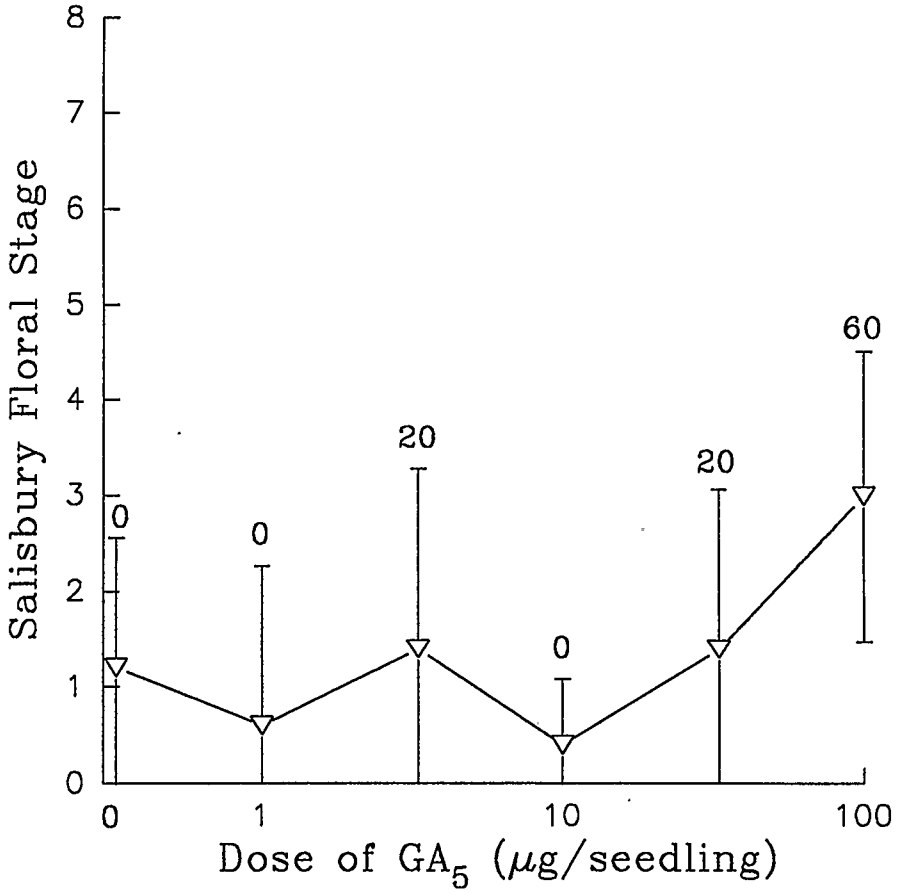

Figure B-7 Efficacy of GA_5 on floral evocation in the Xanthium seedling bioassay. Doses of GA_5 were applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

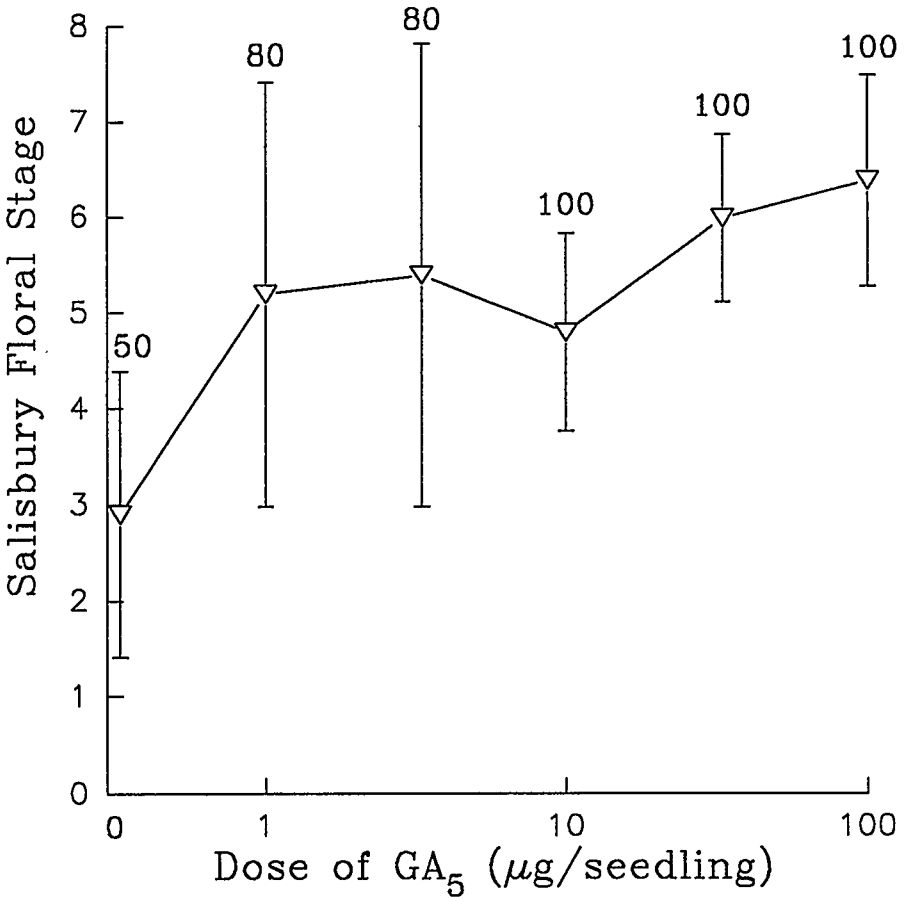

Figure B-8 Efficacy of GA_5 on floral evocation in the Xanthium seedling bioassay. Doses of GA_5 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

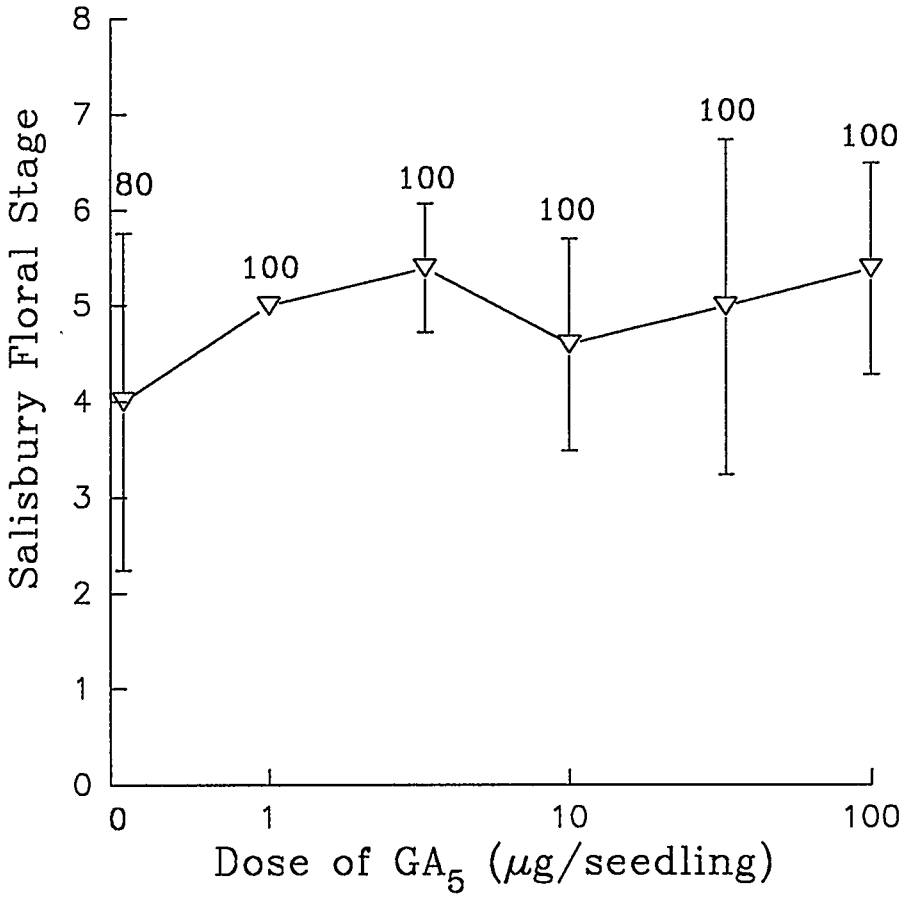

Figure B-9 Efficacy of GA_5 on floral evocation in the Xanthium seedling bioassay. Doses of GA_5 were applied at 1200 h, 8 h before induction with a single marginally-inductive long night (12 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

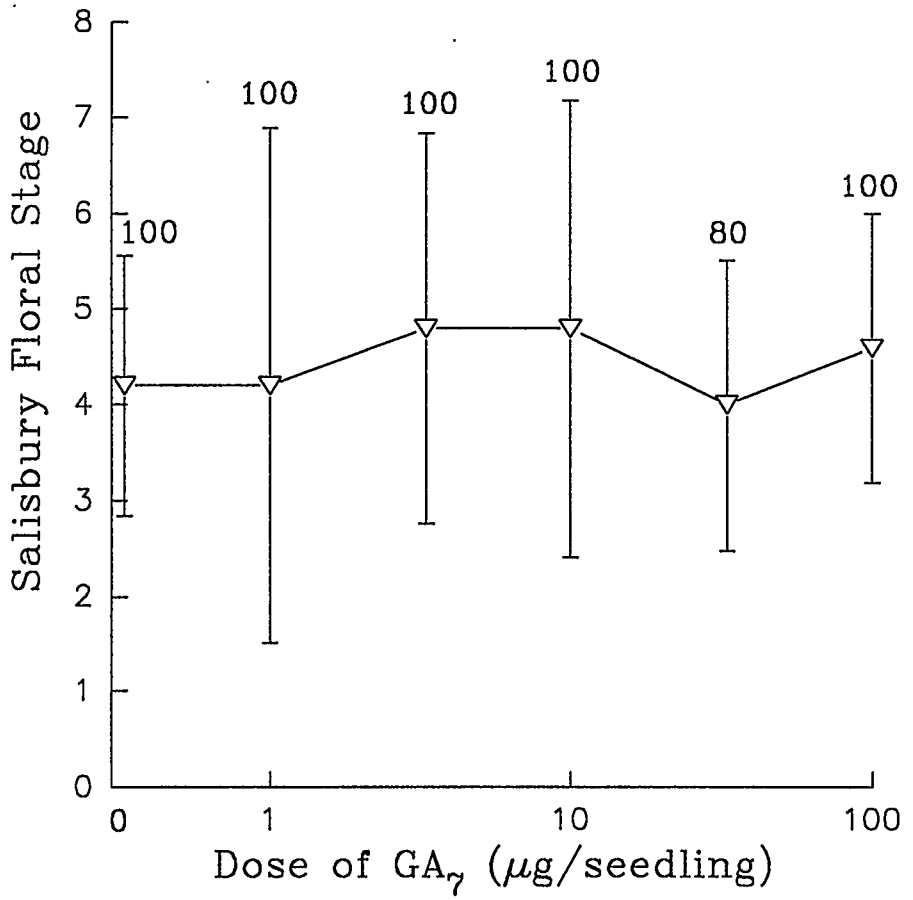

Figure B-10 Efficacy of GA_7 on floral evocation in the Xanthium seedling bioassay. Doses of GA_7 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

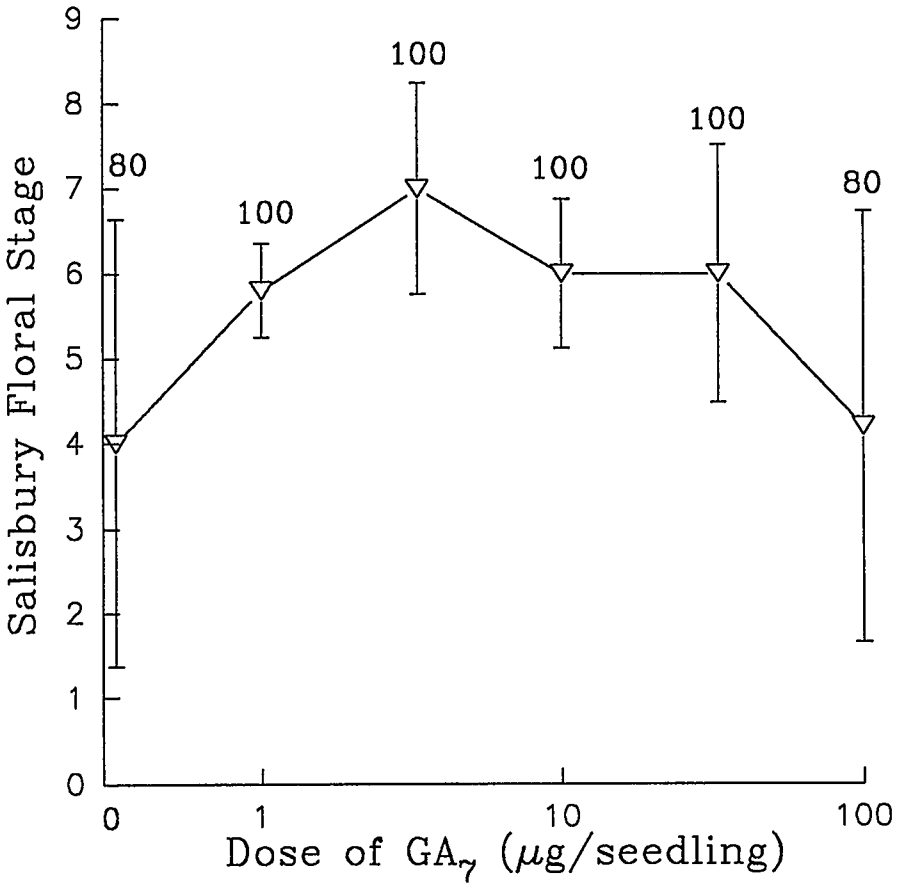

Figure B-11 Efficacy of GA_7 on floral evocation in the Xanthium seedling bioassay. Doses of GA_7 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

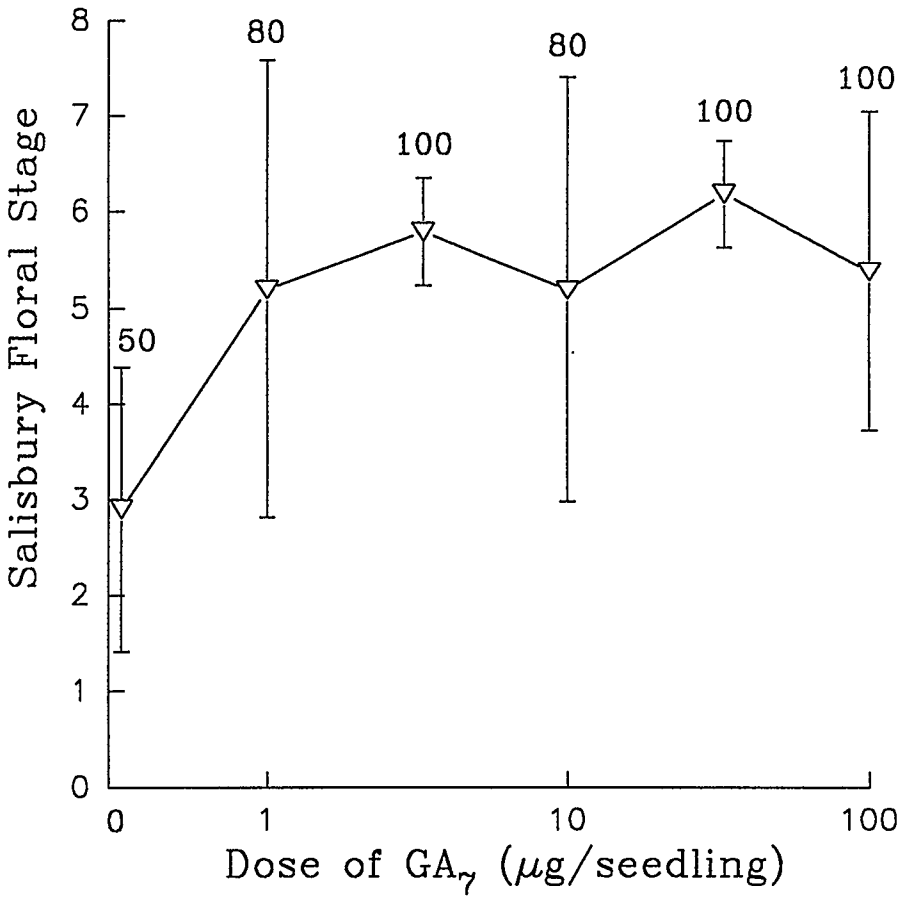

Figure B-12 Efficacy of GA_7 on floral evocation in the Xanthium seedling bioassay. Doses of GA_7 were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

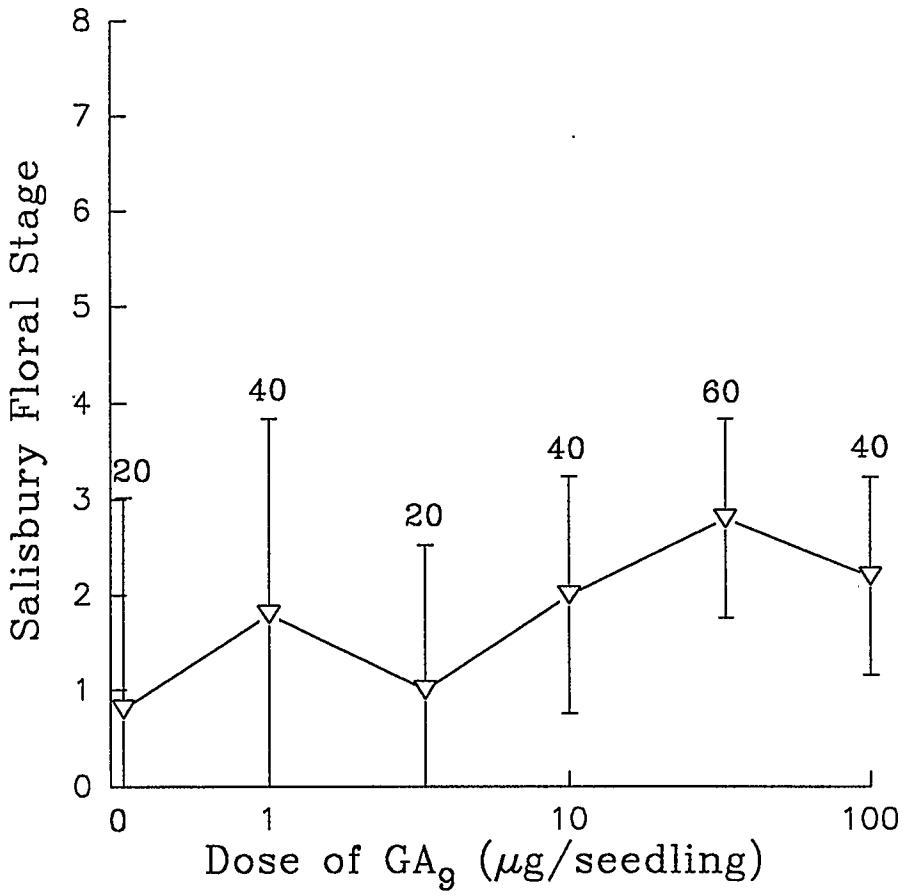

Figure B-13 Efficacy of GA_9 on floral evocation in the Xanthium seedling bioassay. Doses of GA_9 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

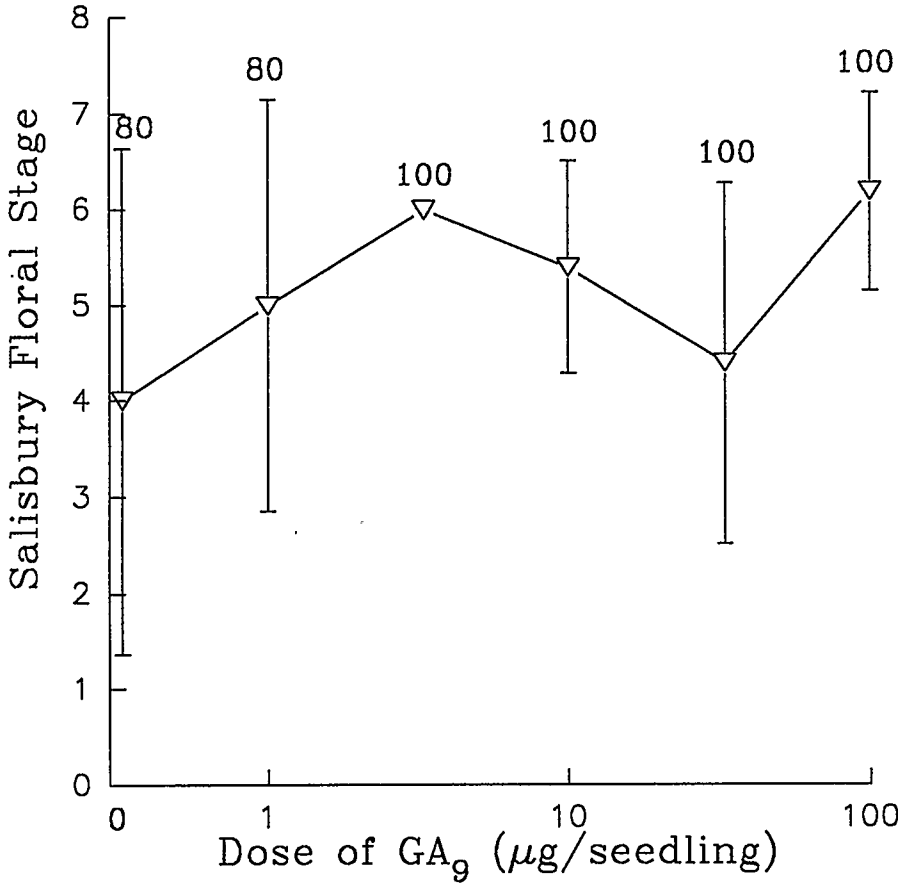

Figure B-14 Efficacy of GA_9 on floral evocation in the Xanthium seedling bioassay. Doses of GA_9 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

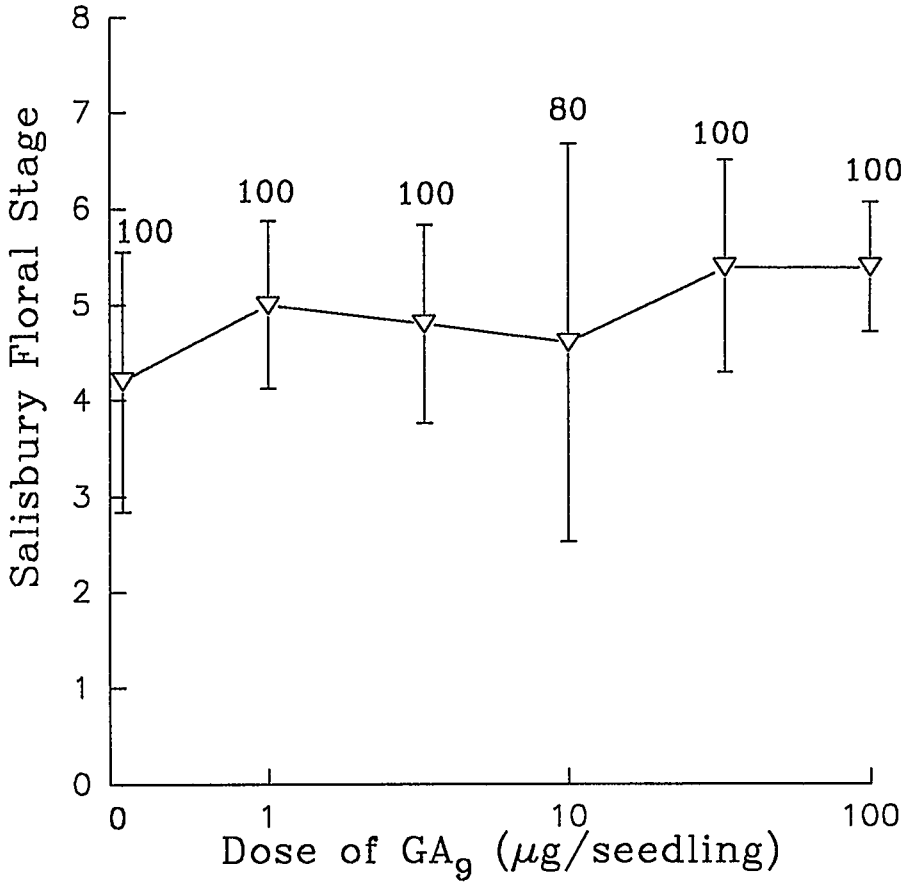

Figure B-15 Efficacy of GA_9 on floral evocation in the Xanthium seedling bioassay. Doses of GA_9 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

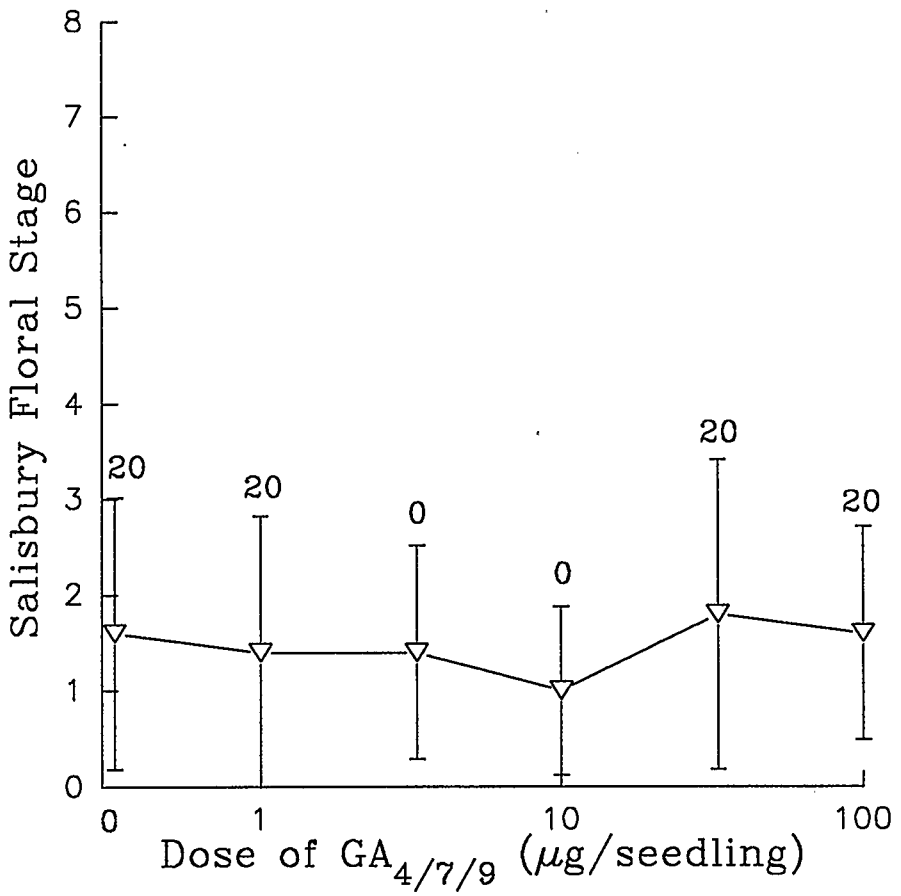

Figure B-16 Efficacy of $GA_{4/7/9}$ (62:30:8) on floral evocation in the Xanthium seedling bioassay. Doses of $GA_{4/7/9}$ were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

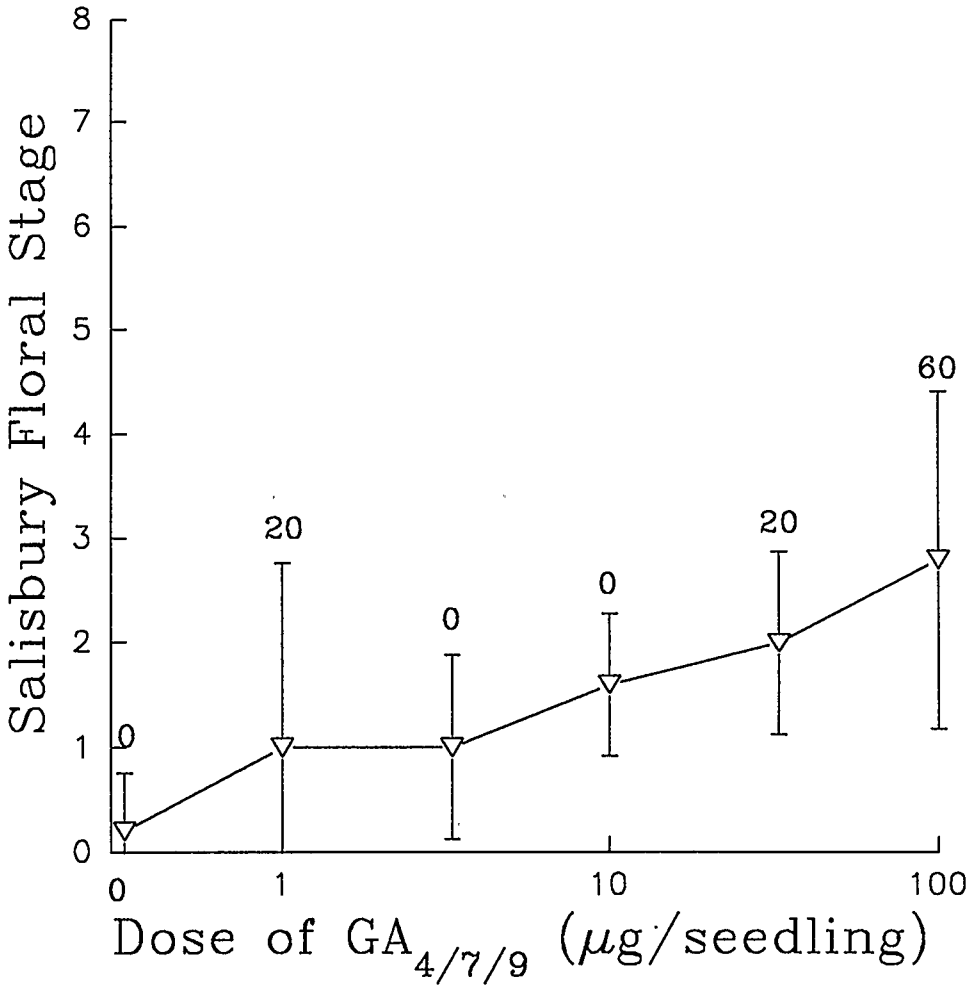

Figure B-17 Efficacy of $GA_{4/7/9}$ (62:30:8) on floral evocation in the Xanthium seedling bioassay. Doses of $GA_{4/7/9}$ were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

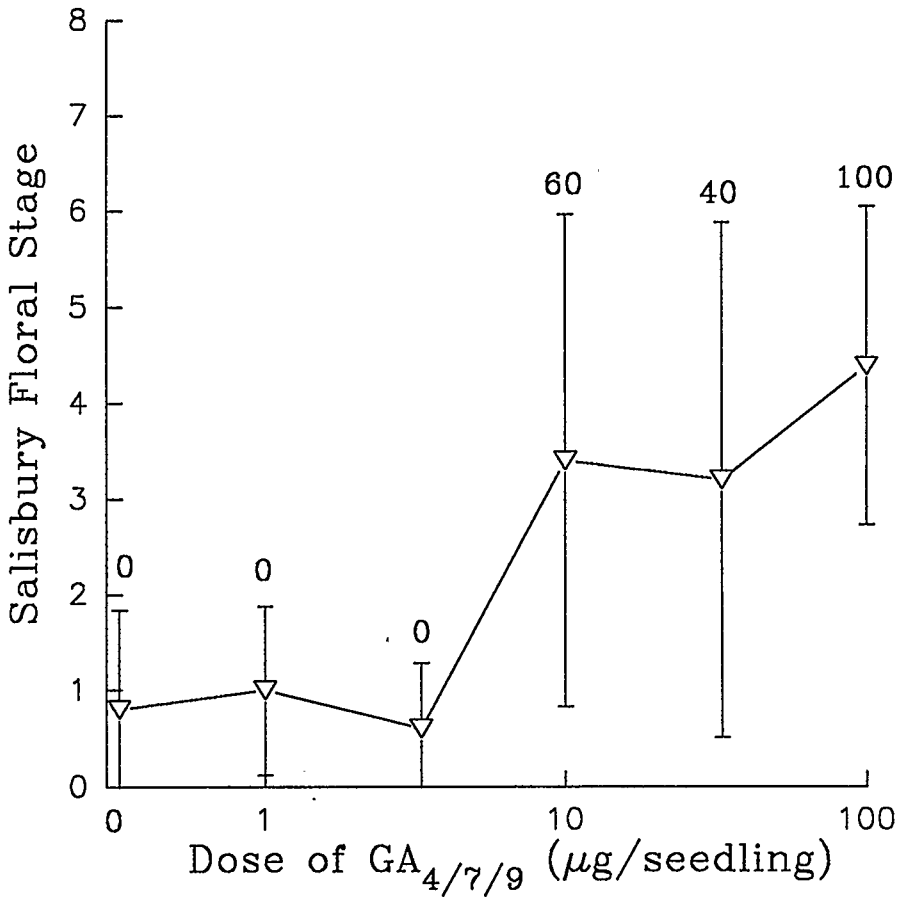

Figure B-18 Efficacy of $GA_{4/7/9}$ (62:30:8) on floral evocation in the Xanthium seedling bioassay. Doses of $GA_{4/7/9}$ were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

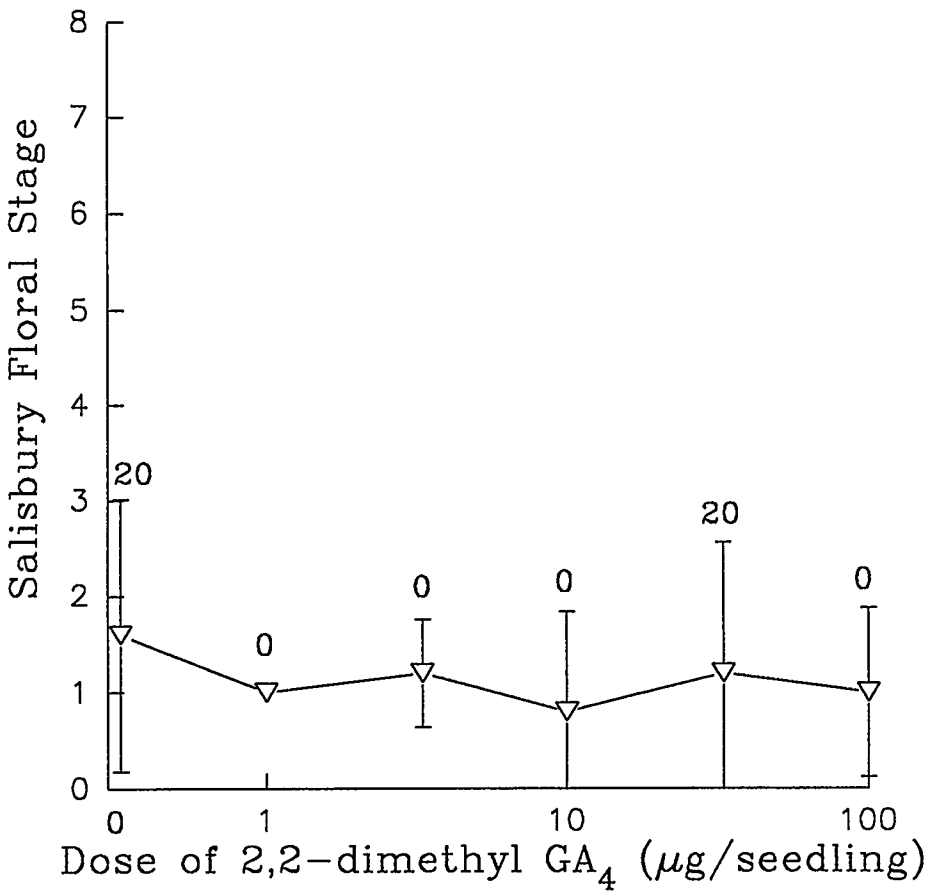

Figure B-19 Efficacy of 2,2-dimethyl GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of 2,2-dimethyl GA_4 were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

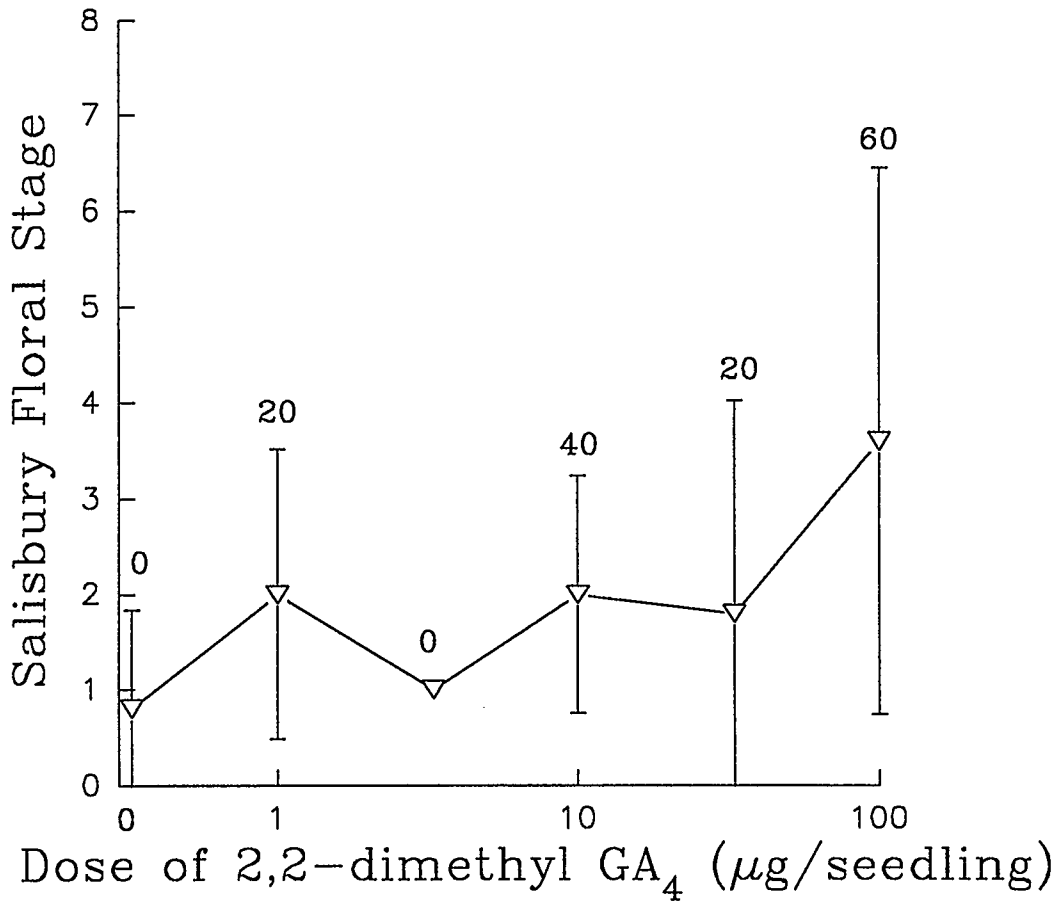

Figure B-20 Efficacy of 2,2-dimethyl GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of 2,2-dimethyl GA_4 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

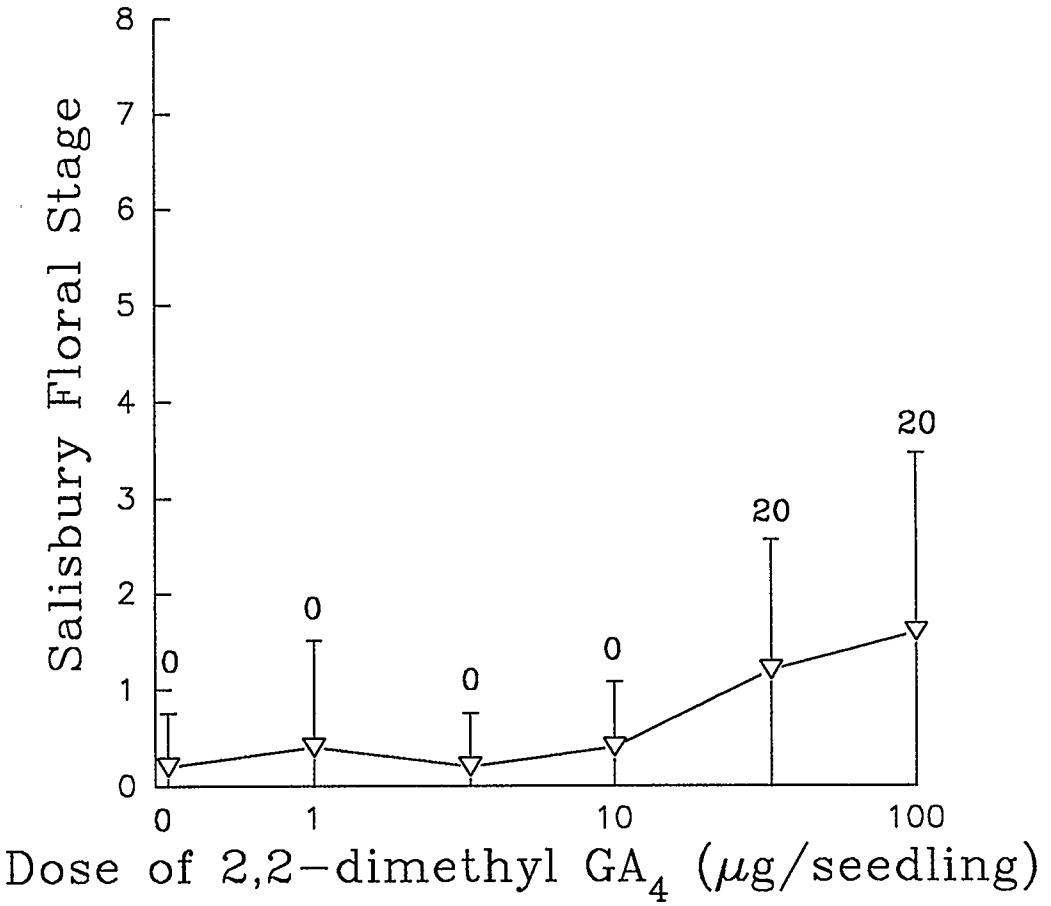

Figure B-21 Efficacy of 2,2-dimethyl GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of 2,2-dimethyl GA_4 were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

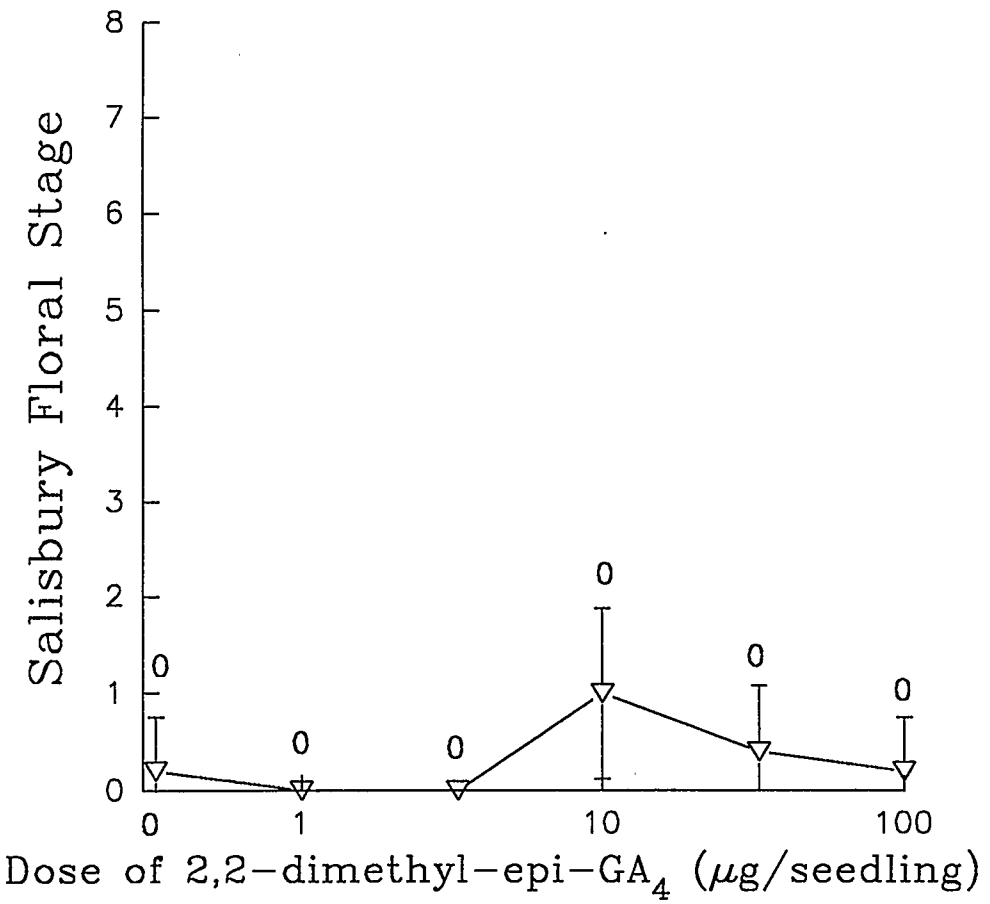

Figure B-22 Efficacy of 2,2-dimethyl-epi-GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of 2,2-dimethyl-epi-GA_4 were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

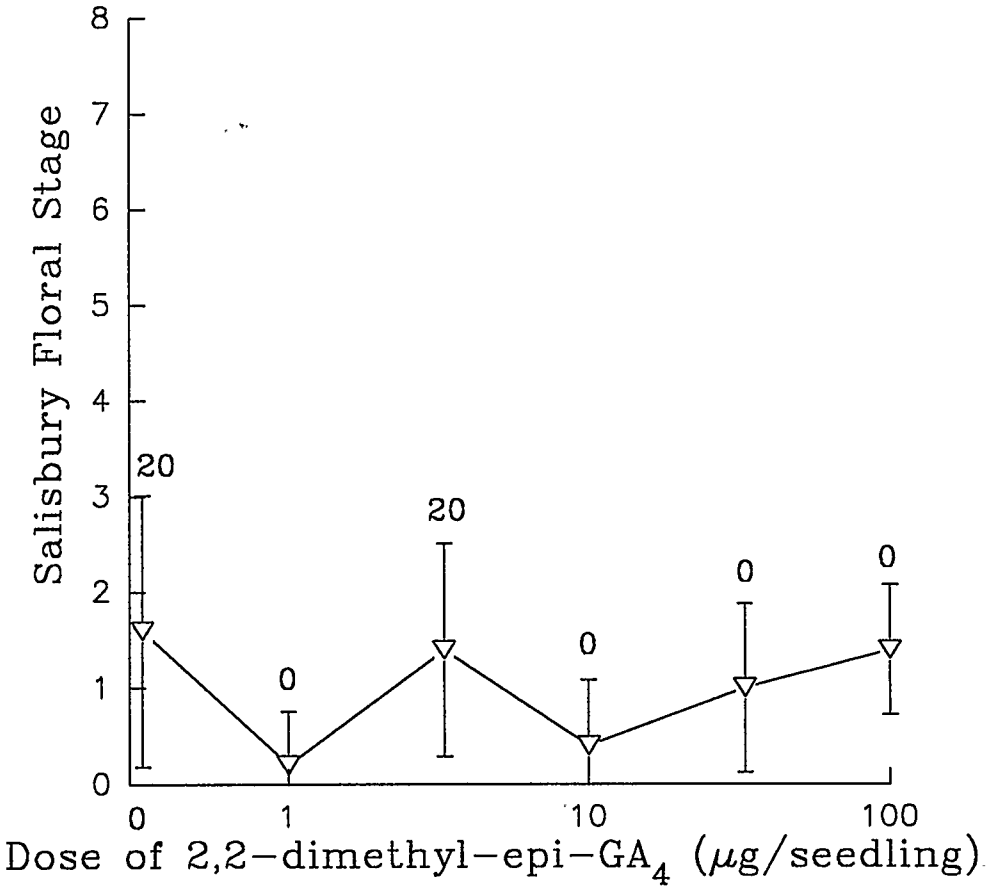

Figure B-23 Efficacy of 2,2-dimethyl-epi-GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of 2,2-dimethyl-epi-GA_4 were applied at 1200 h, 9.5 h before induction with a single marginally-inductive long night (10.5 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.

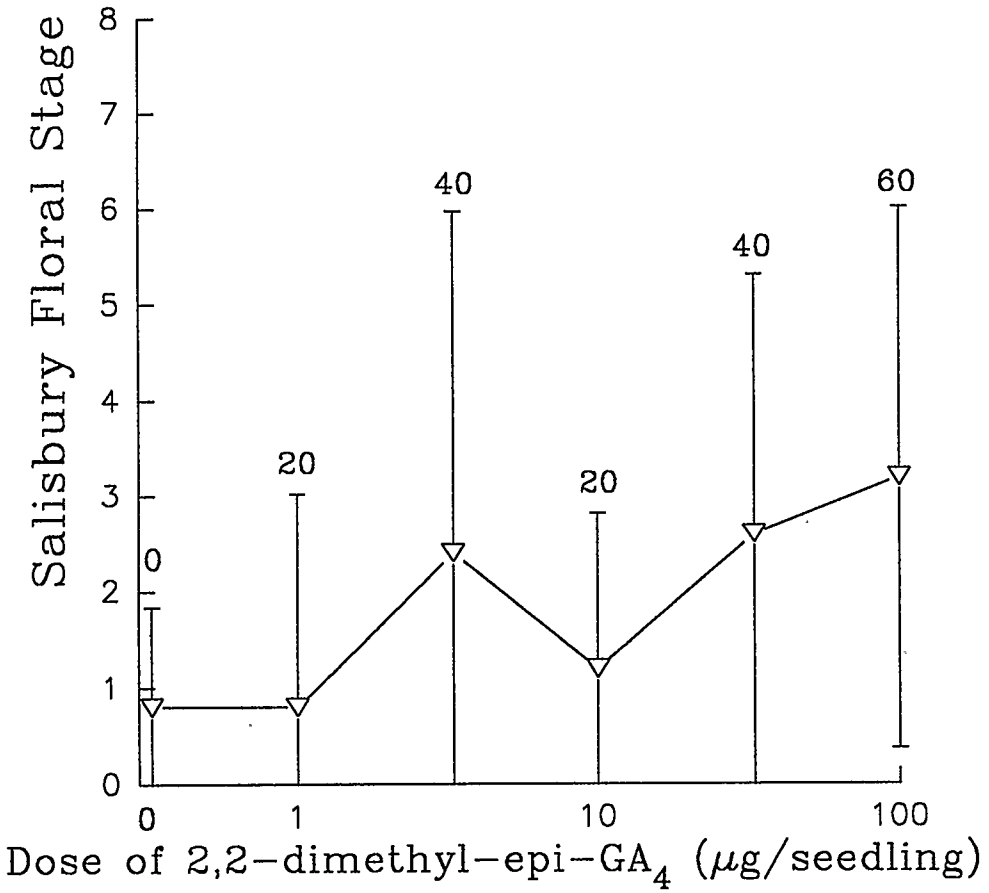

Figure B-24 Efficacy of 2,2-dimethyl-epi-GA_4 on floral evocation in the Xanthium seedling bioassay. Doses of 2,2-dimethyl-epi-GA_4 were applied at 1200 h, 9 h before induction with a single marginally-inductive long night (11 h dark). Values represent the mean of 5 seedlings (Salisbury Floral stages) scored 9 days post-induction. Error bars represent 95% confidence intervals. Flowering quotients are indicated above the error bars.