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Studies on the Role of Abscisic Acid in the Initiation of Bud Dormancy in *Alnus glutinosa* and *Betula pubescens**

T. J. Hocking** and J. R. Hillman

Department of Botany, University of Glasgow, Glasgow, G12 8QQ, U.K.

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Summary. The effects of leaf-applied (±)-abscisic acid on the growth and dormancy of *Betula pubescens* Ehrh. and *Alnus glutinosa* Gaertn. growing under long days provide no evidence that leaf-applied abscisic acid induces or promotes the formation of resting buds in these species. Radiotracer studies show that a small percentage of the radioactivity applied as [2-^{14}C]abscisic acid to the leaves accumulates in the apical region of the shoot. Of the radioactivity that was recovered from this region after 8 days, less than 10% was chromatographically similar to [2-^{14}C]abscisic acid. The significance of these results with respect to the role of abscisic acid in regulating the induction of bud dormancy is discussed.

Introduction

The concept that growth inhibitors and in particular, abscisic acid (ABA), are involved in the photoperiodic induction of bud dormancy in woody species originated largely from two experimental approaches. One involved the bioassay of endogenous inhibitor levels (inhibitor β or ABA-like activity) in tissue extracts from the leaves and buds of plants maintained under long or short days (Robinson and Wareing, 1964). Subsequently, Milborrow (1967) showed that ABA was an active component of the inhibitor β fraction.

A second approach concerned the effects of applying inhibitors to plants. Eagles and Wareing (1963) reported that application of inhibitor extracts from birch plants (*Betula pubescens*) maintained under short days to the leaves of birch plants maintained under 14.5 h photoperiods caused the formation of resting buds. El-Antably *et al.* (1967) found that application of ABA solution to birch plants growing in long days also caused a cessation of growth and formation of typical resting buds.

More recently, however, Lenton *et al.* (1972) have measured the ABA contents of birch (*B. pubescens*) and sycamore (*Acer pseudoplatanus*) using gas chromatography. They observed no increase in the ABA content of extracts when the plants were transferred to dormancy-inducing conditions. Loveys *et al.* (1974) reported that ABA levels in leaves of *B. lutea* were lowered when plants were transferred to short days but that the metabolism of exogenous [2-^{14}C]ABA was unaffected. Alvin and Saunders (1974), working with *Salix viminalis*, found

* *Abbreviation:* ABA = abscisic acid.

** *Present address:* Department of Biological Sciences, The Polytechnic, Wolverhampton, WV1 1LY, U.K.

no significant evidence that photoperiodically mediated dormancy was associated with changes in ABA levels.

In application studies, Cathey (1968) reported that growth responses induced by exogenous ABA in certain ornamental plants only partially mimicked the growth characteristics induced by short days and that relatively high dosages (>100 ppm) were required for activity. Moreover, Perry and Hellmers (1973) found that while exogenous ABA suspended stem growth under long days in northern races of red maple (*Acer rubrum*), normal winter buds did not develop. Although some of these apparent discrepancies can be attributed to the use of more specific quantitative techniques, clearly further investigation is required. The availability of radioactively-labelled ABA has enabled us to re-examine and extend studies on the effects of exogenous ABA on the growth and dormancy of *B. pubescens*, and in addition, *Alnus glutinosa*.

Materials and Methods

Seedlings of northern biotypes of birch (*Betula pubescens* Ehrh.) and alder (*Alnus glutinosa* Gaertn.) were raised in a heated greenhouse under long days. Before each experiment 3-month old plants were transferred to growth cabinets and maintained for 4 days under long photoperiods consisting of 8 h of high intensity irradiation from a bank of alternate daylight/warm white fluorescent tubes and 8 h of low intensity irradiation from two tubes. For short photoperiods plants were exposed to 8 h of high intensity radiation only. All cabinets were maintained at $20 \pm 1°$ C. Plants were watered regularly to ensure that they were at no time subjected to water stress.

The experimental techniques used for the application of ABA were similar to those used originally by Eagles (1962) for inhibitor extracts. Duplicate experiments were carried out for each species, with at least 15 replicates for each treatment. In each experiment a rolled-up, upper, expanded leaf was immersed in a small vial containing water or a solution of unlabelled or radioactively-labelled ABA. (±)-ABA and [2-^{14}C](±)-ABA (specific activity 45 μCi mg^{-1}) were kindly donated by Hoffmann-La Roche Ltd., Basel, Switzerland. As the plants grew during the experiment further applications were made to successive fully-expanded leaves; the day of application is indicated on the appropriate graph. A set of plants were left untreated as additional controls. Plant height and node number were measured for each plant at regular intervals before and during treatment.

For uptake and distribution studies plants supplied with [2-^{14}C]ABA were removed from their pots and the roots washed free of soil. Each entire plant was then divided into small portions and each portion was placed in a scintillation vial containing 2 cm^3 of 95% ethanol. The tissue was extracted for 48 hours at 10° C, reduced to dryness *in vacuo* and 10 cm^3 of a scintillation mixture comprising 2,5-diphenyl oxazole (PPO) dissolved in toluene (4 gl^{-1}) were added to each vial. The samples were counted on a Packard "Tri-Carb" liquid scintillation spectrometer with an automatic activity analyser, correcting for quenching and printing-out data in disintegrations per minute (dpm), to obtain a quantitative estimation of the distribution of radioactivity throughout the plant.

For chromatographic analysis, extensive purification was necessary prior to chromatography, following in part the method of Lenton *et al.* (1971). Homogenised tissue was extracted in 80% methanol, reduced to aqueous solution, frozen, thawed and centrifuged to remove suspended material. The supernatant was extracted at pH 3.5 with ether. In some cases the ether extract was reduced to dryness and the residue dissolved in methanol for chromatography. For extracts requiring further purification the ether phase was extracted alternately at pH 8.0 four times with $^1/_4$ volume of 5% sodium bicarbonate and $^1/_4$ volume water. The combined aqueous extracts were re-extracted at pH 3.5 with ether, the ether removed and the final extract dried over sodium sulphate. The residue was dissolved in methanol, applied to Whatman No. 3 chromatography paper and the chromatogram developed in n-butanol: n-propanol: ammonia: water:: 2:6:1:2 v/v. After development and drying the chromatogram was divided into 0.05 Rf zones and assayed using liquid scintillation spectrophotometry.

Results

1. Photoinduction of Bud Dormancy in Alnus glutinosa and Betula pubescens

The heights of plants maintained under long or short days at 20° C were measured at regular intervals over a period of 25 days (Fig. 1). By the 13th day (*A. glutinosa*) and the 12th day (*B. pubescens*) stem elongation had ceased in short day plants. Resting buds were also beginning to form by this time. In *A. glutinosa* the formation of resting buds was characterised by a reddening of the bud scales. By day 23 all buds on short day plants were dormant (Fig. 2).

2. Effect of Leaf Application of ABA on the Growth of A. glutinosa and B. pubescens

Plant heights and node numbers were recorded for control and treatment plants at regular intervals (Figs. 3, 4). The final % increases in height and node number are given in Table 1. The increases in height were approximately linear with time. There were no marked differences between treatments, although there was evidence of slight growth promotion compared to the control with the lowest concentration of ABA used.

Thus, in these experiments there was no evidence of any inhibition of growth by leaf-applied ABA.

3. Uptake, distribution and analysis of ^{14}C in *A. glutinosa*. In the above experiments with *A. glutinosa*, replicate plants were supplied with 5×10^{-7} M [2-^{14}C]ABA in exactly the same way and at the same time as the other treatments. These plants were harvested at regular intervals for radioassay of uptake and distribution of ^{14}C (Table 2) and for chromatographic analysis of tissue extracts (Table 3). Uptake and distribution were complicated by the fact that after 8 and 16 days further applications of [2-^{14}C]ABA were made to successive leaves. The uptake after 8 days was lower than after 4 days. The percentage of the uptake

Table 1. Mean % increase in height and mean increase in node number, 24 days after application of ABA to the leaves of *A. glutinosa* and *B. pubescens* growing in long days

	Untreated control	H_2O control	ABA treatment		
			2×10^{-4} M	10^{-5} M	5×10^{-7}M ABA
A. glutinosa					
% increase in height	36.4±4.2[a]	37.5±5.8	39.9±3.5	44.7±6.0	48.4±6.4
Increase in node no.	4.3±0.2	5.1±0.4	4.4±0.3	5.0±0.2	5.25±0.2
B. pubescens					
% increase in height	61.2±6.8	66.8±5.2	57.6±5.0		
Increase in node no.	7.8±0.3	8.3±0.5	8.0±0.4		

[a] Mean value ± standard error, $n = 15$; % values transformed using angular transformation.

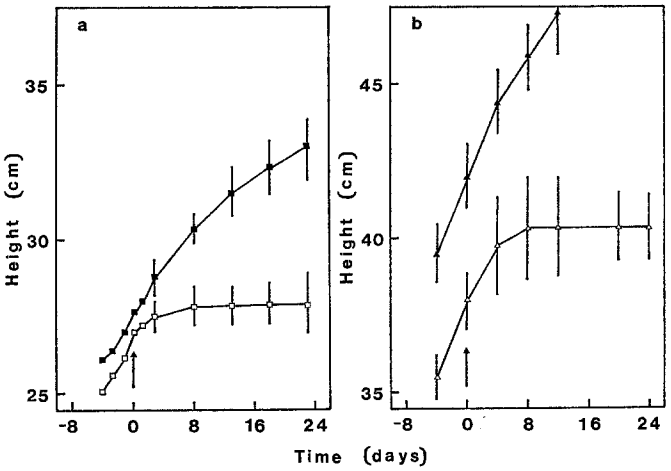

Fig. 1. Effect of long days (solid symbols) or short days (open symbols) on the height of plants of (a) *Alnus glutinosa* and (b) *Betula pubescens*. The arrow indicates the time of transfer of plants to short days. Vertical bars represent twice the standard error of the mean

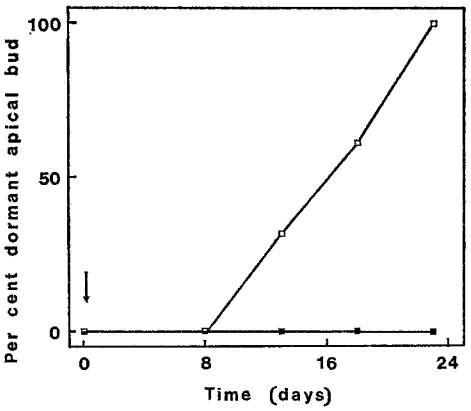

Fig. 2. Effect of long days (solid symbols) or short days (open symbols) on the mean per cent dormant buds formed on plants of *Alnus glutinosa*. The arrow indicates the time of transfer of plants to short days

exported from the donor leaf (—ves) decreased with time; i.e. the more that was taken up, the more that was immobilised in the leaf. Consequently, the mean distribution in the plant was dominated by the % dpm remaining in the donor leaf (—ves). The % dpm in the apical region of the plant reached 11.2 after the first 4 days; this represents a concentration of 1847 dpm g^{-1}. Nonetheless, the accumulation in the apical region was markedly higher than elsewhere, especially the roots, where accumulation was low. This was possibly a reflection of the application being made to an upper expanded leaf which would show a greater tendency to export to the upper regions of the plant.

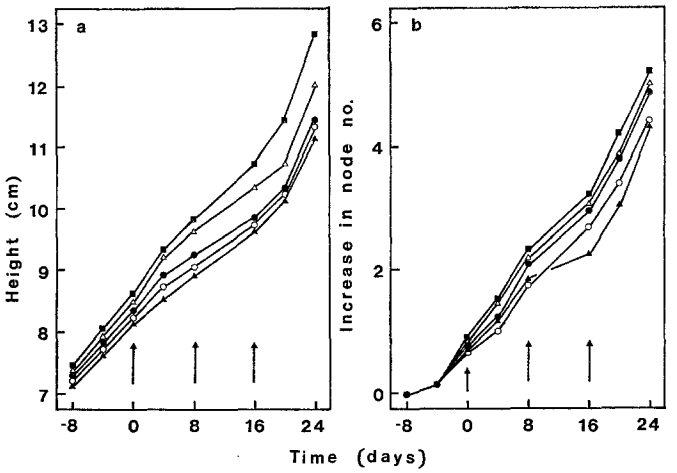

Fig. 3. Effect of leaf-applied ABA on (a) the height (b) the increase in node number of plants of *Alnus glutinosa* growing in long days at 20° C. The arrows indicate the time of application of —●— H_2O, —○— 2×10^{-4} M ABA, —▲— 10^{-5} M ABA, —■— 5×10^{-7} M ABA, —▲— untreated controls

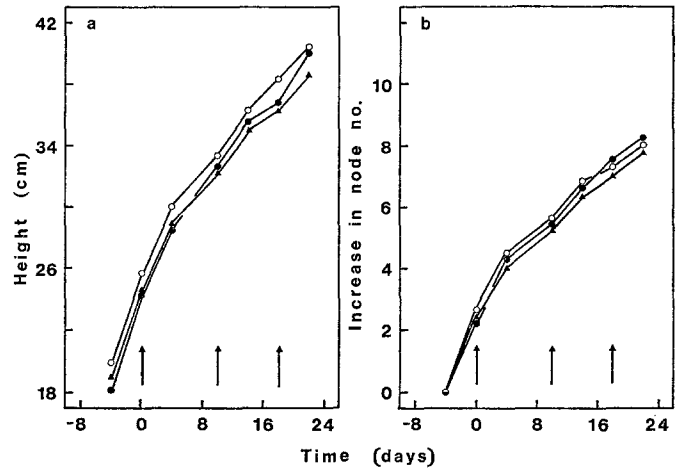

Fig. 4. Effect of leaf-applied ABA on (a) the height and (b) the increase in node number of plants of *B. pubescens* growing in long days at 20° C. The arrows indicate the time of application of —●— H_2O, —○— 2×10^{-4} M ABA, —▲— untreated controls

The results from the chromatographic analysis show that only a small proportion of the radioactivity recovered from the apical region of the shoot was chromatographically similar to [2-^{14}C]ABA. The major peak of radioactivity in extracts from apical regions occurred at Rf 0.7–0.75, 0.05 to 0.1 Rf lower than [2-^{14}C]ABA in 2:6:1:2; a result which was found consistently in tissue extracts and is probably not an artefact of chromatography (Hocking, 1973).

Table 2. Uptake and distribution of ^{14}C in *A. glutinosa* growing in long days, following leaf application of 5×10^{-7} M [2-^{14}C]ABA

Days after application	Uptake (dpm)	% exported[a]	Distribution[b] shoot	donor leaves			shoot	roots
			above	1st	2nd	3rd	below	
4	3675[c]	18.4	11.2	81.6	—	—	5.5	1.7
8	2824	8.1	5.3	91.9	—	—	1.8	1.0
16	6513	4.3	3.0	74.2	21.5	—	1.3	0.0
24	12853	3.6	3.3	47.4	34.0	15.0	0.3	0.0

[a] % dpm (of total uptake) moved out of donor leaf (-ves).
[b] % dpm (of total uptake).
[c] Mean values, $n = 5$.

Table 3. Chromatographic analysis of methanolic extracts from *A. glutinosa* growing in long days, following leaf application of 5×10^{-7} M [2-^{14}C]ABA
Paper chromatography using n-butanol:n-propanol:ammonia:water: 2:6:1:2 (v/v)

Extract	Rf of major peak	% dpm recovered at ABA Rf
[2-^{14}C]ABA	0.75–0.85	73.0
8 day apex	0.70–0.75	9.5
8 day donor leaf	0.75–0.85	35.9
8 day [2-^{14}C]ABA	0.75–0.85	63.1
24 day apex	0.70–0.75	12.3
24 day donor leaf	0.75–0.85	44.4

Discussion

The effects of leaf-applied ABA on the growth of birch are very similar to those on alder and conflict to some extent with the work of Eagles (1962), Eagles and Wareing (1963) and El-Antably *et al.* (1967). Two deviations from the technique used by Eagles must be emphasised. Firstly, he applied an inhibitor extract to the leaves, whereas pure ABA solution was used in the present study. While it is known that inhibitor extracts of the kind used do contain ABA, they may obviously contain other inhibitory factors (Lenton *et al.*, 1972). It is possible that one or more of these factors may be responsible, or required in addition to ABA, for the observed effects on bud dormancy.

Secondly, Eagles maintained the experimental plants under a $14^1/_2$ hour photoperiod, thought to be just above the critical photoperiod for birch, which caused the plants to grow less actively than the plants used in the present study. It is possible that the levels of growth promoters, such as the gibberellins, have to be low before ABA is effective; i.e. a hormonal balance is more important than the level of a single hormone. This possibility has been suggested by Wareing and Saunders (1971) and Lenton *et al.* (1972). El-Antably *et al.*, however, used 18 hour photoperiods in their study and reported inhibition of growth by repeated applications of ABA to the leaves and the apical regions of birch plants. The

present study indicates that repeated spraying of the apical regions directly with ABA may be necessary to achieve an observable effect on growth, as only a small proportion of the leaf-applied ABA appears to reach the apical regions unaltered. It should be noted that the concentration of ABA they supplied to the leaves was half the maximum concentration used in the present study.

In summary, leaf-applied ABA does not appear to induce bud dormancy in birch and alder growing in long days. Together with the observations of Lenton *et al.* (1972) and Loveys *et al.* (1974) it may be argued that endogenous ABA in the leaves does not have a direct controlling effect on the formation of resting buds. On the other hand, the endogenous ABA produced in the leaves may have a role under natural conditions, but the results obtained using exogenous ABA are confounded by other factors, e.g. gibberellins, cytokinins, or problems of penetration, compartmentation, distribution and metabolism. Some of these problems have been investigated further and will be considered elsewhere.

It must be stressed that these observations do not preclude a role for free and bound ABA in controlling other aspects of bud dormancy (see Lesham *et al.*, 1974). Nevertheless, short-term fluctuations in environmental conditions can cause large increases in the level of ABA (see Milborrow, 1974) and it is difficult to envisage a controlling system for bud dormancy in which changes in ABA alone regulate the initiation and progress of dormancy. Critical evaluation of the rates of ABA synthesis and inactivation as well as sequestration and other means of removal from the receptor sites must be made during the initiation, maintenance and breaking of the dormant condition.

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