ANALYSIS OF PLANT GROWTH SUBSTANCES
IN RELATION TO SEEDLING AND PLANT GROWTH

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ABSTRACT

Despite a relatively large literature discussing the roles of auxins, gibberellins, cytokinins, and inhibitors (abscisic acid) in the physiology of woody perennial plants, relatively few data relate these compounds to seedling vigour. Possible reasons for this deficiency include an inability to detect accurately the low concentrations of these compounds, especially the gibberellins and cytokinins, and an inability to distinguish between physiologically active hormones and similar but bound compounds. However, the past decade has seen the development of analytical techniques with potential for detecting picogram quantities of growth substances. Such precision may reveal definite relationships between seedling growth vigour and levels of plant hormones.

INTRODUCTION

Although literature concerning plant growth substances is voluminous, much of it relates to annual herbaceous plants, growth of cells or organs, or floral induction, and little is even marginally germane to vigour of tree seedlings.

Tree seedling vigour after outplanting appears to correlate strongly with the environment during the dormant period and the speed of growth initiation in the spring. This paper summarizes current data relating each of the five classes of plant growth regulators, auxin, ethylene, gibberellin, cytokinin, and abscisic acid, to dormancy and growth initiation. Unfortunately, many reports imprecisely identify the hormones to be discussed. Therefore, part of this paper describes current research which will potentially aid evaluation of hormones at far lower levels than previously possible.

GROWTH SUBSTANCES

Auxin

Although auxin was the first plant-growth substance to be identified (Thimann, 1972), few reports relate levels of this hormone to seedling physiology during or immediately after dormancy. Further, few authors discussing indole-3-acetic acid (IAA) identify the compound satisfactorily (Bandurski and Schulze, 1974). DeYoe and Zaerr (1976) report that levels of indole-3-acetic acid in actively elongating shoots of Douglas fir *(Pseudotsuga menziesii* (Mirb.) Franco) are at least 100-fold that found in dormant (December) material. Similarly, Nelson (1978) demonstrated that levels of IAA in western hemlock *(Tsuga heterophylla* (Raf.) Sarg.) were highest during spring and lowest in late summer and early autumn. Caruso *et al.* (1978) reported similar levels in seedling and mature material. Unfortunately, these are

the only papers known to us which unambiguously evaluate levels of IAA in forest tree seedling tissue. Alden (1971) and Dunberg (1976) found a similar pattern with Scots pine *(Pinus silvestris* L.) and *Picea abies* (L.) Karst, respectively, as did earlier workers who relied on bioassays to estimate IAA levels. Though data relate high levels of IAA to the spring growth initiation period (Zaerr, 1967), no reports demonstrate differential levels of IAA with differing degrees of shoot-growth vigour. On the contrary, Sweet and Rook (1972) suggest that relative levels of inhibitor, not auxin, may be associated with differing growth response. It has been suggested, however, by several workers (Stone and Norberg, 1979; Jenkinson and Nelson, 1978; Lavender, 1964; Wakely, 1954), that vigorous root growth after spring planting is a hallmark of healthy seedlings. And Carlson and Larson (1977) found that IAA added to the pruned root system of red oak *(Quercus rubra* L.) seedlings stimulated a significant increase in elongation of roots.

The lack of correlation of endogenous levels of IAA with seedling vigour may be due to analysis procedures which do not correctly identify IAA, extraction procedures which destroy IAA (Little *et al.,* 1978), or work in which such correlations were not an objective. Therefore, our present state of knowledge leaves the role of IAA in forest tree seedlings in the realm of speculation.

Ethylene

Long before it was known that plants themselves produced it (Thimann, 1972), ethylene was found to have damaging effects upon plants as a component of illuminating gas (in 1901) and tobacco smoke (in 1913). The literature is concerned primarily with the role of this gas in stimulating the ripening of fruit and with the growth responses of annual herbaceous plants, in which ethylene is often reported to be associated with auxin.

We have found no publication relating ethylene to coniferous seedling vigour, but current work at a nursery of the Industrial Forestry Association[1] and at Oregon State University in Corvallis shows that when Douglas fir and western hemlock seedlings are stored in darkness for one or more months at approximately 1°C in the presence of 5 ppm ethylene, the incidence of physiological decline is greatly reduced for autumn-lifted seedlings. The growth response of such seedlings compared to Douglas fir control seedlings stored without ethylene shows significantly more vigorous shoot and root growth after outplanting and a mortality of only 7% (1 Aug. 1979) as opposed to 63%.

Gibberellins (GA)

Gibberellins have been characterised (Paleg and West, 1972) by their capacity to stimulate dramatic extension growth. Low-vigour seedlings of Douglas fir frequently show extreme "planting shock" characterised by a "bottle brush" appearance, short needles clustered closely on stems that elongate little after bud burst. Pharis (1976, p. 293) notes, "Thus, for the known growth hormones, it would appear that only GA's can currently be considered with certainty to play a controlling or limiting factor role in shoot elongation of conifers." But, though workers have related increased levels of endogenous or exogenous gibberellins at the time of bud break to increased stem growth or extended periods of rapid elongation (Pharis and Kuo, 1977; Pharis, 1976; Dunberg, 1976; Lorenzi *et al.,* 1975; Juntilla, 1976; Jensen and Dochinger, 1972; Farmer, 1966) a cause and effect relation between this hormone and seedling vigour is difficult to establish. The reasons suggested before for the lack of correlation between auxin levels and rate of shoot elongation apply equally to the gibberellins.

The biochemistry of the various gibberellin species is poorly understood. The more polar

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[1] J. Blake, Toledo, Washington, 1979, unpublished data.

gibberellins appear to be associated with shoot elongation, although the work of Wample *et al.* (1975) suggests that the relatively non-polar GA_4 is deactivated by being metabolized to the relatively polar GA_{34}. Even so, our present understanding prohibits correlation between data of Crozier *et al.* (1970) and Lavender *et al.*[2], identifying several gibberellin-like compounds wherein most gibberellin activity varied randomly with tree age, season of the year, and observed growth.

A final factor currently obscuring the relation of gibberellin content to seedling vigour is that most studies identify gibberellin-like compounds only by bioassay. Inhibiting substances, which commonly are difficult to separate from the gibberellins with chromatographic methods may mask gibberellin effect on the experimental material.

Inhibitors (ABA)

The possible role of inhibitory substances in dormancy of perennial plants was first suggested by Hemberg in 1949 (Eagles and Wareing, 1963). Early studies (Robinson *et al.*, 1963) used bioassays to quantify the inhibitor and demonstrated an apparent variation with photoperiod or season (Phillips and Wareing, 1958; Kawase, 1966; Tinklin and Schwabe, 1970). Later work isolated and identified the main component of the inhibitor complex as abscisic acid (ABA).

The identification of ABA led to trials of endogenous applications with both positive and negative results concerning dormancy induction in experimental populations (El-Antably *et al.*, 1967; Cathey, 1968; Perry and Hellmers, 1973; Hocking and Hillman, 1975; Noodén and Weber, 1978). Such trial results do not, of course, necessarily indicate the role of endogeneous ABA, as they may be modified by factors such as penetration, degradation of applied material, or concentrations of other regulators.

Milborrow (1974) reported laboratory techniques other than bioassay procedures which yielded much more accurate data on levels of endogenous ABA than were previously available. ABA concentration was shown to be affected more dramatically by moisture stress than by photoperiod and was also shown to be associated with stomatal behaviour (Blake and Ferrell, 1977).

Although ABA is no longer generally thought to be the dormancy-inducing compound that early workers suggested, the work of Blake and Ferrell should not be interpreted as negating a possible role for ABA in dormancy induction and release. Moisture stress itself often triggers dormancy (Vegis, 1964). Certainly, recent trials have shown a pattern of seasonal changes in ABA level. The greatest concentrations occur during dormancy induction in late summer and early autumn and the lowest levels at about bud break (Davison and Young, 1974; Brown, 1976; Webber *et al.*, 1979; Nelson, 1978; Dumbroff *et al.*, 1979). Further, Harrison and Saunders (1975) note a reduction in free (presumably active form) but not total ABA *Betula verrucosa* Ehrh. buds with increased speed of bud break, and Wright (1975) reports highest free/bound ratio of ABA at bud set and highest bound/free ratio at bud break. And Bacon (1978) reports a correlation between levels of ABA and its metabolities and reduced growth of *Pinus caribaea* var. *hondurensis* seedlings.

Sweet and Rook (1972) have demonstrated an inverse correlation between the growth vigour of *Pinus radiata* D.Don roots and the quantities of a growth inhibitor, presumably ABA. They caution against assuming a causal relationship, however, because the data reflect the quantity of inhibitor extracted rather than the quantity produced or consumed. Clearly, the techniques for precise measurement of ABA in plant material exist, but more careful research is necessary to define the role of this substance in seedling vigour.   ʼ

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[2] D. Lavender et al., Oregon State University, Corvallis, 1972, unpublished data.

Cytokinins

Kinetin was first isolated and identified in 1955 (Skoog and Schmitz, 1972). Since that time, natural substances such as zeatin, zeatinriboside, isopentenyl adenine, and isopentenyl adenosine, and artificial compounds such as SD8339 and benzyladenine, have been shown to possess similar regulatory capacities. The group is called "cytokinins." These regulators have been implicated in cell division and enlargement, bud and root formation, protein biosynthesis, and apical dominance. However, their principal interest to this discussion is their interaction with other growth regulators and their apparent involvement in activating dormant buds.

Several publications (Morris, 1979; Dumbroff and Brown, 1976; Lorenzi *et al.*, 1975; Hewitt and Wareing, 1974; Taylor and Dumbroff, 1975; Reid and Burrows, 1968) suggest that cytokinin activity in buds is greatest during the spring. Weinberger (1969) and Leike (1967) have shown that applications of cytokinins will speed bud break if the buds have had at least partial chilling. However, lack of a definitive bioassay has prevented accurate quantitation of these regulators. Gas-chromatography mass-spectrometry (Morris *et al.*, 1976; Purse *et al.*, 1976; Horgan *et al.*, 1973) now permits more exact measurement of these hormones. Further refinement of technique will allow characterisation of the role of cytokinins in seedling vigour.

We have so far evaluated present knowledge of the role of individual plant growth regulators in the annual growth cycle of perennial woody plants. As Khan (1975) and Bachelard and Wightman (1974) suggest, however, plant growth processes are probably modulated by a changing balance of several plant hormones and inhibitors. Such substances, by definition, occur in minute quantities and, further, are often difficult to separate from other compounds that mask their presence and confuse the analysis.

Recently developed techniques should permit more accurate evaluation of hormone levels and, consequently, a more critical evaluation of the role of plant growth regulatory substances in seedling vigour.

ADVANCES IN ANALYTICAL METHODS

Recent advances in analytical capacity and other techniques will make possible better detection and measurement of hormones in gram quantities of tissue. Bioassays are still needed to detect hormones in some instances, but their routine use to quantitate known hormones is no longer necessary. Because the equipment and technology necessary for analysis of hormones is becoming more specialised, sophisticated, and expensive, fewer laboratories are able to conduct hormone analyses. But, though fewer in number, the experiments forthcoming should be very useful because they can be conducted rapidly on small quantities of plant tissue.

Abscisic acid can be analysed by rather simple procedures. Solvent extraction, solvent partitioning, separation on a high-performance liquid chromatograph (HPLC), methylation, and detection and quantitation by a gas-liquid chromatograph (GLC) with an electron capture detector produce reliable results with a variety of tissues.

Indole-3-acetic acid can be measured by simple clean-up procedures and HPLC with a fluorescence detector. Since IAA fluoresces naturally, no derivatisation is necessary. Using this system, we have detected quantities of IAA as low as one picogram from extracts of Douglas fir shoots and from xylem sap[3].

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[3] A. Crozier, K. Loferski, J. Zaerr and R. Morris. Manuscript in preparation

One of the most serious problems encountered when analysing gibberellins or cytokinins by GC or GC-MS is the large quantities of impurities contained in the sample to be analysed. Conventional methods of purification often result in large losses of hormones. Efficient sample purification is the key to analysis of hormones in small samples.

Electrophoretic elution (EE) of GA's and cytokinins are presently under development in the Oregon State University Forest Research Laboratory. Results indicate that free GA's are eluted from a C_{18} Porasil column at 800 V and 2.5 mA. Use with 100 ml of Douglas fir sap spiked with tritiated gibberellin standards gives a reduction in dry weight and UV absorbance greater than 90% but achieves over 85% recovery of the standards. Because of the adsorption mechanism (hydrophobic interaction) and differences in the polarity of the various GA's, some fractionation occurs during electrophoresis although there is still overlap between the elution volumes for the several gibberellins. For instance, GA_1 is eluted after 2 hours, GA_4 and GA_{20} after 10 hours, and GA_9 after 30 hours. This variation in elution times would seem to preclude routine use of the technique as a group separatory procedure for GA's, although future developments may overcome the difficulty. The technique is promising for cytokinins, which are retained by the C_{18} support while electrophoresis removes large amounts of impurities within 2 hours and subsequently can be removed in purified form by elution with ethanol. Electrophoretic elution is a simple procedure that may remove large quantities (up to 96%) of interfering substances and still permit high (sometimes more than 90%) recovery of ABA, GA's, and cytokinins (DeYoe and Zaerr, manuscript in preparation).

Techniques for enzyme-linked immuno-specific adsorption (ELISA) offer even greater promise for purification and recovery of GA's and cytokinins. Although ELISA was developed as an analytical tool for virus research, the technique could be the basis for a one-or-two-step cleanup procedure before HPLC.

These two potentially powerful purification systems, EE and ELISA, are being developed in the Oregon State University Forest Research Laboratory and, when perfected, should eliminate much of the tedious cleanup and massive loss characteristic of contemporary hormone analysis. The more conventional GC-MS techniques currently in use will be easier to apply once a relatively clean sample is obtained.

Crown ether catalysis made possible the synthesising of GA esters that could be detected in smaller amounts than previously possible. Studies in this laboratory utilised p-bromophenacyl GA esters detectable in amounts as low as 5 ng (Morris and Zaerr, 1978; Zaerr and Morris, 1978). More recently we have used GA methoxycoumaryl esters (GACE's) synthesised from 4-bromoethyl-7-methoxycoumarin by Crown ether catalysis. These derivatives are strongly fluorescent (having excitation maximum at 320 nm and emission maximum at 400 nm) and can be detected at the low picogram level with an on-line spectrofluorometric detector after HPLC on a C_{18} support (Fig. 1) (Crozier et al., manuscript in preparation).

A wide range of HPLC columns, including the silica gel adsorption system originally used by Reeve and Crozier (1978) for GA benzyl esters, can be used to purify and analyse GACE's. The recoveries from adsorption systems rarely exceed 60-70%, therefore, they have to some extent been superseded by the recent proliferation of supports which chemically bonded stationary phases which offer recoveries of more than 90%. Our preliminary investigations show that high performance-gel permeation chromatography (HP-GPC) on a cross-linked divinyl benzene copolymer eluted with tetrahydrofuran is also attractive because the separatory mechanism is different from other HPLC systems and because samples weighing several milligrams can be processed without loss of resolution. HP-GPC is therefore an ideal first step in a multistage HPLC analysis for either GA's, cytokinins, or their respective derivatives.

Also encouraging is the use of 5 μm spherical supports that achieve high efficiencies on a

routine basis. Figure 2 illustrates the type of resolution achieved when a standard mixture of GACE's are chromatographed on a reverse phase column having 14,000 theoretical plates (Johnson and Stevenson, 1978). Recent data show that four 250 x 4.4 mm 5 μm Ultrasphere C_{18} columns in series make possible generation of 100,000 theoretical plates with a 1 ml min^{-1} flow and 8,000 psi pressure.
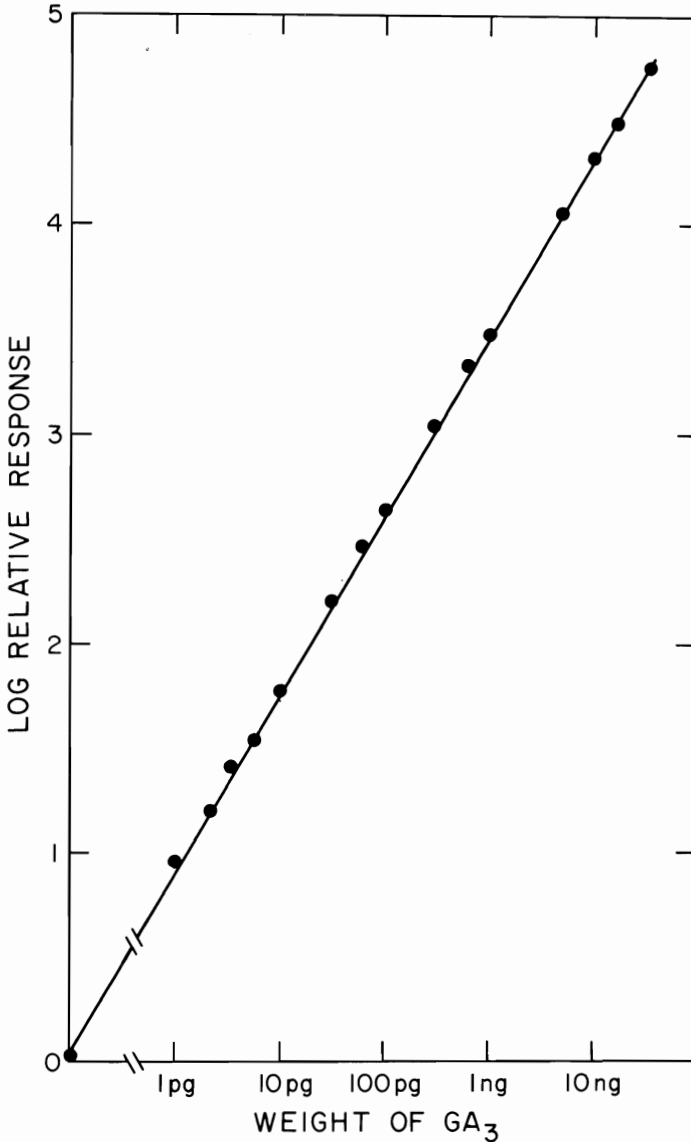

FIG. 1. — Spectrophotofluorimeter response to methoxycoumaryl ester of GA_3. Excitation 320 nm, emission 400 nm.
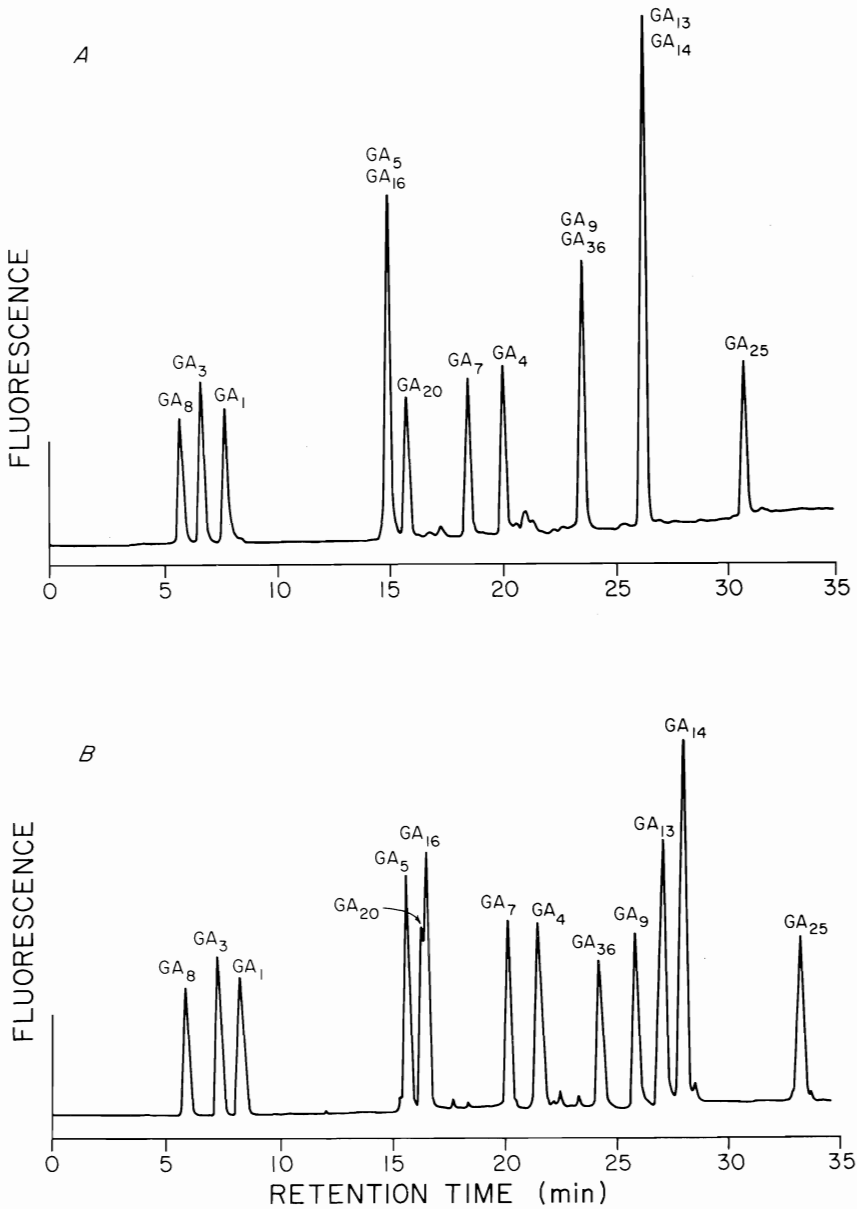
FIG. 2. — Reverse phase HPLC on methoxycoumaryl esters of gibberellin. Column: 5 x 250 mm Shandon Hypersil ODS: Mobile phase: 30 min gradient (A) 60-100% methanol in 0.02M, pH 3.5 ammonium acetate buffer; (B) 40-80% ethanol in 0.02M, pH 3.5 ammonium acetate buffer; Flow rate: 1 ml min $^{-1}$. Sample: methoxycoumaryl esters of gibberellin A_1, A_3, A_4, A_5, A_7, A_8, A_9, A_{13}, A_{14}, A_{16}, A_{20}, A_{25} A_{36}, ca 9 ng mono, 4.5 ng bis, and 3.0 ng tris esters. Detector; Perkin Elmer 650 LC spectrophotofluorimeter, excitation 320 nm, emission 400 nm.

CONCLUSIONS

Since the discovery of plant hormones, scientists have attempted to explain growth processes in terms of known hormones. As more are discovered, new explanations are required. At present we know that many occur in forest trees and that the levels appear to vary seasonally. Limitation in analytical methods in the past has prohibited satisfactory understanding of hormones in trees today. Advances in analytical techniques, however, promise more rigorous identification and quantitation of hormones on small samples of plant tissue and should produce more lucid explanations of how they control plant-growth processes. Exciting surprises — new hormones and change of old ideas — lie in store for forest tree physiologists.

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