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Review

The role of endogenous auxin in root initiation

Part I: Evidence from studies on auxin application, and analysis of endogenous levels

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Abstract. This paper describes the process of the formation of adventitious roots. There appears to be good agreement that this consists of four stages, dedifferentiation coupled with the formation of a meristematic locus, cell division to form a radially symmetrical cluster of cells, further divisions coupled with organisation into a bilaterally symmetrical meristem and finally growth of cells in the basal part of the meristem which causes its protursion through the epidermis. Evidence for the involvement of auxins in these various stages is reviewed and the extent to which rooting of easy- and hard-to-root species can be accounted for in terms of auxin content discussed. Peaks of IAA occur soon after excision of cuttings in some species and there is some evidence suggesting that this is correlated with changes in peroxidase activity. The possible involvement of cytokinins with auxins is briefly considered.

1. Introduction

The purpose of this review is to consider the involvement of auxins in the process of root initiation, particularly the relationship between endogenous levels of IAA and the early events of root formation. The data reviewed comes from studies on auxin application and endogenous auxin analysis in cuttings.

The concept of plant tissue sensitivity to plant growth substances was brought to the fore by Trewavas in 1981 [66], and has since been the subject of considerable debate [67]. The second part of this review will consider sensitivity, and more recent evidence on auxin involvement in root initiation from studies on morphogenesis in transgenic plants.

2. Definition of adventitious root primordia

In this first part, adventitious roots are defined as those originating in locations other than the primary root system. Consequently a cutting, perhaps a piece of shoot material excised from the parent plant may, under certain conditions, produce adventitious roots and hence a new root system and a complete plant [22]. In spite of the differing origin of the two types of root, their physiological functions are essentially the same. Whilst acting as supports, they both offer a means of extracting water and nutrients from their surroundings. This review will concentrate on the formation of adventitious roots on cuttings of woody and non-woody plants. There are many more specialised adventitious roots such as the aerial roots of epiphytes and the tabular roots which buttress tropical trees. The functions and evolution of these specialised adventitious roots have been discussed elsewhere [4].

2.1 Preformed root primordia

It is pertinent at this point to make a distinction between adventitious root primordia that have been induced de novo and those that are preformed. Preformed root initials are formed in the intact plant in the normal development of that plant [30]. They are usually very slow to develop, are found almost exclusively in woody plants and occasionally become evident externally as overt or aerial roots. The site of formation of preformed root primordia varies between species, although they have frequently been associated with ray tissue (*Thuja* species [23]) and nodal regions (Malaceae [12]). In stem cuttings of *Salix fragilis*, preformed roots are typically associated with nodal regions [13, 28]. They are not found in the first three nodes, but in subsequent nodes a predictable growth response can be observed. Root primordia associated with nodes of the same age are always at the same developmental stage [28].

Subsequent discussion in this paper will refer mainly to induced adventitious root primordia, i.e. those formed in response to wounding the shoot by severance, often by 'taking a cutting'.

2.2 Anatomical development of adventitious roots

2.2.1 Location

Anatomical studies on a wide range of plant material have shown that adventitious root formation usually occurs in the vicinity of differentiating vascular tissues [31], but within this generalisation there are many sites of origin. In a recent review Lovell and White [45] cited examples of 34 different sites of origin in woody plant stems and 19 sites of origin in the stems of herbaceous plants. It is possible with the large number of woody species investigated to identify certain favoured sites. Similarly to preformed root primordia, these sites are rays associated with phloem and cambium, or in bud or leaf gaps. Although induced root primordia are found in internodal as well as nodal regions, they may also occur in callus tissue produced at a wound surface.

2.2.2 Early anatomical events in the development of an adventitious root primordium/initial

In the most simplistic terms the process of adventitious root formation can be divided into two stages: the initiation of the root meristem, and its subsequent growth. Anatomically and biochemically there are a number of different stages in root initiation but the exact sequence of events in time and space is difficult to define unequivocally. This is due to the different observations in different species and to inconsistent use of terminology between investigators. There are still relatively few histological studies that present clear details of the time of initiation and the subsequent developmental sequences and the majority of those reported concern herbaceous cuttings. Several workers have attempted to define distinct stages in adventitious root formation [19, 24, 58], all of which essentially include the following:

(1) Dedifferentiation and the formation of a new meristematic locus:

this is the primary event of adventitious root initiation.

(2) Early cell divisions:

these produce a cluster of cells which show no polarity and are essentially radially symmetrical. At this stage the cells may not yet be determined in their new developmental pathway.

(3) Later cell divisions to form an organised determined root meristem:

it has been reported that the minimum size for a determined root meristem is 1500 cells [65, 73]. This is characterised by its bilateral symmetry.

(4) Formation of the root by extension growth of cells produced by the meristem:

this stage includes the formation of the vascular union between the new meristem and the stem, and is the stage at which the root first becomes visible externally.

Smith and Thorpe [58] examined the development of adventitious roots in *Pinus radiata* hypocotyls, which originate on the margins of differentiating resin ducts or in the parenchymatous tissue. The first visible sign of a new meristematic locus was the expansion of a single cell, with simultaneous nuclear swelling. The cytoplasm of such cells was much denser than that of adjacent cells. These early events seem characteristic of a meristematic locus, and have been reported in other species, for example tomato [16], *Phaseolus vulgaris* [55] and *P. aureus* [11]. Four days after taking hypocotyl cuttings of *Pinus radiata*, Smith and Thorpe [58] were able to demonstrate the presence of a meristematic locus by staining with dihydroxyphenylalanine. Within 10 days the meristemoids had developed into root primordia. A similar sequence of events leading to adventitious root formation is found in *P. aureus* hypocotyl cuttings, although the time scale is very different [11]. Here the first cell division occurred within 24 h and by 48 h well developed root primordia were evident. Although little work has been carried out with woody cuttings, the results of Davis et al. [18] with *Ficus pumila* suggest that the development of root initials in such material is similar to that described above.

3. The role of auxin

3.1 Circumstantial evidence

The notion that substances which promote rooting are present in leaves and that they are transported to the base of a cutting, originated towards the end of the last century [57]. It became more popular following the work of van der Lek [68] with willow, poplar and grape. He demonstrated that active buds produced a substance(s) which promoted rooting, since rooting was inhibited after an incision was made through the vascular system between the bud and the site of root initiation. Thimann and Went [64] discovered that 'heteroauxin', isolated from urine [39] stimulated adventitious rooting in the pea test developed for rhizocaline [72]. Synthetic IAA was also shown to stimulate adventitious rooting in this bioassay [63]. The practical significance of this work was quickly realised as IAA applied in lanolin was shown to stimulate the rooting of cuttings of a number of species including leafless lemon and *Tradescantia* [17]. Following the discovery of IAA and its association with adventitious rooting, many trials were carried out to see whether the application of synthetic auxin could solve the problems of difficult-to-root species. Audus [1] compiled an extensive list of the rooting responses of a wide range of species to applied auxins, and found that the majority responded positively. In discussion, Audus stressed the positive nature of these results, and suggested that auxin is one of the determinants although its effectiveness when applied externally may be affected by endogenous factors such as nutrients and other plant growth substances.

3.2 Exogenous application of auxin

For many years the only evidence of the involvement of auxins in the initiation of adventitious roots came from studies on exogenous applications. As indicated above, a number of workers have investigated the source of auxin in cuttings. Leaves and buds, known sites of auxin production [50, 74] have been found necessary for root initiation in a number of species. Replacement of buds and/or leaves by added auxin has been partly or completely successful in stimulating adventitious rooting in Douglas Fir [56]. *Pisum* [21] and *Salix fragilis* [29]. Further, auxin movement is preferentially basipetal [20, 51], which is consistent with the observed polarity of root formation [70]; roots forming at the base of cuttings even when they are held in an inverted position. However there is considerable evidence that auxin is not needed during all the stages of adventitious root production. Indeed some evidence suggests an inhibitory action at a later stage. Nahlawi and Howard [52] found that plum rootstock cuttings rooted better with an initial application of indole-3-butyric acid (IBA), than with repeated applications. The plum rootstock 'Pixy' failed to root in the absence of IBA [35]. Leafy pea stem cuttings were shown to be sensitive to auxin only during the first 3 days after the cutting was taken [48]. In a more critical evaluation of changing sensitivity, Smith and Thorpe [59] discovered that IBA was essential for two separate processes in the initiation of adventitious roots in *Pinus radiata* hypocotyls. IBA was necessary over the 4 day period immediately prior to the formation of a meristematic locus, and also over the next 2 days, during which the first cell divisions occured. Batten and Goodwin [5] did not make the distinction between the first cell divisions of root primordium formation. They found that exposure to a solution of IBA or IAA was necessary for 16 h to achieve maximal root initiation in etiolated *Phaseolus aureus* cuttings. Cell division was observed within this 16 h period. A similar effect of added auxin on the rooting of *Azukia* stem cuttings has been reported [47]. James [34] reported that 12 h of IBA exposure stimulated rooting of M9 apple shoots in vitro, but 48 h treatment increased the rooting three fold. Although the length of the auxin 'sensitive' phase is to some extent dependent upon the type and concentration of the auxin, and the plant species, this evidence suggests an early role for endogenous auxin in the initiation of root primordia. Auxin may not be an absolute requirement for later developmental stages, as indicated by the studies on the growth and development of excised root cultures [25]. Thimann [62] showed that IAA can be inhibitory to root development at all but the very lowest concentrations.

3.3 Endogenous auxin

Considering the large volume of literature pertaining to the effects on rooting by applied auxin, relatively few workers have attempted to correlate endogenous auxin levels with adventitious root formation in cuttings. It is very surprising that this opinion could have been expressed more than 25 years ago by Doré [19] and yet still be true today. However in the past 5 years a number of workers have published results of auxin analyses using reliable physico-chemical techniques.

3.3.1 Estimation at the time of cutting excision

Endogenous auxin levels associated with the rooting of different species [53], cultivars of the same species [7, 40], seasonal variations [69] and age differences [32] have been estimated. The early work on endogenous auxin levels and adventitious rooting largely utilised bioassays, and often considered the auxin status of the cutting at the time of excision. The results published in these systems are contradictory. Odom and Carpenter [53] found that auxin levels at the base of seedling cuttings of *Alternanthera*, *Chrysanthemum* and *Coleus* correlated well with the vigour and rapidity of rooting. No differences were found in the auxin levels in an easy and a hard-to-root cultivar of *Dahlia* [7], sugar maple [27], *Chrysanthemum* [61] and more recently *Rhododendron* [75]. In contrast a positive correlation between ease of rooting and auxin levels has been reported with two cultivars of grapevine rootstock [40]. Much higher levels of auxin were found in the easy-to-root cultivar, although the bioassay used for this estimation was not reported.

Rooting differences occuring within the same species arguably provide a better system for investigation. This system removes the chance of genetic causes for differing auxin measurements. Hengst [32] showed that the variation in rooting of *Streptocarpus* leaves of different ages was closely correlated with variation in auxin content of the whole plant. Other workers have investigated seasonal rooting patterns and associated auxin levels and found no correlation between the two [27, 38]. In contrast, Smith and Wareing [60] reported a decline in auxin levels in the stem of *Populus* × *robusta* cuttings taken over the growing season and this decline correlated well with a gradual decrease in the rooting ability of the cuttings. The authors suggested that these declines were under the influence of photoperiod. Cuttings of *Cotinus coggygria* root well in the spring, but very poorly in the summer and autumn [10]. The levels of IAA in the rooting zone (stem base) of cuttings taken in the spring was significantly higher than in the summer. The level of conjugated IAA was significantly higher in the summer cuttings. Consequently the ratio of free IAA to total IAA varied markedly at the two times of year. Spring cuttings were taken at the beginning of the period of rapid growth (early June) whilst summer cuttings were taken at the end of the growth period (late July). If IAA is associated with the onset of cambial activity [2, 44] and increased growth rate [3], then it might be expected that in the summer cuttings, the concentration of free IAA would be much less than in the spring. An attempt was made to see how the levels of IAA in the spring cuttings of *Cotinus* changed with time after excision [10]. Over the first 14 days the level of IAA in the rooting zone was found to decline significantly. This decline was accompanied by the differentiation and growth of the root primordia.

The problems of determining the association between endogenous plant growth regulators and root initiation in woody plant material are considerable. More recently workers have concentrated on using non-woody or hypocotyl explants to investigate these problems, considering the changes in the levels of endogenous auxin during the rooting period.

3.3.2 Estimation during the rooting period

It has been reported that free IAA, measured using ELISA techniques, increases in whole terminal cuttings of *Chrysanthemum moriflorum* until the time when the first adventitious roots become visible [71]. From the data presented it is not possible to judge whether the peak of IAA occurs before or after the primary event of root initiation. Ideally the levels of IAA should be measured several times before the primary event of root initiation.

A modified HPLC technique [9] was used in our laboratories in a preliminary investigation into the changes in endogenous hormone levels during the early stages of root initiation in *Phaseolus aureus* [8]. Root initiation and emergence in 6-day-old *P. aureus* hypocotyls is very rapid. The first cell division takes place within 24 h, well developed root primordia are visible within 60 h and roots emerge after 100 h. Endogenous hormones were analysed during the 35 h following excision. In the rooting zone at the base of the hypocotyl, a sharp peak of free IAA was detected in the first 10 h following excision. The level of IAA then declined below that at the time of excision. There was very little change in the levels of IAA in the remainder of the hypocotyl. These results suggest that a transient rise in the levels of IAA occurs before the first detectable event in root initiation, but falls prior to subsequent cell divisions. Moncousin et al. [49] recently reported changes in the levels of IAA in in vitro cuttings of grapevine, on a non-hormone medium. Using ELISA, in the basal part of the shoots they found a clear increase in IAA after 12 h, followed by a sharp decrease to a minimum after 36 h, then a very slow recovery. This early peak of IAA was not found in the apical part of the shoots. It confirmed the view that a peak of IAA can precede root formation. Moncousin et al. [49] noted that some early cytological events such as nuclear swelling were visible at the time of the auxin decline but not before. Therefore the peak of auxin concentration coincided with the termination of the induction period. The period of low auxin corresponded to the early events of the initiation phase. Further support for the requirement of an early peak in IAA comes from the work of Maldiney et al. [46] with hypocotyl cuttings of Craigella and Craigella lateral suppressor tomatoes. Using ELISA they also identified a peak of IAA, although the peak occurred after 72 h. Root primordia were detectable in cleared hypocotyl sections after 96 h. The authors suggested that the primary event of root initiation occurred concomitant with the rise in the IAA level. A similar finding was reported by Label et al. [42] during in vitro rooting of *Prunus avium* explants. Growth of the primordia occurred as the level of IAA declined. Berthon et al. [6] reported that adventitious root initiation in *Sequoiadendron giganteum* cuttings in vitro was accompanied by a fall in IAA, measured by ELISA. Micro-cuttings were placed on a medium containing NAA. Root primordia began to form after 6 days but the timing of sampling dates was such that an increase in IAA could easily have been missed.

4. Discussion

The fact that auxin is involved in the process of adventitious root initiation is well established. However, much of the data to support this is circumstantial. Measurement of endogenous levels of IAA in the rooting zone of cuttings should provide more direct evidence and indicate how changing auxin levels control or influence the different phases of root initiation. Unfortunately there are still relatively few studies on this process using unequivocal physico-chemical techniques.

The first stage of root initiation is dedifferentiation and the formation of a new meristem at the locus. Several workers have found a peak in the level of IAA in the rooting zone associated with this primary event [8, 46, 49]. In each case the level of IAA subsequently declined. There are other instances where no increase in IAA concentration was observed, only a gradual decline [6, 10]. Earlier sampling might have revealed a peak of IAA in these studies. Auxin is transported basipetally in the shoot, consequently when a cutting is excised, auxin might be expected to accumulate at the base. Several resports show that this accumulation is aided by reduced activity of IAA oxidase/peroxidase in the rooting zone [6, 15, 49]. Vine cuttings show a transitory decrease in peroxidase after excision and this has been shown to coincide with an increase in IAA concentration [49]. The IAA concentration subsequently declined, accompanied by increased peroxidase activity. Gasper, however, proposed in 1981 [26] that the primary event of root initiation is characterised by a decrease in the level of IAA, although this was proposed before unequivocal evidence of the early accumulation of IAA had been obtained. More recently Jarvis [36] proposed a model in which auxin accumulation is associated both with the formation of the meristematic locus and the early cell divisions of the second phase. The third phase (continued cell divisions to form an organised meristem) is associated with low levels of IAA. The data available to date do not allow us to judge whether phase one and phase two both require an increased concentration of auxin. Many workers have studied the cytological and biochemical changes associated with the early cell divisions in root formation. The first visible signs of initiation in *Phaseolus aureus* hypocotyl cuttings are the cytoplasm and nucleus becoming dense. This occurs within the first 24 h, with cell division following within the next 48 h [14, 73]. Jarvis et al. [37] reported a phase of diminished RNA synthesis within the first 20 h. A subsequent increase in RNA synthesis which would correspond to the first cell divisions has also been demonstrated [43]. Both of these studies suggested that the increased synthesis of RNA was caused by an increased auxin concentration.

Severance of a shoot from its root system will inevitably have considerable effects on movement and localised accumulation of assimilates and plant growth substances. An intact plant is considered to be transporting cytokinin from the root to the shoot. Excision of the shoot removes this source of cytokinin and might, therefore be expected to lead to a reduced cytokinin concentration at the base of the shoot. Preliminary studies of cytokinin levels in cuttings of *Cotinus* and *Phaseolus aureus* [8] showed that whilst cytokinins were easily detectable at the time of excision, their concentration fell dramatically to very low levels on subsequent harvest times. Similar findings have been reported in *Xanthium* and tomato [33, 46]. An increase in cytokinin concentration was reported in willow and poplar cuttings during the rooting period [41, 54] but the timing of this was such that it may well have been due to synthesis within the newly developed root primordia. When cuttings are excised, cells at the base are likely to be exposed to changing ratios of auxin to cytokinin which might stimulate the start of the events of the cell cycle.

It is possible to erect quite complex hypotheses for the involvement of auxin in adventitious root initiation. But in the opinion of the authors there is disagreement between reports, and still very few studies accurately measuring levels of auxin around the crucial time of the 'primary' event of root initiation, in or close to the cells that are involved.

This review has dealt largely with the role of absolute levels of endogenous auxin in the process of adventitious root initiation, and the evidence for that involvement. However it would be unwise to ignore the role of sensitivity in the response of cells to auxin. Several recent studies on rooting have been interpreted in these terms [12, 34]. The second part of this review discusses sensitivity, and further evidence for the role of auxin in root initiation arising from studies on transgenic plants.

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