

## Adventitious root formation in ornamental plants: II. The role of plant hormones

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## **Abstract**

Adventitious root formation (ARF) is an important step in the vegetative propagation of plants for forestry, horticulture, and agriculture. As a physiological process, adventitious rooting is regulated by multiple plant hormones that partake in a complex web of interactions. Further, the exogenous application of synthetic and natural plant hormones is common practice for inducing ARF in plant propagation ~~industry~~. In this review, we revisit the established, central function of auxins in ARF, and discuss the mechanism of their activity. We also discuss the effects of two other plant hormone classes with established roles in adventitious rooting, ethylene and cytokinins, and summarize our present understanding of how these three hormone classes interact. The effects of abscisic acid, gibberellins, brassinosteroids, salicylic and jasmonic acids, and nitric oxide on ARF are also presented. Throughout, we emphasize the interactions between hormone classes and how these affect ARF. This review will provide a better understanding of adventitious rooting as an intricate physiological process, and will demonstrate the complexity of induction of ARF by exogenous hormonal treatments.

**Key words:** adventitious roots, auxins, cytokinins, ethylene, plant hormones, nitric oxide

## INTRODUCTION

Adventitious root formation (ARF, or rhizogenesis) is the process whereby roots develop outside of their normal developmental sequence or at an unusual location (Esau 1977). Forestry, horticulture, and agriculture all take advantage of ARF from cuttings, where this is a necessary step in asexual plant propagation. While cuttings have been used to propagate plants for ages, the discovery of auxins and other plant hormone classes has made ARF a much more efficient and productive strategy for plant propagation over the course of the last two centuries (Haissig and Davis 1994). As such, the effect of auxins on ARF has been studied in-depth by scientists, and its application in industry is now widespread.

Applied auxins have a predictable and consistent effect across plant taxa in inducing ARF. Other plant hormone classes, such as gibberellins (GAs), cytokinins (CKs), brassinosteroids (BRs), abscisic acid (ABA), and ethylene also have demonstrated effects on ARF, though they are not commonly used in industry. However, their effects cannot be ignored. Plant hormones regulate virtually all aspects of plant growth and development, and generally exert their effect through interactions with other hormones (Davies 2010). Though the ability of applied auxin to induce adventitious root growth is common, its mechanism and its interactions with the other hormone classes are not generally considered or understood. In our previous review, we documented successful induction of ARF in a wide variety of ornamental species using plant growth regulators and growth media (Oinam et al. 2011). In this review, we compile new information regarding the mechanism of auxin's activity and its interaction with cytokinins and ethylene. The effects of all of the above major hormone classes on ARF are discussed individually, as well as the less-well understood effects of the emerging hormones salicylic acid, jasmonic acid, and nitric oxide.

## AUXINS AND ADVENTITIOUS ROOT GROWTH

Auxins are a group of plant hormones defined by their activity *in planta*; primarily, they promote cellular elongation, induce cell division when applied with a cytokinin, induce ethylene synthesis, and induce adventitious root growth (Normanly et al. 2010). Auxins have been shown to be involved in arguably every aspect of root formation, including induction of cellular rhizogenic competence, root apical meristem differentiation, development of the root cap and vasculature, and tropic responses (Konieczny et al. 2009). Because of auxin's critical role in root development and its use in industry, the effect of different auxins has been a major theme in ARF research. It is well established that the basipetal transport and accumulation of auxin at the base of stem cuttings causes ARF (Liu and Reid 1992 a,b; Hausman et al. 1995; Guerrero et al. 1999). Adventitious rooting is induced by the activation, differentiation, and elongation of phloem parenchyma cells adjacent to vascular bundles in the stem (Lund et al. 1996, De Klerk et al. 1999, see Oinam et al. 2011). However, these effects are only observed when auxin is applied to induce ARF; auxin becomes inhibitory to ARF when applied during later development (De Klerk et al. 1999).

As a "phytohormone class", auxins include both naturally occurring and synthetic chemicals. Natural auxins include indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA), indole-3-butyric acid (IBA) and phenylacetic acid (PAA). 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA) are examples of well-studied synthetic auxins (Simon and Petrasek 2010). Figure 1 presents chemical structures of different naturally occurring and synthetic auxins.

In horticultural and agricultural industries, IBA is widely used as a rooting agent. Although IAA is the most abundant naturally occurring auxin and was the first auxin to be used

to stimulate adventitious root formation on stem cuttings (Cooper 1935), it is generally not utilized in industry because of its tendency to degrade rapidly with light. IBA is commonly applied as it has greater stability than IAA, and is less susceptible to *in vivo* catabolism and conjugation to sugar and amino acid molecules that render it inactive. IBA is the second most abundant natural auxin, making up 25-30% of the auxin pool in *Arabidopsis thaliana* seedlings (Ludwig-Muller et al. 1993). Although it has been shown that IBA can be synthesized independently, the majority of IBA in plant tissues is synthesized from IAA (Ludwig-Muller 2000). The conversion of IBA to IAA has been shown in *Pinus sylvestris* (Dunberg et al. 1981), *Vitis vinifera* (Epstein and Lavee 1984), *Malus pumila* (Alvarez et al. 1989), *Pyrus communis* (Baraldi et al. 1993). In stem cuttings of *Arabidopsis thaliana* the application of IBA causes an increase in the endogenous IAA levels (Figure 2) suggesting the conversion of IBA to IAA. That IBA can also be converted back to IAA (Zolman et al. 2008) indicates that IBA might not itself be a bioactive auxin; however, this remains a matter of debate (Normanly et al. 2010, Poupart and Waddell 2000, Zolman et al. 2000, Ludwig-Muller et al. 2005). Observations that the effects of IBA and IAA can vary suggest that they do have slightly different activities. For example, in *A. thaliana* seedlings, conversion of IBA to IAA leads to induction of root hair elongation, whereas IAA from other sources induces hypocotyl elongation (Strader et al. 2010). In rice, the induction of lateral root development can be accomplished by application of both IAA and IBA, however the amount of applied IAA has to be about 20-fold higher than the amount of applied IBA to achieve a similar response (Chhun et al. 2003, 2004). It is not absolutely clear whether applied IBA is bioactive on its own in inducing ARF or if it must first be converted to IAA. That IBA can be converted to IAA while other auxins can induce ARF without being converted to IAA together suggest that applied IBA can induce ARF both directly and indirectly.

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Applied IBA has also been shown to have a higher root inducing capacity than IAA itself despite demonstrated conversion to the latter (Zimmerman and Wilcoxon 1935, Chao et al. 2001). In *Vigna radiata*, for example, ARF was induced by exogenous IBA application, but not by IAA application (Riov and Yang 1989). In *Malus pumila* cuttings, applied IBA was much more potent in inducing adventitious root formation than IAA, but the conversion of IBA to IAA was very limited (~1%) (Alvarez et al. 1989). Alternatively, the greater ability of IBA over IAA to promote ARF can be attributed to its higher stability in plant tissues and growth medium (Nordstrom et al., 1991).

Differences in auxin metabolism, in particular conjugation of auxins to inactive forms, can significantly alter the plant's ability to form new adventitious roots. For example, in studies with two cultivars of *Prunus avium*, where cuttings of one cultivar had a greater ability to produce new roots than another cultivar, it was shown that the cultivar with greater rooting ability conjugated IBA at a lesser rate than the other (Epstein et al. 1993). Further, application of a specific inhibitor of IBA conjugation to a *P. avium* cultivar with a lower rooting ability rapidly increased root formation (Epstein et al. 1993).

NAA is also used to induce rooting, but is not as common as IBA as it is a synthetic product and can be toxic at high concentrations. 2,4-D is toxic at much lower concentrations than is NAA and is often applied as a herbicide, not a rooting agent, as it causes uncontrolled cell division.

The method of application of exogenous auxin to plant cuttings or intact plants requires careful consideration, as auxin can be applied at lower concentrations (micromolar range) during a period ranging from few days to even few weeks or it can be applied at higher concentrations (millimolar range) during short (range of minutes) exposure (Hartmann et al. 1990). The use of

lower concentrations of applied auxin is preferable for juvenile plant cuttings which require a longer period to form new roots and for overall growth. The use of higher concentrations of applied auxin is preferable for mature plant cuttings which benefit from a short exposure to exogenous auxin (dip or a one-time spray) before replanting. The success in induction of adventitious rooting can also depend on the auxin used, as discussed above.

It has been shown that some plants are capable of producing new roots on cuttings spontaneously, without applied auxin (Nordstrom and Eliasson 1991). However, the production of new roots on these cuttings was demonstrated to still be under auxin control. The removal of the shoot apex reduced endogenous auxin content, resulting in a decrease in the number of new roots relative to control cuttings. (Nordstrom and Eliasson 1991). Conversely, the application of auxin to cuttings with removed shoot apices significantly increased the number of new roots formed (Nordstrom et al. 1991, Liu and Reid, 1992 a,b).

#### **THE ROLE OF ETHYLENE IN ADVENTITIOUS ROOTING**

In addition to auxin, numerous studies have found that the simple gas molecule ethylene plays an important role in adventitious root growth (Biondi et al. 1990, Clark et al. 1999). In wounded sunflower hypocotyls, increased ethylene production has been shown to stimulate the formation of root primordia (Liu et al. 1990). Interestingly, this was only true in the lower portion of the hypocotyl; increased ethylene levels in the upper sunflower hypocotyl inhibited rooting (Liu et al. 1990). Application of 1-aminocyclopropane-1-carboxylic acid (ACC), the biosynthetic precursor to ethylene, to apple leaf disks has also been shown to induce root initiation. This treatment failed when it was applied to agar-grown cuttings due to accumulation of excessively high ethylene levels (De Klerk et al. 1999). These effects of ethylene on root

initiation are not surprising considering that auxin-ethylene cross-talk is expected to take place at biosynthetic, signaling, and response levels. This cross-talk can have either synergistic or antagonistic effects, depending on the location and the response in question (Stepanova et al. 2007). For example, increased ethylene levels in soil flooded plants induced tissue sensitivity to IAA and thus promoted initiation of adventitious rooting (Lorbicke and Sauter 1999). Also, inhibition of ethylene biosynthesis or auxin transport in soil-flooded plants was demonstrated to decrease the number of adventitious roots (Visser et al. 1996).

Ethylene has been shown to affect both auxin transport (Suttle 1988) and perception (Bertell et al. 1990), as well as to stimulate an increase in auxin biosynthesis (Seifert et al., 2004). Auxins can in turn increase the rate of ethylene biosynthesis (Riov and Yang 1989). Transcription of ACC synthase genes, which catalyze the regulatory committed step in ethylene synthesis, is specifically induced by auxins (Abel et al. 1995, Tsuchisaka and Theologis 2004). Further, auxin-induced ethylene production has been shown to stimulate adventitious rooting in mung bean hypocotyl cuttings (Pan et al. 2002). Under other conditions, auxins restrict ethylene production post-transcriptionally by blocking the ACC-synthase catalyzed conversion of S-adenosyl methionine (SAM) to ACC (Stepanova et al. 2007).

Another interaction scheme between auxin and ethylene was recently described by Konieczny et al. (2009), wherein ethylene promotes auxin polar transport by activating transcription of PIN and AUX auxin transport proteins. Because localized auxin accumulation is a key factor in root formation and patterning, and because auxin accumulation is determined by localized PIN carrier proteins, this interaction favors localized AR growth. The authors incorporate this information into a larger model describing hormonal control of adventitious rooting. They also include cytokinins in their scheme, as cytokinins and auxins synergistically

increase ethylene biosynthesis. However, cytokinins predominantly counteract auxin-induced root formation by a number of processes, such as down-regulation of root-specific cyclin-dependent kinases, polar auxin transport disruption by repression of the expression of PIN proteins, and the direct down-regulation of auxin biosynthesis. Cytokinin concentration is greatest at the formation stage of ARF when increased rates of cell divisions are required. Reduced ethylene signaling and, potentially, biosynthesis induced by increased sucrose concentration, may account for high cytokinin levels (Yanagisawa et al. 2003, Mortley et al. 2008).

## CYTOKININS

Cytokinins (CKs) are a class of plant hormones that promote cell division, shoot development, and have a strong effect on apical dominance. CKs appear to play an antagonistic role in multiple stages of AR development in many species, including *Arabidopsis*, *Medicago*, tobacco and rice (De Klerk et al. 1997, Werner et al. 2003, Rani Debi et al. 2005, Gonzalez-Rizzo et al. 2006, Li et al. 2006, Laplaze et al. 2007) but are required for the formation stage when cell divisions occur. Application of CK to stem cuttings during AR establishment strongly inhibits their development (De Klerk et al. 1999). A biologically active and naturally occurring CK, *trans*-zeatin riboside, was identified in cucumber root xylem sap as a potent inhibitor of ARF in hypocotyls (Kuroha et al. 2002). In sunflower hypocotyl cuttings, application of CK at low concentrations induced root primordia initiation, whereas CK applied at higher concentrations inhibited root primordia initiation (Fabijan et al. 1981). In *Lactuca sativa*, application of CKs at physiological concentrations inhibited root formation and mitigated the effects of IAA application (Zhang and Hasenstein 1999). An *A. thaliana* line overexpressing

cytokinin oxidase/dehydrogenase (CKX) genes with demonstrated reduced endogenous CK levels showed increased frequency of ARF (Werner et al. 2003). Opposite patterns in endogenous levels of auxins and CKs have been detected in numerous plant species during ARF (Ramirez-Carvajal et al., 2009); auxin concentrations are high and CK concentrations are low during early stages of AR development, while auxin concentration are low and CK concentrations are high during later development when cell divisions are occurring (Blakesley et al. 1985, Maldiney et al. 1986, Kevers et al. 1997). Further, histological studies have shown CK to inhibit the differentiation of primordia in pea cuttings during early developmental stages (Bollmark and Eliasson, 1986). Finally, ethylene has been shown to promote rooting by stimulating cytokinin catabolism (Bollmark and Eliasson 1990). See Fig. 3 for a summary of auxin, cytokinin, and ethylene interactions during ARF.

## GIBBERELLINS

Similar to auxins and cytokinins, gibberellins (GAs) as a group of plant hormones are defined by the processes they affect *in planta*, including stem elongation, seed dormancy and germination, flowering, and senescence. Products of GA biosynthesis have been detected in root tips of different plants and GA signalling is required for primary root growth in *A. thaliana* and rice (Fu and Harberd 2003, Kaneko et al. 2003), however, research suggests that GAs generally restrict adventitious rooting. Rice mutants overexpressing *GA2oxs*, which is responsible for catabolism of growth-active GAs, show a phenotype typical of GA deficiency and exhibit increased adventitious rooting (Lo et al. 2008). Application of growth-active GA inhibited ARF in wild type rice plants (Lo et al. 2008); similarly, transgenic poplar trees with GA insensitivity show increased adventitious rooting, whereas application of gibberellic acid inhibited AR growth

(Busov et al. 2006). In deep water rice plants GAs were shown to act synergistically with ethylene to promote initiation and growth of ARs (Steffens et al. 2006); however, this synergism is likely unique to submerged plants. It is still unknown if auxins interact with GAs in regulating ARF, either by directly down-regulating GA biosynthesis or via an interaction with ethylene.

## BRASSINOSTEROIDS

Brassinosteroids (BR) can promote cell expansion and elongation, vascular differentiation, senescence, and assist in plant adaptation to cold and drought stress. Application of BR in *A. thaliana*, promoted root growth at low concentrations, but was inhibitory at higher concentrations (Mussig et al. 2003). As brassinosteroids often stimulate a physiological response through their interaction with auxin (Nemhauser et al. 2004), it was suggested that BRs also interact with auxin to promote lateral root development (Bao et al. 2004). An effect of endogenous BR levels on auxin signalling was demonstrated through investigation of an *A.thaliana* line with a root-specific deficiency in BR signalling. These plants exhibited a short and highly branched root system, and auxin-responsive transcription was globally impaired (Mouchel et al. 2006). Rice mutants affected in BR biosynthesis (*brassinosteroid-dependent 1* [*brd1*]) show a significant reduction in BR and abnormal root morphology phenotypes. Normal root growth could be restored by application of BR (Mori et al. 2002). Also in rice, a casein kinase 1 gene, *OsCKI*, was shown to be upregulated by BR and was able to induce adventitious root formation, likely by increasing endogenous auxin levels (Liu et al. 2003). Thus, although the precise mechanism of their activity is unclear, BRs appear to promote lateral root development and ARF through interactions with endogenous auxins.

## **ABASICIS ACID**

Abscisic acid has subtle roles in many plant processes, but is especially important in dormancy, germination, abscission, and stomatal regulation. The specific function of abscisic acid (ABA) in ARF is not clear, but several pieces of evidence suggest that it might be involved. In *A. thaliana* seedlings, exogenously applied ABA significantly inhibited primary and lateral root development (Beaudoin et al. 2000, De Smet et al. 2003), and ABA inhibition of lateral root development appeared to be auxin independent (De Smet et al. 2003). Also in *A. thaliana*, the ABA-insensitive line *abi3* has decreased sensitivity to auxin-induced root initiation, suggesting that an interaction between auxin and ABA promotes lateral root development (Brady et al., 2003). Similarly, in rice, ABA was shown to induce lateral root development (Chen et al. 2006).

## **SALICYLIC AND JASMONIC ACIDS**

Although the involvement of the defence-related hormones salicylic acid (SA) and jasmonic acid (JA) in ARF is not well studied, there are some reports that both hormones have an effect on this process. Kling et. al. (1983) reported that a combination of applied IAA with SA at 0.1 mM stimulated ARF at higher rate than IAA applied alone. Li (1995) reported SA-induced ARF on hypocotyl cuttings of *Vigna radiata*. It was later suggested that SA may induce ARF by stimulating oxidation of auxin (De Klerk et. al. 1997). Finally, Singh (1993) found that application of SA induced root formation in young shoots of some ornamental plants. Similarly, jasmonic acid was shown to stimulate ARF in *Vigna radiata* (Zimmerman and Vick 1983) and potato stem cuttings (Ravnikar et al. 1990, 1992).

## **NITRIC OXIDE**

Nitric oxide (NO) is a diffusible lipophilic gas which has three forms in both animals and plants: NO<sup>+</sup> (nitrosonium cation), NO<sup>-</sup> (nitroxyl anion) and NO<sup>.</sup> (nitric oxide radical). Its lipophilic nature allows it to easily diffuse through biological membranes, and so it is not surprising that it serves as an intercellular signalling messenger in mammals. NO has recently emerged as a cellular messenger in numerous physiological processes in plants through interactions with plant hormones (Lamattina et al. 2003), including interaction with auxin during AR development (Gouvea et al. 1997). Increased endogenous IAA content was shown to increase NO concentration during adventitious rooting, and it was therefore proposed that NO operates as a second messenger downstream of IAA (Pagnussat et al. 2002, 2003). Since then, NO has been shown to be involved in the induction of lateral root development (Correa-Aragunde et al. 2004), in the root gravitropic response (Hu et al., 2005), and in the growth and development of root hairs (Lombardo et al. 2006). In-depth studies have investigated the mechanism of NO's effect on ARF. It has been proposed that increased NO levels induce lateral root development via both a cGMP and a cGMP-independent MAPK cascade. (Pagnussat et al., 2003, 2004).

## CONCLUSIONS

Many different plant hormone classes are involved in ARF. This is not surprising, given that variable levels of many plant hormones are present in any given plant cell. Thus, changes in the concentration of plant hormones and sensitivity of plant tissues to these hormones are causal in hormone-mediated and/or -regulated developmental events. Understanding which plant hormones are active at a given stage of plant development and how they interact with each other is a complicated issue especially since endogenous concentrations of plant hormones can rapidly

influence each others' biosynthesis. While it is possible to determine the mode of action and interactions between two plant hormone classes, the introduction of a third class makes it almost impossible to properly interpret physiological responses. It appears that during the induction stage of root development there is a requirement for higher auxin concentration at the site of the induction, leading to increased ethylene production, which in turn inhibits CK biosynthesis. At the later ~~formation~~ stage of AR development endogenous auxin concentrations are reduced, which likely leads to a decrease in ethylene production and thus ceases the inhibition of CK biosynthesis. How other plant hormone classes affect adventitious rooting and how they interact with these hormones is less clear, but will doubtless be the subject of future research. Full understanding of the endogenous signals that control ARF will give us a better appreciation of AR development in general, and can be applied in the manipulation of adventitious rooting for plant propagation industry.

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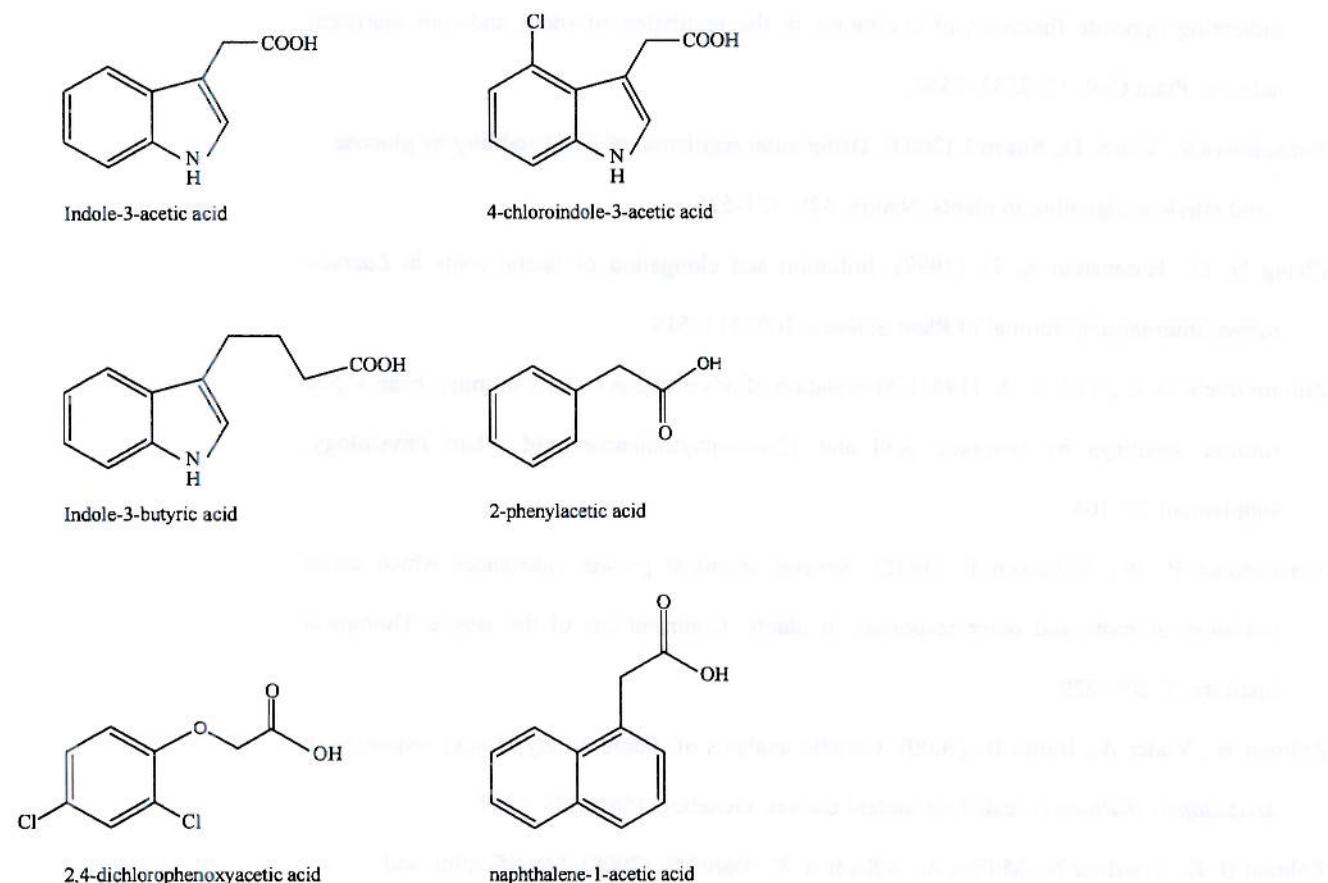
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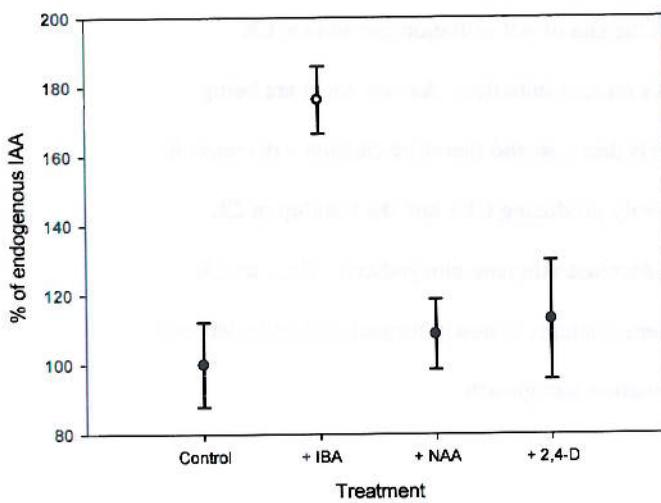
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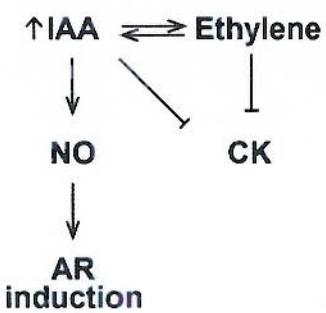
**Figure 1:** Chemical structures of natural and synthetic auxins: indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA), indole-3-butryic acid (IBA), phenylacetic acid (PAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA).



**Figure 2.** Percentage of endogenous IAA in 2 wk old *Arabidopsis thaliana* plants with hypocotyl and roots removed, at day 2 following the treatment with control solution (no auxin added, set at 100% endogenous IAA) or various auxins: IBA, NAA and 2,4-D applied at  $10^{-4}$  M. The error bars indicate S.E.M. and data is from three independent biological experiments (Kurepin and Yeung, unpublished data). Only applied IBA resulted in an increase in endogenous IAA levels, and this treatment was the most efficient (comparing to Control, + NAA or +2,4-D) in promoting ARF.



**Figure 3.** The proposed model of auxin, ethylene, and cytokinin interactions during adventitious root induction (A) and formation (B) in stem cuttings. At the site of stem cutting, the mechanical stress induces localized ethylene production, which in turn can block IAA transport resulting in a local increase in IAA. The increase in endogenous IAA concentration causes an increase in ethylene production, which in turn promotes an increase in IAA biosynthesis in a positive feedback loop. This build up in IAA causes increased cell division and then, via NO signaling, AR initiation. CK levels, which can be inhibitory for AR initiation at physiological concentrations, are low in stem cuttings as the main site for CK biosynthesis, the roots, is not present. Additionally, the high ethylene concentrations at the site of AR initiation can induce CK catabolism, therefore removing the bioactive CKs on root initiation. As new roots are being formed during AR formation stage, ethylene levels decrease and therefore causing a decrease in IAA levels. The newly formed root cells are actively producing CKs and the buildup in CK levels antagonizes IAA levels which in turn can decrease ethylene biosynthesis. Thus, as CK concentrations increase while the IAA and ethylene contents in newly formed root cells decrease, this CK to IAA/ethylene ratio allows for AR formation and growth.

**A. Induction Stage****B. Formation Stage**