Understanding Leads to Significant Improvements in Microcuttings

The roots of **rooting**

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Cuttings produced in tissue culture can be rooted ex vitro in the same way as conventional cuttings, for example, by a dip in rooting powder. Alternatively, they can be rooted in tissue culture (in vitro) on a nutrient medium with a moderate auxin concentration. In vitro rooting often results in far better performance during acclimatization. The special conditions during in vitro rooting should be considered to achieve optimal in vitro rooting.

Rooting is a cornerstone of the horticultural industry. Some crops, in particular monocotyledons such as bulbous crops, root easily and usually do not need a special rooting treatment. Cuttings of woody plants are at the other end of the spectrum and often fail to root. Poor rooting has vast economic consequences. In conventional propagation, losses are estimated to be 10-25% with nursery crops and 5% with ornamental crops. These figures refer to survival rates and additional losses occur because of poor growth of cuttings and the need for intensive chemical protection. In micropropagation, an additional economic aspect plays a major part: rooting can be carried out in vitro or ex vitro. From the economic point of view, ex vitro rooting is preferable because in vitro rooting increases the costs per microcutting considerably. However, the choice between in vitro and ex vitro rooting depends on a complete outline of costs, including the performance after planting. The procedures for ex vitro rooting are the same as for rooting of conventional cuttings. With in vitro rooting, there are two new aspects that are usually unrecognized, namely the choice of auxin (is IBA the best auxin just as with ex vitro rooting?) and ethylene. They are discussed below.

Auxin

In the late 1920s, research on plant hormones began with the discovery of the auxin IAA by the Dutch plant physiologist, Frits Went. Soon after, it was demonstrated that auxin promotes rooting. The next findings crucial for the practical use of auxin in rooting were made in the 1930s: the discovery of IBA as an alternative for IAA, and the use of talcum powder as carrier of auxin enabling "rooting powder". Progress in fundamental research, though, was limited. Much of the research concerned the screening of newly discovered plant growth regulators and auxin analogues for their effect on rooting. A major problem for biochemical and molecular research is that only very few cells in a stem, far less than 0.1 %, are involved in the process. So when tissues are analyzed biochemically, their characteristics are swamped by the surrounding cells. A recent step forward was the discovery that rooting, just as other regeneration processes, consists of successive developmental steps. Auxin has distinctive effects in these steps. It has already been known for a long time, that auxin is required for the formation of root meristems, but inhibits their outgrowth. In apple microcuttings rooted in vitro using IBA, about half of the induced root meristems did not develop any further when IBA was not removed. The timing of the action of auxin was established in detail by giving 24-hour pulses with auxin on various days after excision of microcuttings. Thus, samples of 30 microcuttings were transferred to medium with auxin at various times after excision. Up to this transfer, they were cultured at auxin-free medium. After 24 hours at the auxin-containing medium, they were transferred back to auxin-free medium. During the 24-hour pulse, the auxin concentration in the tissue increases sharply and after the pulse it decreases to the original level within a few hours. These pulses showed that in apple microcuttings, auxin was active as a rhizogenic signal from 24-96 hours after excision. This timing was validated by pulses with the genuine anti-auxin PCIB (PCIB competes with auxin for the auxin receptor) and the cytokinin BAP (cytokinins are auxin antagonists). A summary of the timing in apple is shown in Figure 1. In Arabidopsis, mutants have been isolated for the successive steps.

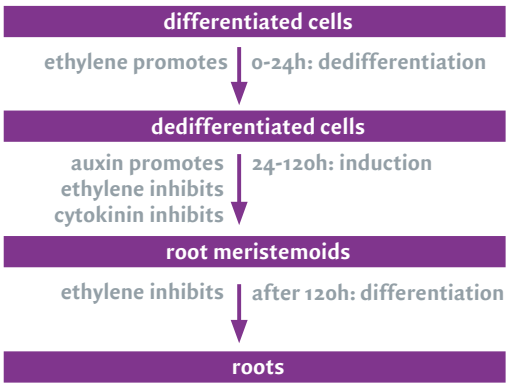

Fig. 1. Timing of the successive steps in the rooting process. The effect of plant hormones is indicated

Choice of the type of auxin

For rooting ex vitro, microcuttings are dipped in

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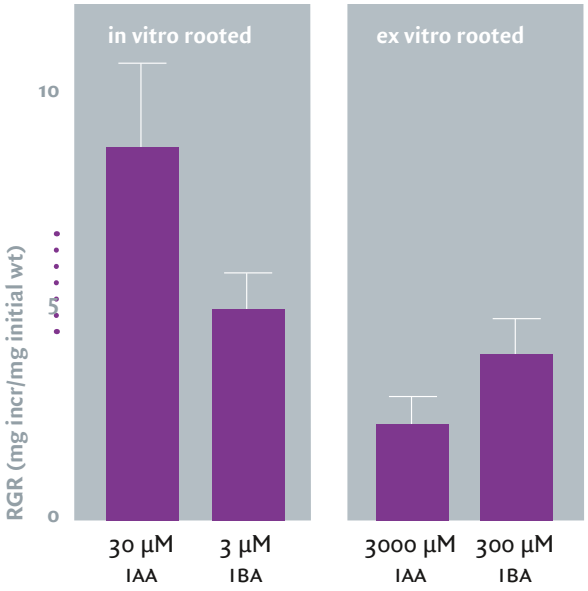
Fig. 2. Performance after in vitro and ex vitro rooting with IBA or IAA. For each, a range of concentrations was tested. In the bar graph, only the optimal concentrations are shown. The performance was measured as the RGR (relative growth rate)



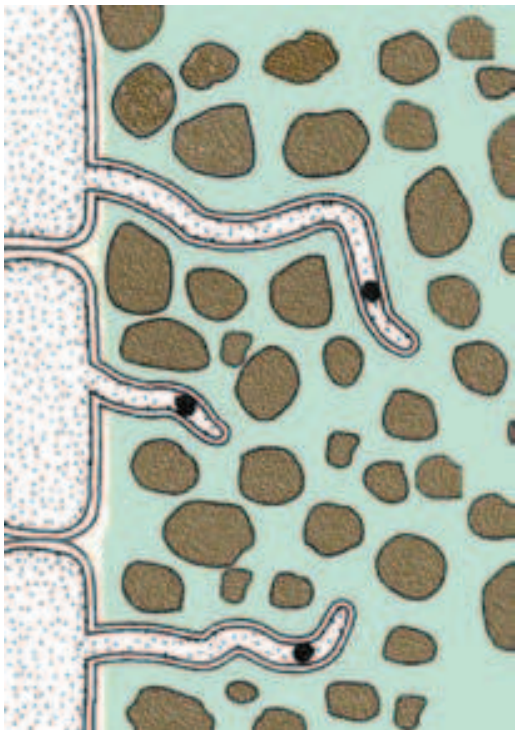


Fig. 3. Root hair that has grown in between soil particles. Root hairs formed after planting will grow in this way

rooting powder just as conventional cuttings. To the best of our knowledge, the kinetics of auxin uptake from rooting powder has never been determined. Expectedly, uptake occurs only during the first hours: auxin is rapidly metabolized by soil micro-organisms and moved away by water flow. In other procedures of ex vitro rooting, submersion (complete cuttings are submersed in auxin solution) or absorption (cuttings are placed with their basal end in an auxin solution), uptake also occurs as an early pulse. As noted above, the rhizogenic action of auxin takes place some days later, so a stable auxin like IBA should be used to keep auxin at a sufficiently high-level during this period. During in vitro rooting, on the other hand, microcuttings are cultured for a period of several days on "rooting medium". This is nutrient medium with a moderate concentration of auxin (in the µM range and not in the mM range, as used for ex vitro rooting). Auxin is taken up from the medium continuously, also during the period when it is needed for its rhizogenic action. Auxin is harmful after the meristems have been formed: it reduces root and shoot growth and promotes callus growth. Therefore, an unstable auxin like IAA that does not persist in the medium is advantageous. When the shoots are kept in the dark for the first week and are transferred to the light after that, IAA will be rapidly photo-oxidized. In this way, the persistence of IAA is minimized. Thus, on the basis of theoretical grounds, for in vitro rooting IAA may be preferable. For in vitro rooting of apple microcuttings, IAA was indeed far better than IBA. For ex vitro rooting, IBA was better (Figure 2). The rate of catabolism of IAA depends on the genotype, so with other crops IAA may be too unstable. For example, when a crop produces a high level of ROS (reactive oxygen species) at wounding (taking a cutting involves wounding), IAA may be catabolized at a high rate and an auxin that is resistant to oxidation is preferable.

Do in vitro roots function after planting in soil?

In scientific literature, there has been some debate about the functioning of in vitro roots after transfer to soil. Some researchers argue that they do not function. In vitro formed root tissues undoubtedly function poorly with respect to uptake of water and nutrients, because this depends on well-performing root hairs. Root hairs are tiny, hair-like outgrowths from surface cells of plant roots. They penetrate the soil and greatly increase the area available for the uptake of water and inorganics (Figure 3). They survive for a few days only. Root hairs are found just behind the root tip and are continuously being formed. Root hairs formed in vitro will not function after transfer to soil, since they are very vulnerable

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and will also not be located in between soil particles. The advantage of in vitro formed roots is another one. When microcuttings with roots are transferred to soil, the roots appear to resume growth immediately and generate new root hairs quickly. Therefore, the roots will fully function again shortly after the transfer. In ex vitro rooting, roots still have to be initiated in soil: the new roots emerge from the shoot after more than a week, and only after that will they start to form root hairs and start to function.

Ethylene

Auxin is the main hormonal actor in rooting. Cytokinins are inhibitory but are still required but with a very low concentration. Another hormonal player is ethylene. The action of this gaseous hormone during rooting is complex. It should first be noted that auxin firmly promotes ethylene synthesis. Furthermore, ethylene accumulates in submerged tissues, thus also in the section of a stem that is placed into the medium. In plant tissues, ethylene is barely metabolized, if at all. Plants regulate endogenous ethylene levels simply by releasing it into the atmosphere. This release is blocked in submerged tissues. There are various tools available to establish the role of

Fig. 4. Various ways to inhibit or promote ethylene in tissue culture

Adddition

> Ethephon releases ethylene

Synthesis

> AVG blocks synthesis (expensive)

> ACC is a direct precursor and is metabolized to ethylene

Perception

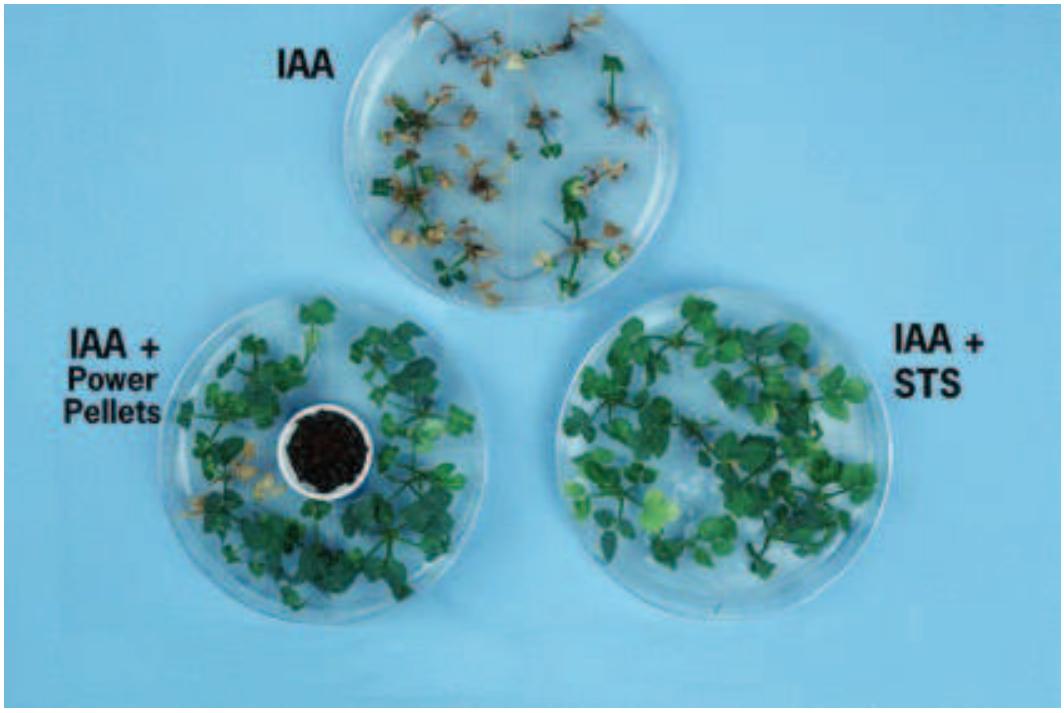
> STS (a solute) blocks perception (contains Ag which is a heavy metal)

> 1-MCP (a gas) blocks the ethylene receptor (inconvenient)

Removal from the gas-phase

> grains coated with $KMnO_4$, e.g. Power Pellets (cheap)

Fig. 5. Rose shoots rooted in vitro with IAA. The detrimental effect of ethylene was negated by adding STS (an ethylene inhibitor) or by removing ethylene from the headspace with Power Pellets. The Power Pellets are in the small vessel in the middle



ethylene (Figure 4). They have shown that ethylene is promotive during the dedifferentiation phase of the rooting process and inhibitory after that, during both the induction step and root growth. Finally, ethylene is needed for root hair formation. During in vitro rooting, the promotion of ethylene synthesis by the auxin treatment may cause problems, because ethylene accumulates in the headspace. In rose, this leads to leaf senescence (Figure 5). Senescence was prevented by adding STS to the medium, but this also resulted in poor rooting. When ethylene was removed from the headspace by $KMnO_4$, using "Power Pellets" (Ryan Instruments, Sassenheim, The Netherlands; several similar products are commercially available), senescence was inhibited. At the same time, rooting was not affected, because the Power Pellets did not remove ethylene from the tissue that was submerged in the medium, but only from the headspace. This treatment led to improved acclimatization.

Future

The results presented above are useful for growers, but do not solve the problem of recalcitrance to root. In rooting research during the past seven decades, the most promising progress about recalcitrance was not found in the rooting process itself, but in an improvement of the ability of cuttings to root. This was achieved by rejuvenation and stem elongation/etiolation. At Plant Research International, we have started research into the background of this enhancement. We are also developing an adequate system for rooting research in Arabidopsis. This allows the use of mutants and also to exploit the knowledge about the formation of lateral roots in Arabidopsis.