Timing of the phases in adventitious root formation in apple microcuttings

Geert-Jan De Klerk1,3, Marina Keppel1,4, Jolanda Ter Brugge1 and Herman Meekes5,6

1 Centre for Plant Tissue Culture Research, PO Box 85, 2160 AB Lisse, The Netherlands
2 Department of Experimental Botany, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Received 5 December 1994; Accepted 6 April 1995

Abstract

As with other regeneration processes, adventitious root formation may be divided into three phases, namely dedifferentiation, induction and differentiation. We assumed that the appropriate hormonal conditions for rooting, in particular a high level of auxin and a low level of cytokinin, are required only during the induction phase. Hence, the effect of 24 h pulses with indolebutyric acid (IBA) or benzylaminopurine (BAP) should be maximal during this phase. On this assumption, the timing of the three phases was determined in microcuttings of Malus ‘Jork 9′. The promotion of rooting was largest for 24 h IBA-pulses given 24-48 h or 48-72 h after the onset of culture. During culture of shoots on IBA-containing medium 24 h BAP-pulses were given. Inhibition of rooting was maximal for the BAP-pulses given between 24 and 96 h. An analysis of the kinetics of root emergence also indicated that added IBA acted from 24 h onwards: emergence of roots from the tissue occurred simultaneously for the IBA pulses at 0-24 h and 24-48 h, but lagged 1 d behind for the 48-72 h pulse and 2 d for the 72-96 h pulse. We concluded that dedifferentiation occurred from 0 to 24 h, induction from 24 to 72 or 96 h, and after that differentiation. Histological observations showed that after 24 h, cells with swollen nuclei and dense cytoplasm had appeared in the regions of the stem where the roots were formed. The first cell divisions were observed after 48 h. After 96 h, meristemoids of c. 30 cells had been formed. After the BAP-pulses at 24-48 h or 48-72 h, these meristematic cells formed callus.

Key words: Adventitious rooting, apple, auxin, cytokinin, Malus, regeneration.

Introduction

The regeneration of adventitious roots has been divided into various phases based on histological (Lovell and White, 1986) and biochemical (Gaspar, 1981) observations. Phases may also be identified physiologically by changes in the reaction to added hormones. Such changes are well known for adventitious root formation: auxins are required during the first period of root formation, but block root growth (Thimann, 1936) and outgrowth of root primordia (De Klerk et al., 1990), whereas the inhibition of rooting by cytokinins occurs specifically during the early period of the rooting process (Eriksen, 1974; Fabijan et al.,, 1981; Bollmark and Eliasson, 1986), Christianson and Warnick (1983) divided the regeneration of adventitious shoots from leaf explants of Convolvulus into three phases based on the reaction to hormones. They supposed that differentiated cells first require dedifferentiation to become competent to respond to the organogenic signal. During this initial phase, the hormonal composition of the medium may be varied over a wide range. In the second phase, the proper hormonal signal must be supplied. In this induction phase, the tissue becomes committed to shoot formation. In the third phase, morphological differentiation occurs. During this latter phase, the hormone concentration may again be varied over a wide range.

In the present article, we examine the timing of the phases of the rooting process in microcuttings of Malus ‘Jork 9′ by giving pulses with auxin (indolebutyric acid,
IBA) or cytokinin (benzylaminopurine, BAP). We assume that the appropriate hormonal conditions for rooting, i.e. a high level of auxin and a low level of cytokinin, are only required during the induction phase. We also relate the period during which the microcuttings have a high responsiveness to these hormones, with histological observations.

Materials and methods

Plant material
Shoot production of *Malus Jork 9* was maintained as described previously (De Klerk et al., 1990). Shoots of 1 cm in length were transferred to culture tubes with 15 ml of a modified Murashige-Skoog medium (Murashige and Skoog, 1962) to which 4.4 μM BAP and 0.5 μM IBA had been added. After 6 weeks proliferation at 20°C with a 16 h photoperiod (35 μmol m⁻² s⁻¹) provided by cool white fluorescent lamps, shoots were counted daily. Shoots of 1-1.5 cm in length were cultured in the dark on a modified Murashige-Skoog medium with KNO₃ and MgSO₄ at half concentration, NH₄NO₃ at 200 mg l⁻¹, KH₂PO₄ at 135 mg l⁻¹, and Ca(NO₃)₂·4H₂O at 600 mg l⁻¹ and without CaCl₂ (De Klerk et al., 1990). After 7 d, the shoots were transferred to the light. To establish the timing of a cytokinin-sensitive phase, shoots were transferred at the indicated times to medium with an addition of 2 μM IBA and after 24 h back to IBA-free medium. Rooting was determined at the indicated times as percentage of shoots that rooted, and as mean number of roots per rooted shoot. Rooting was determined in this way, since the number of roots often does not show a normal distribution when the non-rooted shoots are included (G.J. De Klerk and J. Ter Brugge, unpublished observation). For each determination 30 shoots were used.

The kinetics of rooting was examined with 1 mm stem slices cut from shoots of 1.5-2 cm. The slices were cultured with the apical side down on a nylon mesh (4 × 4 cm) on 25 ml hormone-free medium in a 9 cm Petri dish (Van Der Krieken et al., 1993). The Petri dish was incubated upside down in the dark in a culture room at 25°C. At various times, the nylon mesh with the slices attached, was transferred to medium with 25 μM IBA (Welander and Pawlacki, 1993), cultured for 24 h on this medium and then transferred back to hormone-free medium and to the light. At both transfers, excess liquid was removed by placing the mesh on filter paper (5 s). In each Petri dish, 30 slices originating from six shoots were cultured. For each determination, 90 slices were used. Roots were counted daily with a dissecting microscope.

Results

IBA-pulses
During culture of shoots on medium with 1 μM IBA, first emergence of roots from the stems occurred after 9 d. Figure 1 shows that 24 h pulses with IBA were most effective when given 24–48 or 48–72 h after excision. When counted after 10 d, the 0–24 h pulse resulted in a significantly lower rooting percentage than the 24–48 (P<0.05) and 48–72 h (P<0.01) pulses (Fig. 1A). When counted after 21 d, all pulses resulted in a high rooting percentage (83–90%) with the exception of the 120–144 h pulse. The rooting percentage of the latter pulse (53%) was significantly lower than the other rooting percentages (P<0.05). The number of roots per rooted shoot counted after 21 d (Fig. 1B), was significantly higher after the 24–48 and 48–72 h pulses than after the other four pulses, with one exception (48–72 versus 96–120 h: P<0.1). The 120–144 h pulse induced the lowest number of roots per rooted shoot.

The data of Fig. 1 suggest that the induction phase did not start immediately upon excision, but c. 24 h after excision. If this assumption is true, the kinetics of root formation should be similar when the IBA-pulse is given at 0–24 or 24–48 h. (For the IBA-pulse at 0–24 h, auxin carried-over after the pulse will act as an organogenetic stimulus from 24 h onwards, whereas IBA given during the pulse at 24–48 h will act immediately.) Thus, we determined the kinetics of root emergence after IBA-pulses given at various times after excision. To avoid interaction by auxin synthesized in the upper part of the shoot and to facilitate observation with a dissecting microscope, we used 1 mm slices cut from stems and
Fig. 1. Rooting of *Malus 'Jork 9'* shoots after 24 h pulses with IBA at the indicated times. The shoots were cultured on IBA-free medium and received a 24 h pulse with 2 \( \mu \)M IBA at the indicated times. (A) Percentage of rooted shoots counted after 10, 14 and 21 d; (B) mean number of roots per rooted shoot counted after 21 d.

cultured in Petri dishes (Van Der Krieken *et al.*, 1993). Figure 2 shows that root formation in slices after pulses at 0–24 h or 24–48 h had initially the same kinetics. The pulses at 48–72 h and 72–96 h lagged 1 d and 2 d behind, respectively. Figure 2 also shows that during preculture on hormone-free medium the slices rapidly lost the capability to root.

**BAP-pulses**

Pulses of 1 \( \mu \)M BAP given at 24–48, 48–72 or 72–96 h during a rooting treatment with 1 \( \mu \)M IBA, strongly reduced both the rooting percentage and the number of roots per rooted shoot (Fig. 3A, B). The pulse at 0–24 h and the pulses after 96 h had less or no effect. When counted after 14 d, the rooting percentage after the 48–72 h pulse was significantly different from all other percentages \((P<0.01\) or \(P<0.05\)). Pulses with 2 \( \mu \)M BAP gave similar results (data not shown). In other experiments, inhibition by the pulses of 24–48 and 48–72 h was

Fig. 2. The cumulative rooting percentages of 1 mm slices excised from stems of *Malus 'Jork 9'* shoots. The slices were cultured on a mesh in a Petri dish on IBA-free medium and received a 24 h pulse with 25 \( \mu \)M IBA at the indicated times. The number of slices with roots was determined every day.

Fig. 3. Rooting of *Malus 'Jork 9'* shoots after 24 h pulses with BAP given at the indicated period during the rooting treatment. The shoots were cultured on medium with 1 \( \mu \)M IBA and received a 24 h pulse with 1 \( \mu \)M BAP at the indicated times. Roots were counted after 14 and 28 d; c = control that had not received a BAP-pulse. (A) Percentage of rooted shoots; (B) mean number of roots per rooted shoot.
De Klerk et al.

Fig. 4. Rooting of Malus 'Jork 9' shoots after 24 h pulses with a range of BAP concentrations given at 24-48 h or at 48-72 h. The shoots were cultured on medium with IBA and received a 24 h pulse with BAP at 24-48 or 48-72 h. Roots were counted after 28 d. (A) Percentage of rooted shoots; (B) mean number of roots per rooted shoot.

usually the same. This was also observed in an experiment in which pulses with a range of BAP-concentrations were given either at 24-48 or 48-72 h (Fig. 4). A reduction of the rooting percentage by c. 50% was reached at 1 μM of BAP.

Histological analysis

Root primordia in apple microcuttings are formed in the area of the stem close to the interfascicular cambium (Hicks, 1987). In microcuttings of Malus 'Jork 9', the first histological changes after the onset of the standard rooting treatment (Plate 1A) were visible after 24 h, when outward derivatives of the interfascicular cambium adja-

cent to the vascular bundles had developed enlarged nuclei and dense cytoplasm (Plate 1B). At 48 h, several cells with declining vacuoles occurred in short radial rows, and occasionally, cell divisions were observed (Plate 1C). After 72 h, small meristemoids of apparently dividing cells with dense cytoplasm occurred (Plate 1D). After 96 h, meristemoids of c. 30 cells (in cross-section) had been formed (Plate 1E). After 7 d, the meristems had grown outward into the cortex, leaving a trace of developing cells between their original position and themselves (Plate 1F).

In shoots treated with BAP from 24 to 48 h, the meristemoids were still visible after 72 h, even though they were less distinct (Plate 2A). After 120 h, the meristemoid cells were still recognizable, but they lacked a visible organization (Plate 2B). Still later, these areas were occupied by calliform cells with many vacuoles (Plate 2C). Adjacent cortical cells also joined in callus formation. The same results were obtained when the BAP-pulse was from 48 to 72 h.

Discussion

In this article, we determined the timing of the successive phases in adventitious root formation in apple microcuttings on the principle that the phases have specific hormonal requirements, in particular, with respect to auxin and cytokinin. Thus, during rooting, pulses with IBA or BAP were given to achieve a transient increase in the level of free hormone in the tissue. A possible pitfall in these experiments is carry-over after transfer from hormone-containing to hormone-free medium. However, both IBA (in Malus microcuttings, Van Der Krieken et al., 1992a, b, 1993) and BAP (in Malus microcuttings, Nordström and Eliasson, 1986; in tobacco epidermal strips, Van Der Krieken et al., 1990) are rapidly metabolized after uptake, mostly by conjugation. In control experiments (unpublished observation), we found that there were no effects of light (during the transfer from one medium to the other, the shoots were shortly exposed to the light) and of diffusion of compounds from the tissue into the medium. Furthermore, long-lasting effects of the pulses by disturbance of the metabolism of hormones are minor (Palni et al., 1988; W. Van Der Krieken, personal communication). Thus, the main effect of the pulses was a sharp rise in the level of free hormone during

Plate 1. Micrographs of cross-sections of apple microcuttings at various stages of standard rooting treatment (7 d, 1 μM IBA). P = phloem; K = cortical cells; S = sclerenchyma cells; C = cambial zone; M = meristemoid; >> indicate first initials. Unless otherwise specified, the cortical side is at the top of the micrograph and the medullar area at the bottom. All bars are 50 μm. (A) At the start of the rooting treatment (0 h). Note the distinct sclerenchyma caps of the phloem groups, which facilitate visual orientation in the section. (B) After 24 h rooting treatment. Derivatives of the interfascicular cambium adjacent to the phloem groups have developed more cytoplasm and larger nuclei. (C) After 48 h. The first initials have developed rows of redifferentiating cells in the phloem ray area. (D) After 72 h. Small coherent groups of dividing cells, meristemoids, have developed. (E) After 96 h. The meristemoids have enlarged, causing lateral compression in the phloem cells lying in between. (F) After 7 d. The meristemoid has fully developed and protruded into the cortex. Note the position of the sclerenchyma cells that have been left behind. The cortical area is at the left-hand side of the micrograph.
Phases in adventitious root formation
the pulse and during a short period (a few hours) after the pulse.

The increased responsiveness to auxin (stimulation, Fig. 1) and cytokinin (inhibition, Fig. 3) from 24 to 96 h after the start of the rooting treatment show that induction occurred during this period (as defined by Christianson and Warnick, 1983; see Introduction). Increased rooting after postponement of the auxin treatment has been observed previously (Shibaoka, 1971; Mohammed and Eriksen, 1974). The similar initial kinetics of root emergence after the pulses at 0–24 and 24–48 h, and the 24 or 48 h delay of root emergence after the 48–72 or 72–96 h pulses, respectively, also indicate that auxin only has an effect from 24 h onwards.

The phase-specific promotion by IBA-pulses was not as evident as the phase-specific inhibition by BAP-pulses (compare Figs 1 and 3). This is likely due to a different mode of action of these pulses. The BAP-pulse redirected the process as shown by the callus formation from the meristemoids after this pulse (Plate 2). Withholding auxin and culturing the shoots on hormone-free medium, resulted in a standstill of the process as shown by the kinetics of root emergence after IBA-pulses given at different times (Fig. 2). When IBA would have had only this effect, the final rooting percentages and root numbers should have been the same for all pulses except for the pulse of 0–24 h (because this pulse only acted by carry-over). However, rooting after the late pulses was low. This shows that, in addition, withholding auxin also resulted in redirection of the process. Slices lost the capability to root very fast (Fig. 2). This explains why in slices, rooting after the IBA-pulse at 24–48 h was lower than after the 0–24 h pulse, even though the latter pulse only acted by carry-over. A decrease in the capability to regenerate after culture on hormone-free medium has been observed before for adventitious root, shoot and flower bud formation (Attfield and Evans, 1991; Smulders et al., 1990).

Root primordia may be formed directly from stem cells, or indirectly via callus (Hartmann et al., 1990). At 24 h after the start of the rooting treatment, some cells became densely cytoplasmic, having swollen nuclei (Plate 1B). Induction (24 to 72 or 96 h; Plate 1C–E) coincided with the first cell divisions and the formation of a meristemoid. Thus, in apple microcuttings, induction was not preceded by unorganized cell divisions on a large scale.

In conclusion, the timing of the phases of adventitious root formation in apple (dedifferentiation, induction and differentiation) has been established. This enables a study of the role of the various classes of plant growth regulators and other factors in the three phases. This work is now in progress.

Acknowledgement

We thank Drs Ton Croes, Luud Gillissen and Wim Van Der Krieken for the critical reading of the manuscript. This work was carried out in the framework of the COST 8.7 working group 'Root Regeneration'.

References


Nordström A-C, Eliasson L. 1986. Uptake and translocation of [14C]-labelled benzylaminopurine in apple shoots grown in

Plate 2. Micrographs of cross-sections of apple microcuttings treated during the standard rooting treatment with 1 μM BAP at 24–48 h. All indications as in Plate 1. (A) 72 h after the start of the rooting treatment. The meristemoid cells are still visible, but their coherence is less apparent. (B) 120 h after the start of the rooting treatment. The meristemoids are still recognizable, but have split up into various new groups. (C) 7 d after the start of the rooting treatment. No more meristemoids can be discerned. The phloem ray area and the adjacent cortex are occupied by rounded cells, somewhat richer in cytoplasm than normal cortical cells, typically found in callus tissue.


