*Plant Cell Tissue Organ Culture 3: 301–311*


# Rooting apple cultivars *in vitro*: Interactions among light, temperature, phloroglucinol and auxin

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*(Received March 14, 1984; in revised form and accepted July 24, 1984)*

**Key words:** micropropagation, *Malus domestica*, etiolation, indolebutyric acid

**Abstract.** 'Delicious' apple (*Malus domestica Borkh.*) and several of its strains, which have been difficult to root in vitro, were successfully propagated with rooting percentages up to 100%. The combination of treatments used to achieve this result included placing the shoots on rooting medium in the dark at 30 °C for the first week of the rooting stage, then moving them to a regime of 16 hr light – 8 hr dark at 25 °C. The rooting medium contained half strength Murashige and Skoog salts plus 1.2 μM thiamine HCl, 0.56 mM myo-inositol, 1 mM phloroglucinol (PG), 1.4 μM indolebutyric acid (IBA), 1.3 μM gibberellic acid ($GA_3$), 87.6 mM sucrose, and 7 g $l^{-1}$ Difco Bacto agar. Dark treatment applied during the proliferation stage (etiolation) was less effective than one applied at the beginning of the rooting stage. The optimum length of dark treatment during rooting was 4 to 7 days. Increasing the temperature from 25 °C to 30 °C improved rooting of 'Delicious', 'Royal Red Delicious', and 'Vermont Spur Delicious' in the absence of PG but generally had less effect in the presence of PG. Further increase in temperature to 35 °C stimulated rooting of 'Royal Red Delicious' but reduced rooting of 'Vermont Spur Delicious'. Transfer of the cuttings to auxin-free medium after 1 week had no effect on percentage rooting and increased the number of roots per cutting for only 1 of 4 cultivars tested and then only in the presence of PG. In general PG stimulated rooting of 'Delicious' and its strains, but had no effect on 'Golden Delicious'.

## Introduction

Despite recent advances in micropropagation techniques, certain cultivars of apple, e.g. 'Delicious', have continued to be difficult to root in vitro. Etiolation of proliferating cultures substantially improved subsequent rooting of 'Supreme Red Delicious', 'Wellspur Delicious' and several other cultivars but the rooted plants were difficult to acclimate in the greenhouse [1, 20]. Dark treatment applied during the rooting stage has improved rooting of apple rootstocks [17], apple cultivars [2, 4] and plum [3]. Rooting improved in two apple cultivars with increasing time in culture but this improvement required more than 30 subcultures for 'Delicious' [16].

Increased temperature during the rooting stage has been reported to improve rooting of apple [12] and Myrobalan plum [3] but to decrease rooting of 'Calita' Japanese plum [14]. Phloroglucinol (PG) has improved the rooting of some apple rootstocks and cultivars [7, 9, 10, 11, 20, 22] while having no effect or inhibiting rooting in others [20, 22].

Transferring apple cuttings on which root initiation has begun from medium containing auxin to one without auxin has been used to improve rooting [2, 7, 17, 18], but in other cases good rooting has been obtained without the necessity of this transfer [10, 11, 20, 22].

The research reported here was designed to examine the effects of dark treatment, temperature, phloroglucinol and auxin and interactions among these factors on the difficult-to-root 'Delicious' apple, several of its strains, and 'Golden Delicious'.

## Materials and methods

Cultures were initiated from actively growing shoot tips collected from trees growing in the orchard or greenhouse and from meristem-tips dissected from buds on dormant or actively growing shoots. Details of the culture establishment procedures have been published [21, 22]. Shoot proliferation was done on a medium consisting of Murashige and Skoog (MS) salts [13] supplemented with 0.56 mM myo-inositol, 1.2 $\mu$M thiamine HCl, 4.4 $\mu$M benzylamino purine (BA), 0.5 $\mu$M IBA, 1.4 $\mu$M $GA_3$, 87.6 mM sucrose and 4.8 g $l^{-1}$ Phytagar. Beginning in July, 1982, the proliferation medium was modified by increasing the agar concentration to 7 g $l^{-1}$ and in October, 1982, Difco Bacto-agar was substituted for Phytagar. Also beginning in July, Fe was supplied at the same concentration by using (ethylenedinitrilo) tetra-acetic acid, ferric sodium salt. For rooting, the MS salts and sucrose concentrations were halved, BA and $GA_3$ were eliminated and the IBA concentration was varied according to the desired treatment. PG was supplied at 1 mM for either proliferation or rooting. All media were autoclaved for 15 min at 121 °C and 1.1 kg/cm$^2$.

Cultures were grown at 25 ° ± 2 °C with 16-hr photoperiods provided by warm white fluorescent lights at a photon flux density of 40–60 $\mu$mol $s^{-1}$ $m^{-2}$.

Most of the cultivars used in these studies were virus-indexed and obtained from the IR-2 repository in Prosser, WA. The cultivars with the IR-2 code numbers and the times at which cultures were initiated are: 'Delicious' (5-1)–July and September, 1981; 'Triple Red Delicious' (246-1)–May, July and September, 1981; 'Golden Delicious' (13-1)–February and June, 1982; 'Vermont Spur Delicious' (204-1)–June, 1982. 'Triple Red Delicious' is indistinguishable from 'Royal Red Delicious' (R.F. Stouffer, personal communication) and will be referred to by the latter name in this paper. Virus-indexed scionwood of 'Redchief Delicious' was obtained from Hilltop Orchards and Nurseries, Inc., Hartford, MI and placed in culture in December, 1981. In addition, cultures were established from non-indexed orchard trees of 'Delicious' (NI) in June, 1979, and 'Redspur Delicious' in July, 1980. The rooting experiments reported here were conducted from August, 1982 through August, 1983.

Terminal shoot cuttings 2 to 3 cm long were prepared from proliferating cultures retaining the leaves only at the apical 1 cm of the cutting. A single cutting was placed in each 25 x 95 mm shell vial containing 10 ml rooting medium.

For etiolation, jars of proliferating cultures were wrapped in aluminum foil for 30 days. This period was reduced to 7–10 days for those experiments comparing dark treatment during proliferation with that during rooting. Dark treatment was given to rooting cuttings by wrapping racks of vials in aluminum foil or by placing unwrapped ones in darkened growth chambers.

A factorial combination of treatments was used in the experiments reported here with 15 to 40 cuttings per treatment. Since roots were not visible until 8 to 12 days after the start of rooting experiments, data on number of cuttings rooted and number of roots per cutting were collected 2, 3, 4 and 5 weeks after experiments were initiated.

## Results

### *Effect of dark treatment during proliferation or rooting*

Shoots of 'Delicious' (NI) that had been proliferated in the dark rooted better than those proliferated in the light whereas the reverse was true for 'Redchief Delicious' (Table 1). PG in the proliferation medium tended to reduce subsequent rooting of 'Delicious' shoots but the effect on 'Redchief Delicious' was inconsistent. The effects of the proliferation pretreatments and the IBA concentration were greater on percentage of cuttings rooted than on the number of roots produced per rooted cutting.

Light and dark treatments applied during proliferation had less effect than the same treatments applied during the first week of the rooting stage. (Table 2). Proliferation in the light produced shoots that rooted better for 'Delicious' (NI) and its two strains. This contrasts with earlier results obtained with 'Delicious' (NI) (Table 1). Inconsistent responses of these cultivars to

Table 1. Rooting response at two IBA concentrations of shoots proliferated in light or dark on medium with or without PG[a].

| | IBA | % Rooting | | | | Mean no. roots per rooted cutting | | | |
|---|---|---|---|---|---|---|---|---|---|
| | (μM) | Light | | Dark | | Light | | Dark | |
| Cultivar | | − PG | + PG | − PG | + PG | − PG | + PG | − PG | + PG |
| Delicious (NI)[b] | 4.9 | 35 | 5 | 70 | 20 | 3.9 ± 1.3[d] | 2.0 ± 0 | 3.3 ± 0.5 | 4.5 ± 0.9 |
| | 15 | 30 | 5 | 35 | 10 | 2.2 ± 0.5 | 1.0 ± 0 | 3.7 ± 1.3 | 1.0 ± 0 |
| Redchief Delicious[c] | 4.9 | 45 | 40 | 5 | 15 | 2.7 ± 0.5 | 2.5 ± 0.8 | 1.0 ± 0 | 4.3 ± 1.7 |
| | 15 | 40 | 25 | 0 | 0 | 2.0 ± 0.6 | 2.2 ± 0.6 | 0 | 0 |

[a]Rooting medium contained PG; 20 cuttings per treatment
[b]Data after 4 weeks
[c]Data after 5 weeks
[d]Standard error of mean

Table 2. Effect on rooting of light (L) or dark (D) treatment either during the last 7 to 10 days of proliferation or during the first 7 days of rooting. Data taken 4 weeks after start of rooting experiment.[a]

| Cultivar | % Rooting | | | | Mean no. roots per rooted cutting | | | |
|---|---|---|---|---|---|---|---|---|
| | L-L[b] | L-D | D-L | D-D | L-L | L-D | D-L | D-D |
| Delicious (NI) | 10 | 55 | 0 | 30 | 1.5 ± 0.5[c] | 2.5 ± 0.4 | 0 | 2.5 ± 0.6 |
| Redchief Delicious | 5 | 40 | 20 | 0 | 1.0 ± 0 | 2.5 ± 0.3 | 1.5 ± 0.5 | 0 |
| Royal Red Delicious | 5 | 30 | 0 | 15 | 1.0 ± 0 | 3.3 ± 1.1 | 0 | 2.7 ± 0.9 |
| Golden Delicious | 40 | 35 | 38 | 48 | 1.8 ± 0.3 | 2.4 ± 0.8 | 1.6 ± 0.2 | 1.9 ± 0.4 |

[a] Rooting medium contained 4.9 μM IBA plus 1 mM PG for 'Delicious' (NI) and 'Redchief Delicious' and 1.5 μM IBA without PG for others. 20 cuttings per treatment except 40 cuttings for dark-proliferated 'Golden Delicious'. Proliferation medium contained no phloroglucinol. Seven days of dark treatment during proliferation for 'Delicious' (NI) and 'Golden Delicious' and 10 days for others.
[b] L-L indicates light during end of proliferation and light during beginning of rooting.
[c] Standard error of mean

etiolation were typical for many other earlier experiments (data not shown). Cuttings rooted better in nearly every case when placed in the dark for the first week of rooting than when maintained continuously in the light (Table 2).

Shoots that rooted after etiolation grew poorly and were difficult to acclimatize, whereas those that rooted after dark treatment in the rooting stage grew more vigorously and were easier to acclimatize.

*Effect of length of dark period during rooting stage*

Cuttings of 'Redchief Delicious' rooted better when given 1 week of dark treatment; less rooting occurred as time in the dark increased up to 3 weeks after which rooting was at the level of the controls (Table 3). For this cultivar, PG in the medium improved rooting with or without a dark treatment (Table 3).

Further testing showed that 4 to 7 days of dark treatment was optimum for rooting of 'Delicious' (5-1) and its strains 'Royal Red' and 'Vermont Spur' (Figures 1, 2) with more effect on th percentage rooting than on the number of roots produced. Again PG in the medium increased the percentage of

Table 3. Effect of dark treatment during rooting stage on rooting of 'Redchief Delicious' cuttings after 5 weeks on rooting medium in presence or absence of PG.[a]

| Weeks in dark | % Rooting | | Mean no. roots per rooted cutting | |
|---|---|---|---|---|
| | − PG | + PG | − PG | + PG |
| 0 | 0 | 25 | – | 4.2 ± 1.0 |
| 1 | 45 | 60 | 2.9 ± 0.6[b] | 4.8 ± 0.6 |
| 2 | 35 | 50 | 3.7 ± 0.8 | 3.8 ± 0.7 |
| 3 | 10 | 20 | 3.0 ± 2.0 | 2.2 ± 0.5 |

[a] Rooting medium contained 4.9 μM IBA; 20 cuttings per treatment.
[b] Standard error of mean

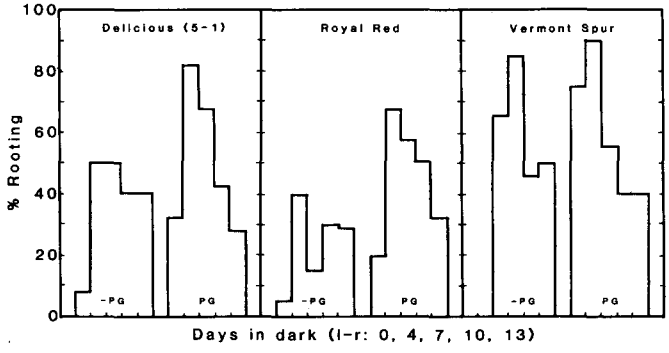


Figure 1. Effect of length of dark period at the beginning of the rooting stage on percentage rooting after 3 weeks. Rooting medium contained 1.5 $\mu$M IBA; 20 cuttings per treatment.

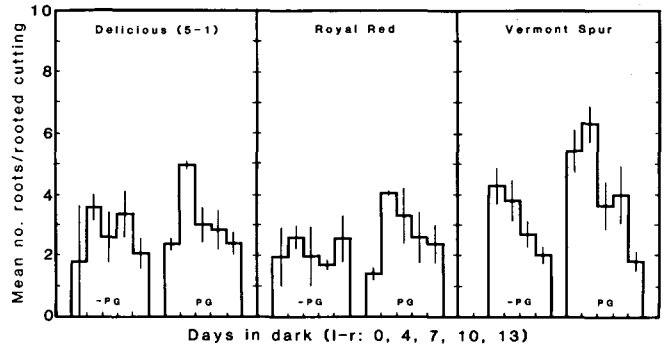


Figure 2. Effect of length of dark period at the beginning of the rooting stage on number of roots produced per rooted cutting after 3 weeks. Rooting medium contained 1.5 $\mu$M IBA: 20 cuttings per treatment.

cuttings rooted, particularly when no dark treatment was given. PG had less effect on the number of roots per cutting.

*Effect of temperature during dark treatment.*

Increasing the temperature during dark treatment from 25 °C to 30 °C increased percentage rooting at 3 weeks of 'Delicious' (NI) and 'Royal Red Delicious' in the absence of PG (Table 4) but had no effect on 'Golden Delicious'. When PG was present, the only effect was on 'Royal Red Delicious' in the presence of 4.9 $\mu$M IBA. Two methods of providing dark treatment at 25 °C gave similar results. PG in the rooting medium increased percentage rooting of 'Delicious' (NI) and 'Royal Red Delicious', particularly at 25 °C, but reduced rooting of 'Golden Delicious' at both 25 °C and 30 °C. In contrast to the percentage rooting data, increasing the temperature from 25 °C to 30 °C increased the number of roots only on 'Royal Red Delicious' in the absence of PG (Table 4). The number of roots per cutting was generally higher for all cultivars when PG was present.

Table 4. Effect of temperature during 1 week dark treatment on percentage rooting and mean number of roots per rooted cutting.[a]

| | % Rooting | | | | Mean no. roots per rooted cutting | | | |
|---|---|---|---|---|---|---|---|---|
| | − PG | | + PG | | − PG | | + PG | |
| Cultivar | 25° | 30° | 25° | 30° | 25° | 30° | 25° | 30° |
| Delicious (NI) | 20 | 50 | 75 | 75 | 3.3 ± 1.5 | 4.6 ± 0.8 | 5.8 ± 0.2 | 5.3 ± 0.7 |
| Royal Red[b] | 10 | 45 | 45 | 75 | 2.0 ± 0 | 3.8 ± 0.7 | 3.8 ± 0.4 | 4.5 ± 1.0 |
| Royal Red | 60 | 90 | 98 | 95 | 3.1 ± 0.2 | 4.6 ± 0.6 | 6.0 ± 0.4 | 5.8 ± 0.7 |
| Golden Delicious | 53 | 53 | 30 | 20 | 2.0 ± 0.7 | 1.2 ± 0.2 | 2.4 ± 0.1 | 1.7 ± 0.3 |

[a]Data for 25° is mean of two methods of providing dark treatment; data for 25° based on 30 (Golden Delicious) or 40 cuttings per treatment and at 30° on half as many cuttings.
[b]Rooting medium with 4.9 μM IBA; the other three trials shown used 1.5 μM IBA.

In a second series of experiments, the effect of a further increase in temperature during dark treatment varied with cultivar (Figure 3). 'Golden Delicious' and 'Delicious' (5-1) were basically unresponsive, whereas 'Delicious' (NI), 'Royal Red Delicious' and 'Vermont Spur Delicious' had increased rooting at 30 °C. Rooting at 35 °C was lower with 'Vermont Spur Delicious' and higher with 'Royal Red Delicious'. However, 'Royal Red Delicious' rooted as well at 30 °C in the earlier comparison (Table 4) as it did at 35 °C in this one (Figure 3). Again, the effectiveness of PG varied with cultivar, stimulating percentage rooting of 'Delicious' (NI), 'Royal Red Delicious' and 'Vermont Spur Delicious', and having little or no effect on 'Delicious' (5-1) and 'Golden Delicious' in these experiments.

A similar, if less striking, effect of temperature during dark treatment was found on the number of roots per cutting (Figure 4). Temperature had little effect on 'Golden Delicious' and 'Vermont Spur Delicious'; PG slightly

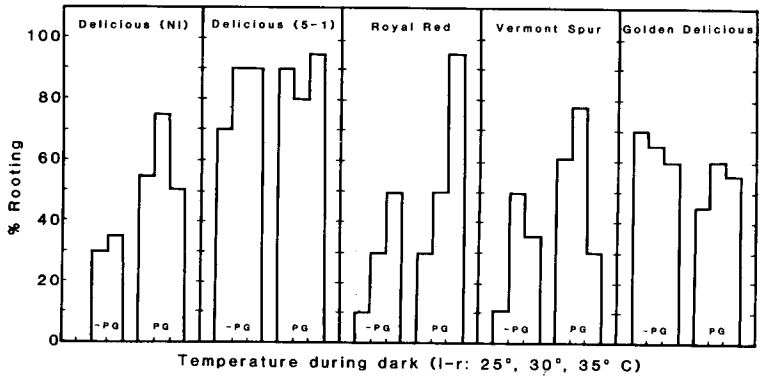


Figure 3. Effect of different temperatures during 1 week dark treatment on percentage rooting after 3 weeks in rooting stage. For 25 °C, dark treatment supplied by wrapping racks of culture tubes in aluminum foil and placing on shelf in lighted growth room; for other temperatures, dark treatment supplied in growth chambers. Rooting medium contained 1.5 μM IBA; 20 cuttings per treatment.

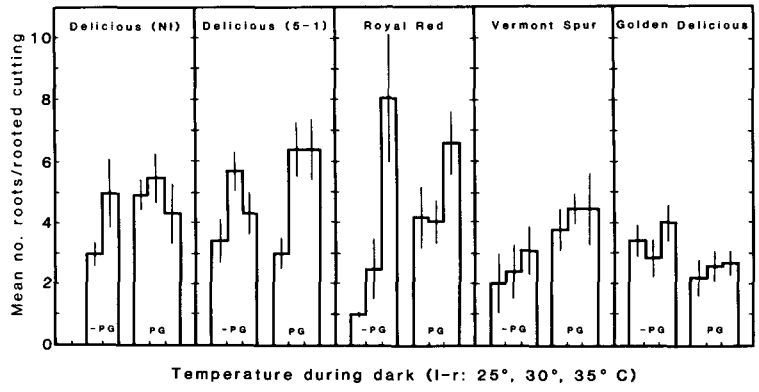


Figure 4. Effect of different temperatures during 1 week dark treatment on mean number of roots per rooted cutting after 3 weeks in rooting stage. For 25 °C, dark treatment supplied by wrapping racks of culture tubes in aluminum foil and placing on shelf in lighted growth room; for other temperatures, dark treatment supplied in growth chambers. Rooting medium contained 1.5 μM IBA; 20 cuttings per treatment.

depressed root number of 'Golden Delicious' and slightly stimulated root number on 'Vermont Spur Delicious'. Increasing temperatures increased root number of 'Delicious' (NI) and 'Royal Red Delicious' in the absence of PG but only 35 °C increased root number when PG was present with 'Royal Red Delicious'.

*Effect of transfer to auxin-free medium*

Moving the cuttings to auxin-free medium after 1 week reduced rooting of 'Delicious' (NI) whether or not PG was present (Table 5). Rooting percentage of 'Delicious' (5-1) and 'Redchief Delicious' was not affected but 'Delicious' (5-1) cuttings produced more roots in the presence of PG when transferred to auxin-free medium. Transfer to auxin-free medium more than doubled the rooting percentage of 'Golden Delicious' in the presence of PG, but equally good rooting was obtained without transfer when no PG was used.

## Discussion

*Effect of dark treatment during proliferation or rooting*

Dark treatment applied to proliferating cultures of 'Delicious' apple increased rooting (Table 1) as was found also with 'Supreme Red Delicious' and 'Wellspur Delicious' [1] and other cultivars [20]. However, the response varied with cultivar [Table 1; 8, 20]. In addition, plants produced from etiolated shoots were weak and difficult to acclimatize, confirming earlier results [1, 20].

Further testing indicated that dark treatment during rooting was more effective than during proliferation (Table 2). A dark treatment during the

first 5–14 days of the rooting stage also improved rooting of M.9 apple rootstock [5] and Myrobalan plum [3] in the presence of IAA and of M.26 apple rootstock and 'Jonagold' [2] and 'Wellspur Delicious' [4] apple when IBA was used. These results correspond well with the findings here that 1 week (Table 3) or less (Figure 1) of dark at the beginning of the rooting stage produced the optimum rooting percentage and usually the greatest number of roots as well.

Druart et al. [2] hypothesize that rooting in tissue cultured apples involves two stages, an inductive phase for which shoots were grown on a medium with $GA_3$ as the sole growth regulator, followed by an initiation phase, for which individual shoots were transferred to an auxin-containing medium. Roots became visible only after 7 to 12 days on this initiation medium. In the studies reported here, cuttings taken for rooting came directly from the proliferation medium, which contained $GA_3$, BA and IBA, with no inductive phase. Nevertheless, roots were visible after 8 to 12 days on the rooting medium, even though no inductive phase had been given. Furthermore, the results with 'Delicious' and 'Royal Red Delicious' (Table 2) show striking increases in rooting percentage and root number when dark treatment was given during the rooting stage and little effect, or a negative one, when dark was given during proliferation. Similarly, the data of Druart et al. [2] show that dark treatment at the beginning of the initiation phase was more effective than dark applied at the end of the inductive phase. Since transfer from a root induction medium to a root initiation medium did not result in more rapid appearance of roots, one must question whether separate induction and initiation phases exist.

*Effect of length of dark period during rooting*

The finding that the optimum length of dark treatment is 4 to 7 days at the beginning of the rooting stage corresponds well with the 5 to 9 days of darkness used for other apple clones by other workers [2, 4, 5, 17]. It is interesting to note that the presence of PG significantly improved rooting when no dark treatment was given (Table 3, Figure 1) but had a lesser, or no, effect with increasing length of dark treatment.

*Effect of temperature during dark*

Increasing the temperature from 25 °C to 30 °C during a dark treatment for the first week of the rooting stage improved rooting in most of the cultivars tested. Further increase in temperature to 35 °C had a demonstrable positive effect only on 'Royal Red Delicious' (Figures 3 and 4), but equally good rooting was obtained at 30 °C with this cultivar in another experiment (Table 4).

No similar treatments have been reported in the literature, but Lane [12] reported that day/night temperatures of 28 °/22 °C were superior to lower ones in rooting shoots of 'McIntosh' seedlings. However, James [5] found no

Table 5. Effects of transferring cuttings to auxin-free medium after 1 week and of PG on rooting of cuttings. Data taken 3 weeks after beginning of experiment.[a]

| | % Rooting | | | | Mean no. roots per rooted cutting | | | |
|---|---|---|---|---|---|---|---|---|
| | Not transferred | | Transferred to auxin-free | | Not transferred | | Transferred to auxin-free | |
| Cultivar | − PG | + PG | − PG | + PG | − PG | + PG | − PG | + PG |
| Delicious (NI) | 15 | 55 | 0 | 35 | 1.7 ± 0.3[b] | 3.5 ± 0.7 | – | 2.9 ± 1.2 |
| Delicious (5-1) | 55 | 95 | 55 | 90 | 4.6 ± 0.7 | 4.6 ± 0.5 | 3.4 ± 0.7 | 6.3 ± 0.8 |
| Redchief Delicious | 5 | 45 | 10 | 45 | 3.0 ± 0 | 2.9 ± 0.6 | 3.0 ± 0 | 3.8 ± 0.6 |
| Golden Delicious | 53 | 20 | 47 | 53 | 1.2 ± 0.2 | 1.7 ± 0.3 | 1.9 ± 0.4 | 1.4 ± 0.3 |

[a]All cuttings were placed in the dark at 30 °C for the first week; rooting medium contained 4.9 μM IBA. Twenty cuttings per treatment except 15 for 'Golden Delicious'.
[b]Standard error of mean

effect of temperatures between 22 °C and 29 °C on root initiation or emergence with M.9 rootstock. In both cases, the temperature treatments were applied to cultures growing on 16 hour photoperiod in contrast to the continuous dark treatment used for the experiments reported here. Similar differences in cultivar responses to temperature treatment have been reported with plum [3, 14].

*Effect of transfer to auxin-free medium*

Transferring shoots to an auxin-free medium after 1 week had essentially no effect on percentage rooting and increased the number of roots only for 'Delicious' (5-1) in the presence of PG (Table 5). These results contrast with those reported for the apple rootstocks M.9 [6], M.26 [17], and MM 104, MM 106 and MM 109 [15]. Transferring shoots to auxin-free medium reduced callus formation [15, 17] but a similar effect could be obtained by reducing the IBA concentration [17] confirming earlier reports on the efficacy of low IBA concentrations for numerous apple cultivars [19, 20, 22].

*Effect of PG*

Phloroglucinol was effective in stimulating rooting of several cultivars, particularly 'Delicious' and some of its strains, in several experiments. This is in accord with the results reported for other cultivars by Jones et al. [9, 10, 11], James and Thurbon [5, 6, 7, 8] and Zimmerman and Broome [22] for 'Spartan'. The fact that PG lacked effect on 'Golden Delicious' reemphasizes the apparent cultivar specificity of its action.

*Other considerations*

The results of these experiments confirm once again the differences in rooting response among different cultivars that have been observed previously [8, 16, 20, 21, 22]. This variability among clones makes it difficult, if not impossible at this time, to generalize about response of apple cultivars to any given root-inducing treatment. It also emphasizes the hazards of trying to generalize results obtained with a single cultivar.

In addition to differences among cultivars, differences in rooting response between two lines of 'Delicious', (NI) and (5-1), were found (Table 5, Figures 3 and 4). Similar differences between lines of M.9 apple rootstocks have been reported [7]. The basis for these differences in rooting response is not known but the virus status of the stock plants of the 'Delicious' lines could be a factor.

Another aspect of the difference in rooting response between the 'Delicious' lines (NI) and (5-1) is the time elapsed between culture establishment and the rooting experiments and, closely related to this time, the number of subcultures. In several experiments in which 'Delicious' (5-1) rooted much better than line (NI) (Table 5, Figure 3), the line (5-1) had been in culture for 15 months (18 subcultures) whereas the line (NI) had been in culture 42 months (39 subcultures). In one case, the difference in rooting between the lines is accentuated in the absence of PG (Figure 3). A similar difference between 'Delicious' (NI) and 'Royal Red Delicious' was noted in the absence of PG at 1.5 $\mu$M IBA but not at 4.9 $\mu$M IBA (Table 4). In this case, 'Delicious' (NI) had been in culture 41 months (38 subcultures) and 'Royal Red Delicious' for 15–16 months (20 and 21 subcultures). Sriskandarajah et al. [16] showed that rooting percentages of 'Jonathan' and 'Delicious' increased with an increasing number of subcultures but that 'Delicious' required many more subcultures than 'Jonathan' before a high rooting percentage was achieved. The results presented here show that the particular strain or line of a cultivar also has a striking influence on the ease with which rooting is accomplished. This fact opens the possibility of selecting lines of a cultivar that are easier to root in vitro and that might differ in other aspects of developmental physiology.

## Acknowledgement

I gratefully acknowledge the excellent assistance and useful comments of Ingrid Fordham.

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