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A Protocol for Rooting and Growing Apple Rootstock Microshoots

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Abstract

Microshoots of apple (*Malus domestica* Borkh.) rootstocks G. 65, G.30 and G.11 were transferred from stage II axillary shoot cultures to stage III rooting media containing 10 levels of indole-butyric acid (IBA) for four weeks to determine optimal conditions for rooting. Microshoots were inverted or left in an upright position. Rootstocks and microshoot position affected rooting and survival; the highest rooting was 30% for G.65 inverted with 2 $mg \cdot L^{-1}$ IBA, 100% for G.30 upright with 3 $mg \cdot L^{-1}$ IBA, and 100% for G.11 inverted with 1 or 2 $mg \cdot L^{-1}$ IBA. No single set of conditions provided consistent rooting in vitro, and plants were not always of high quality. In a second experiment, microshoots were rooted and acclimatized ex vitro in a custom-built fog chamber to assess the effect of two carbon dioxide levels (450 or 1350 $\mu mol \cdot mol^{-1}$) and three light levels (30, 50 or 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$). All rootstocks rooted equally well ex vitro, but survival varied. Plantlets had the highest dry mass, leaf area and growth rates under high light conditions compared to medium light or low light conditions. Supplemental carbon dioxide had no consistent effect. If plants survived fog chamber conditions, subsequent survival in the fog tunnel and greenhouse was 100%. Thus, these rootstocks can be rooted successfully in the fog chamber at high light conditions, which yielded high quality plants. A third experiment was conducted to evaluate the effect of chilling for four weeks at 3.3 °C, or spraying with gibberellic acid 3 (GA_3) on post-rooting dormancy, a problem which frequently occurs with recently rooted apple microshoots. Chilled plants had greater dry mass than control or GA_3 plants, and the GA_3 effect was short-lived. The ex vitro procedure suggested by these experiments could reduce the time associated with rootstock micropropagation to at most 6 months.

Micropropagation of some apple rootstocks can be inefficient and slow (9, 21). This is because microshoots of these rootstocks are difficult to root in vitro (12, 23, 26, 27, 32), and once rooted, often are difficult to acclimate ex vitro (3, 24), or they may set a terminal dormant bud (post-rooting dormancy) that prevents vigorous early growth (11). Three steps in the micropropagation process were examined. The IBA level in the in vitro stage III rooting medium and microshoot position (upright or inverted) that would result in optimal rooting for each of three new apple rootstocks were considered in Experiment 1. Both of these factors are known to influence rooting in other species (26, 28). Profuse callusing of apple is a common problem in the in vitro systems (21, 27). So, the role of light and CO_2 in ex vitro rooting and acclimatization processes was investigated simultaneously in Experiment 2. In *Gerbera jamesonii, Vitis* L. (hybrid) and *Rosa* L. (hybrid), supplemental light and CO_2 have improved rooting and acclimatization (13, 14, 20, 30). Erratic acclimation is characteristic of woody plant microshoots rooted ex vitro for reasons such as small root systems (6, 14), in vitro heterotropism (5, 30), inability to regulate water loss (4, 24), and induced genetic or epigenetic abnormalities (17, 18, 29). Apple microplants typically set a terminal dormant bud shortly after rooting, which may be related to endodormancy (2). Thus, GA_3, chilling and hand defoliation treatments were tested to determine which were more effective at breaking dormancy and stimulating growth (Experiment 3). The goal of this

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research was to identify favorable conditions for accelerating and increasing rooting, survival and growth of three new apple rootstocks over standard protocols.

Materials and Methods

Stage II microshoots of G.65, G.30 and G.11 (difficult, moderate and easy-to-root, respectively; Cummins, J., personal communication) rootstocks were obtained from the New York Agricultural Experiment Station at Geneva, NY. They were subcultured monthly onto fresh Murashige and Skoog (16) basal salt mixture supplemented per liter of medium with: 1 mg each of thiamin, nicotinic acid, pyridoxine, N^6-benzyladenosine (BA), IBA, and GA_3, 100 mg myo-inositol, 30 g lab grade sucrose and 7 g Difco, Bactoagar. The pH was adjusted to 5.6 before dispensing the medium into either glass jars with B-caps (Sigma Chemical Co., St. Louis, MO) or magenta G7 boxes (Magenta Corp., Chicago, IL), then autoclaving for 18 minutes at 121 °C and 100 kPa. Culture vessels were sealed with parafilm and placed in a room maintained at 24 ± 2 °C with ≈ 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation (*PAR*) for 16 hours/day.

Experiment 1: in vitro rooting. In this experiment, 10 levels of IBA (0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 $mg \cdot L^{-1}$) and two microshoot positions were investigated on three apple rootstocks grown in vitro. Microshoots ≈ 1.5 cm long from stage II cultures were rooted in vitro for four weeks on half strength Murashige and Skoog (16) basal salt mixture supplemented per liter of medium with: 1 mg each of thiamin, nicotinic acid, and pyridoxine, 0 to 5 mg IBA, 100 mg myo-inositol, 15 g lab grade sucrose, and 8 g Difco, Bactoagar (Gustafson, H., personal communication).

There were five microshoots per petri dish (2 cm x 10 cm) and four petri dishes per treatment. The cut end of each microshoot was inserted in the medium, petri dishes sealed with parafilm, and placed in the room previously described. Half of the petri dishes were inverted to reverse polarity of microshoots. Inverted microshoots were set upright after two weeks. To examine survival in the greenhouse, rooted microshoots were removed from the petri dishes, transplanted to trays with peat: perlite (2:1, v/v) and acclimatized on a mist bench maintained at 21/17 °C (day/night) temperature with a 16 hour photoperiod.

Experiment 2: ex vitro rooting and survival. The purpose of this experiment was to evaluate two CO_2 and three light levels for ex vitro rooting and acclimatization. Ex vitro rooting was performed in a split plot factorial arrangement, replicated over time. Two CO_2 levels (main plots): 450 ± 50 (low) and 1350 ± 150 (high) $\mu mol \cdot mol^{-1}$ were split by three light levels: 30 ± 5 (low), 50 ± 10 (medium) and 100 ± 20 (high) $\mu mol \cdot m^{-2} \cdot s^{-1}$. The experimental unit was a 3.6 x 2.4 x 0.56 m custom-built fog chamber with six 1.2 x 1.2 m compartments on top of a greenhouse bench. Walls and floors were constructed with 1.25 cm thick plywood, water proof paneling and vinyl. Caulk sealed all inter-connecting joints, and the contact between the lid and the chamber walls was lined with a foam gasket. Two 10 cm diameter holes in each wall had circulation fans (10.2 cm diameter) installed opposing each other to continuously mix the atmosphere inside compartments. A wooden frame lid with Semi-Rigid Flex-O-Pane plastic sheeting (Warp's, Chicago, IL) and a handle covered each compartment (Figure 1).

Fog was generated by four 7.6 L household ultrasonic humidifiers (Sunbeam, Hattiesville, MS) to maintain high relative humidity (RH). Humidifiers outside the fog chamber had short pipes from outlets connected to 1.25 cm diameter PVC pipes and to compartments, creating the fog pipeline (Figure 1). Each outer compartment had two fog inlets, and inner compartments had four fog inlets to ensure even distribution. Pipes were slanted to drain condensate back into humidifiers, which were refilled as needed. Fogging was controlled by a time switch (Tork Model 8301) to 5/5 and 4/6 minutes

(on/off) in the summer and winter, respectively, maintaining 94 to 100% RH for 24 hours.

A carbon dioxide cylinder was connected to three of the compartments via Nalgene tubing with an in-line flow rate meter and a solenoid valve. Two syringe needles were inserted in the Nalgene tubing in each compartment to release CO_2. A Horiba CO_2 monitor (Horiba Instruments Inc., Irvine, CA) regulated the solenoid valve to the on/off mode maintaining CO_2 at 1350 ± 150 $\mu mol \cdot mol^{-1}$ in the enriched chambers. The CO_2 efflux from the enriched compartments to the ambient air elevated CO_2 in the low CO_2 compartments to ≈ 450 ± 50 $\mu mol \cdot mol^{-1}$.

The fog chamber was located in a closed room without windows. It was entirely illuminated by six 2.4 m long General Electric Cool White Power Groove tubes suspended across compartments and connected to a timer (Tork Model 4003-0, Paragon Electrical Company Inc., Two Rivers, WI) (Figure 1). Low light (LL) and medium light (ML) compartments were covered with 3 and 2 layers of Saran shade cloth, respectively; whereas, high light (HL) compartments were not shaded. The room photoperiod was 18 hours of *PAR*, and temperature that was controlled by a ventilator and fans ranged from 22 to 33 °C from fall through summer.

Number 64 trays (40 cm x 30 cm x 2.54 cm; TLC Plymouth, Inc., Plymouth, MN) were filled with moist peat: perlite (1:2, v/v). Microshoots ≈ 2.5 cm long with 3 or 4 leaves were excised and placed in distilled water to avoid wilting. Each microshoot was inserted on the day of excision in a plug (1.25 cm x 1.25 cm x 2.54 cm). Plantlets were introduced to greenhouse conditions with no additional treatment through a fog tunnel, which was a raised wooden frame (3.5 m x 1.0 m x 0.75 m) on top of a greenhouse bench covered with clear polyethylene sheeting. One end of the fog tunnel had a fogger, whereas the other was open to the greenhouse so that a humidity gradient existed along its length. Six incandescent light bulbs suspended above the tunnel extended the photoperiod to 16 hours. Plants remained in the fog tunnel for two weeks during which light and RH were gradually adjusted to ambient levels by moving trays progressively away from the fogger. Thereafter, plants were potted in 10.2 cm diameter standard pots (Kord Products LTD, Bramalea, Ontario) containing peat: perlite (4:1, v/v) and grown in the greenhouse at 21/17 °C (day/night) temperature with a 16-hour photoperiod. Plantlets were fertilized biweekly with 20N-20P-20K Peter's soluble fertilizer at 1 $g \cdot L^{-1}$, beginning soon after rooting. Fertilizer rate was increased to 2 $g \cdot L^{-1}$ from day 70.

The rooting experiment was performed twice for G.30 and G.11, and four times for G.65. Since the effect of time was insignificant in the fog chamber, these data were pooled. Survival was recorded on day 56, and dry mass and leaf area were recorded biweekly on five randomly selected and harvested plants from each treatment combination. Dry mass was transformed to natural logarithm ($\log_e$) for statistical purposes. Data for each rootstock and harvest date were analyzed separately, using Minitab's General Linear Model or two-way analysis of variance, as appropriate. A regression of $\log_e$ dry mass on time provided an estimate (the regression coefficient) of relative growth rate (9). Treatments with no microshoot survival were not analyzed.

Experiment 3: breaking dormancy. This experiment examined factors that might break bud dormancy in rooted plants. Eight-week-old dormant G.65 plants were raised in the fog chamber and potted as described in the ex vitro experiment. Twenty plants were randomly assigned to each of these treatments: 1) distilled water spray, 2) 1000 or 3) 100 ppm GA_3 spray, 4) chilling intact plants, or 5) after hand-defoliation on day 1 of the experiment.

Plants were grown in a greenhouse with 21/17 °C (day/night) temperature and a 16 hour photoperiod. Chilled plants were first placed in a cooler for four weeks to chill at 3.3 °C with 8 hours of incandescent light before moving to the green-

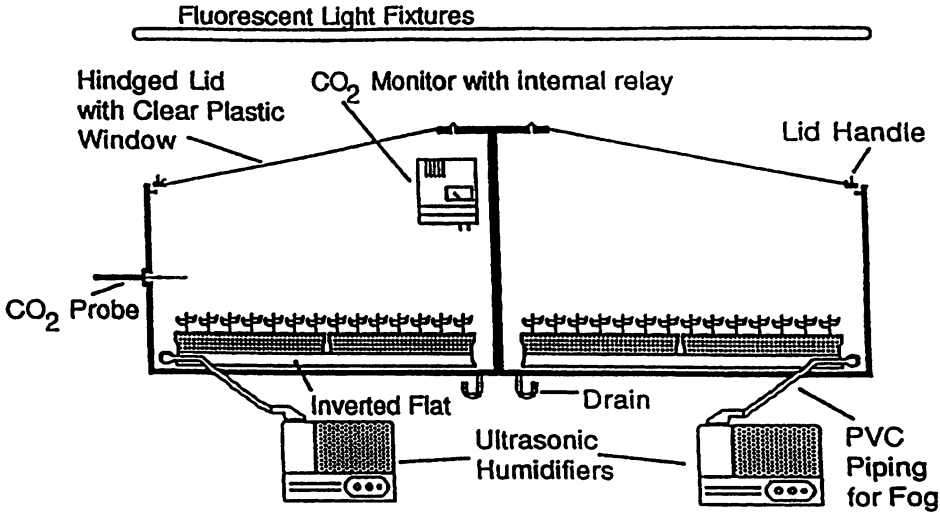


Figure 1. End view of the fog chamber displaying the fluorescent light fixtures, ultrasonic humidifiers, and microshoot arrangement.

house. Plants were watered as needed and fertilized monthly with 20N-20P-20K at 2 $g \cdot L^{-1}$. Five plants were randomly selected and harvested monthly from each treatment to measure dry mass, shoot length and leaf area. Active growth in the cooler was negligible so that chilled plants (treatments 4 and 5) aged 8 and 12 weeks were compared with others (treatments 1, 2 and 3) aged 4 and 8 weeks, respectively.

Results

Experiment 1: in vitro rooting. Microshoot position and IBA level affected in vitro rooting and subsequent survival of plantlets, but the response of rootstocks to these factors varied. G.65 failed to root in the upright position and had the highest rooting (30%) in the inverted position with 2 $mg \cdot L^{-1}$ IBA (Table 1). About 40% of the rooted plantlets of this group survived when transferred to the greenhouse.

In the inverted position, G.30 rooted over a wide range of IBA levels; whereas, in the upright position, it rooted only with 2 to 3.5 $mg \cdot L^{-1}$ IBA (Table 1). In the inverted position, the highest rooting (65%) occurred with 2.5 $mg \cdot L^{-1}$ IBA; 85% of these microshoots survived in the greenhouse. In contrast, rooting was 100% with 3 $mg \cdot L^{-1}$ IBA in the upright position; 80% of these microshoots survived ex vitro.

With G.11, the highest rooting was 100% and 60% in the inverted and upright positions with 1 or 2 and 3 $mg \cdot L^{-1}$ IBA, respectively. Survival of G.11 microshoots in the upright position was higher than of those in the inverted position (Table 1).

Visual observations indicated that root initiation occurred first in G.11, then in G.30 and last in G.65. IBA levels $\geq$ 3 $mg \cdot L^{-1}$ induced more callusing than rooting, and roots were thick (anomalous structure). In addition, root systems formed in vitro had fewer laterals and root hairs.

Experiment 2: ex vitro rooting and survival. Roots were detected at 14 days in the fog chamber. Plantlets deteriorated if left in the fog chamber beyond 42 days, which did not affect survival. The rate of dry mass accumulation was high soon after rooting and during the greenhouse stages, but low at the fog tunnel stage, where some old leaves abscised (Figure 2).

Percent survival recorded at 56 days ranged from 0 to 82%, but data were not analyzed statistically (Table 2). Survival

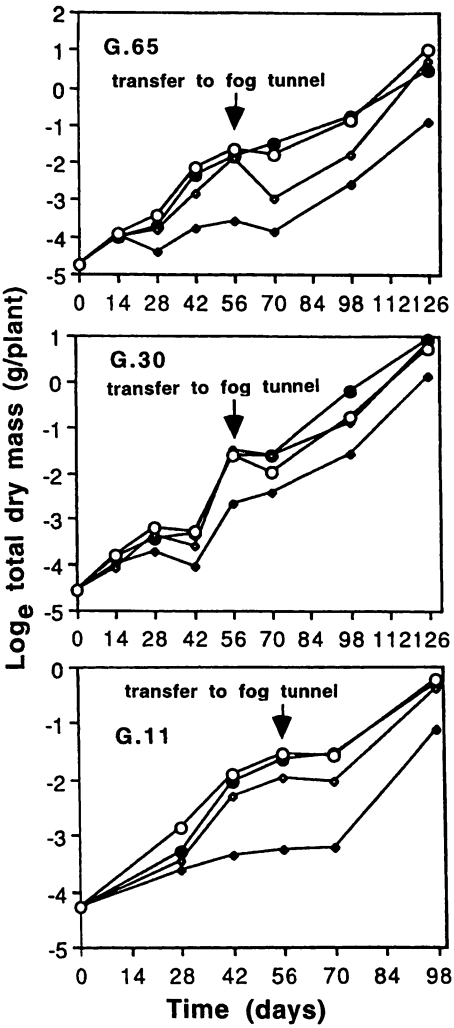


Figure 2. Log$_e$ total dry mass of G.65, G.30 and G.11 microshoots subjected to various levels of light and CO_2, versus time. G.63 and G.30: 50 + 450 (•), 100 + 450 (•); 50 + 1350 (o), 100 + 1350 (O). G.11: 30 + 450 (•), 100 + 450 (•); 50 + 1350 (o), 100 + 1350 (O). 30 = low light, 50 = medium light, 100 = high light; 450 = low CO_2, 1350 = high CO_2.

was 100% in the fog tunnel and greenhouse. The highest survival depended on the rootstock, light and CO_2 treatments. Highest percent survival across CO_2 levels occurred under HL for all rootstocks. Also, it was 82% for G.65 at HL with high CO_2, 77% for G.30 and 65% for G.11 both at ML with low CO_2. Thus, light effects on survival were consistent; whereas, CO_2 effects were inconsistent and depended on the rootstock.

The effects of light, CO_2 and their interaction on root and total dry mass (TDM) varied among rootstocks and were not always maintained over time. Log$_e$-transformed data of TDM are shown in Figure 2; root dry mass data are not presented. With root dry mass (RDM), light had a significant effect at 14, 42 and 98 days for G.65; at 42, 56, 70 and 98 days for G.11, but no significant effect for G.30 ($P \leq 0.05$; Table 3). There was no significant effect of CO_2 on RDM of rootstocks, except G.30 at 70 days ($P \leq 0.05$; Table 3). With TDM, a significant effect of light developed at 42, 56, 98 and 126 days for G.65; at 42, 56 and 70 days for G.11; and did not occur for G.30 ($P \leq 0.05$; Table 3). A significant effect of CO_2 on TDM of: G.30 occurred at 126 days, G.11 at 56 days, and did not occur for G.65 ($P \leq 0.05$; Table 3).

To quantify treatment effects over the entire study period, dry mass was regressed against time and a log-linear model fitted. Estimated growth rates (regression coefficients) for G.65 and G.11 were significantly higher under HL conditions or when ML was enriched with CO_2, but those of G.30 were inconsistent (Table 4). So, light and CO_2 effects on estimated growth rate also varied among rootstocks.

Experiment 3: breaking dormancy. Once plants were in the greenhouse, those chilled intact were not injured, whereas all those chilled after defoliation died (data not shown). Chilling or GA_3 broke dormancy within the first week after treatment. Shoot length of chilled plants was significantly greater than that of control and 100 ppm GA_3 plants; the effect of 1000 ppm GA_3 on shoot length was inconsistent (Table 5). GA_3 plants elongated, but did not accumulate significantly more TDM than controls. TDM of chilled plants was always significantly greater than others. There was no significant difference in RDM of treatments (data not shown).

Table 1. Percent rooting and survival of G.65, G.30 and G.11 microshoots subjected to 10 levels of IBA and either upright or inverted microshoot positions for 4 weeks, then transplanted into a potting mix on a mist bench for acclimatization.

IBA	Percent rooting of microshoots						Percent survival of microshoots					
	Inverted position			Upright position			Inverted position			Upright position		
(mg/L)	G.65	G.30	G.11	G.65	G.30	G.11	G.65	G.30	G.11	G.65	G.30	G.11
0	10	10	0	0	0	0	30	0	—	—	—	—
0.5	0	0	0	0	0	0	—[z]	—	—	—	—	—
1.0	20	20	100	0	0	0	20	0	100	—	—	—
1.5	10	50	80	0	0	0	20	0	50	—	—	—
2.0	30	50	100	0	0	20	40	30	60	—	—	100
2.5	10	65	60	0	20	40	80	85	50	—	0	100
3.0	5	20	20	0	100	60	10	60	0	—	80	100
3.5	0	20	0	0	0	40	—	30	—	—	—	50
4.0	0	20	0	0	0	20	—	60	—	—	—	50
4.5	0	5	0	0	0	50	—	20	—	—	—	60
5.0	0	5	0	0	0	0	—	20	—	—	—	—

[z]No rooting, therefore no microshoot survival.

A leaf cluster formed at the shoot tip of each control and GA_3 plant. Leaves of controls were greener than those of GA_3 or chilled plants, which turned darker green after fertilizing. There were no significant differences in leaf areas of control and GA_3 plants (Table 5). Phytotoxicity (tissue wilting) occurred in shoot tips of the smallest plants sprayed with 1000, but not with 100 ppm GA_3. Axillary buds of the injured plants broke and assumed the terminal role. Regardless, chilling clearly had the most beneficial long-term effect on plant growth.

Discussion

Rooting of microshoots varied among rootstocks, and confirmed that G.65, G.30 and G.11 are, relative to each other, difficult-, moderately difficult- and easy-to-root in vitro, respectively. However, the roots that regenerated in vitro did not establish well in the potting mix regardless of the IBA level in the rooting medium. These results are similar to those of Dunstan (7), Simmonds (21), and Smith et al. (22), who also reported roots of an anomalous structure (thick, few laterals and no root hairs) after in vitro rooting. Simmonds (21) observed poor ex vitro growth of M.26 plants rooted in vitro, then attributed this performance to profuse callusing. In other cases, transfer after a short in vitro period and intensive environmental control improved establishment (7, 27).

A difference also was observed among rootstocks in the optimal IBA level required to induce rooting. The IBA level

Table 2. Percent survival of G.65, G.30 and G.11 microshoots recorded at day 56 in the fog chamber, with various light and CO_2 combinations.[z]

Light ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	CO_2 ($\mu mol \cdot mol^{-1}$)	Rootstock		
		G.65 (n = 4)[y]	G.30 (n = 2)	G.11 (n = 2)
30	450	0	0	48(28)
	1350	69(22)[x]	66(8)	33(2)
50	450	71(12)	77(10)	65(24)
	1350	0	0	31(8)
100	450	66(23)	71(1)	49(15)
	1350	82(13)	59(27)	59(15)

[z]Survival was 100% for all plants beyond 56 days.
[y]Number of times experiments were performed. Data were pooled and averaged.
[x]Values in parenthesis are standard deviations.

Table 3. Significance levels from an analysis of variance table for total dry mass and root dry mass of G.65, G.30 and G.11 at various times during the study.[z]

Clone	Time (d)	Total dry mass (g/plant)[y]			Root dry mass (g/plant)[y]		
		CO_2	Light	CO_2 x Light	CO_2	Light	CO_2 x Light
G.65	14	NS[x]	NS	NS	NS	0.01	NS
	28	NS	NS	NS	NS	NS	NS
	42	NS	0.01	0.04	NS	0.03	NS
	56	NS	0.05	NS	NS	NS	NS
	98	NS	0.02	0.02	NS	0.01	0.01
	126	NS	0.01	0.01	NS	NS	NS
G.30	42	NS	NS	NS	NS	NS	NS
	70	NS	NS	0.05	0.04	NS	NS
	98	NS	NS	NS	NS	NS	NS
	126	0.05	NS	NS	NS	NS	NS
G.11	42	NS	0.03	NS	NS	0.01	0.01
	56	0.03	0.05	NS	NS	0.02	0.04
	70	NS	0.02	0.04	NS	0.01	0.01
	98	NS	NS	NS	NS	0.05	NS

[z]Rootstocks grew for 56 days in the fog chamber, 14 days in the fog tunnel, then 56 days in the greenhouse, except G.11, which grew for 28 days in the greenhouse.
[y]Data were $\log_e$-transformed before analysis of variance.
[x]NS indicates non-significant *P*-values at $P > 0.05$.

often used for rooting these apple microshoots is 1 $mg \cdot L^{-1}$ (Gustafson, H., personal communication). However, we found that this level is optimal only for G.11 (Table 1). Increasing IBA level from 1 to 3 $mg \cdot L^{-1}$ improved rooting of G.65 and G.30; levels greater than 3 $mg \cdot L^{-1}$ were ineffective, thereby confirmed findings of others (26, 28). These levels also inhibited root development and plant survival by causing profuse callusing (21, 27). Thus, optimal levels of IBA for microshoot rooting ranged from 1 to 3 $mg \cdot L^{-1}$, but the specific level depended on the rootstock genotype and microshoot position. In the upright position, they were: 2.0 $mg \cdot L^{-1}$ IBA for G.65, 2.5 $mg \cdot L^{-1}$ IBA for G.11, and 1 or 2.0 $mg \cdot L^{-1}$ IBA for G.11. And in the inverted position, they were: 3.0 $mg \cdot L^{-1}$ IBA for G.30 and G.11, but none for G.65.

Several factors, such as microshoot position, interacted with IBA level to affect

Table 4. Estimated growth rates ($g \cdot plant^{-1} \cdot day^{-1}$) of G.65, G.30 and G.11 under various combinations of light and CO_2 in the fog chamber. Values are slopes of linear regression equations for $\log_e$ total dry mass against time.

Light ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	CO_2 ($\mu mol \cdot mol^{-1}$)	Rootstock		
		G.65	G.30	G.11
30	450	—[z]	—	0.0282 b
	1350	—	—	—
50	450	0.0259 b[y]	0.0354 c	—
	1350	0.0386 a	0.0421 ab	0.0387 a
100	450	0.0403 a	0.0440 a	0.0404 a
	1350	0.0406 a	0.0317 c	0.0392 a
$LSD_{0.05}$		0.0050	0.0047	0.0096

[z]Treatments with no microshoot survival were eliminated from analysis.
[y]Mean separation by Fisher's protected LSD. Values followed by the same letter within a column are not significantly different at $P \leq 0.05$.

Table 5. Shoot length, total dry mass and leaf area of G.65 microplants treated with chilling or GA_3 (ppm) and grown in the greenhouse for eight weeks.

Time	Treatment	Shoot length (cm/plant) Run 1[z]	Shoot length (cm/plant) Run 2	Total dry mass (g/plant) Run 1	Total dry mass (g/plant) Run 2	Leaf area (cm^2/plant) Run 2
Four weeks	Control	4.2 c[y]	2.9 c	0.28 b	0.42 b	34.8 b
	Chilling	7.0 b	13.1 a	0.47 a	0.72 a	138.1 a
	1000 GA_3	16.0 a	—[x]	0.29 b	—	—
	100 GA_3	—	8.4 b	—	0.42 b	61.1 b
	$LSD_{0.05}$	1.3	1.6	0.12	0.01	28.1
Eight weeks	Control	4.4 b	11.6 b	0.56 b	1.37 b	183.6 b
	Chilling	15.3 a	26.1 a	1.65 a	3.79 a	481.0 a
	1000 GA_3	14.8 a	—	0.58 b	—	—
	100 GA_3	—	11.8 b	—	1.70 b	198.8 b
	$LSD_{0.05}$	1.9	5.1	0.19	2.20	222.0

[z]Number of times experiments were performed.
[y]Mean separation by Fisher's protected LSD. Values followed by the same letter within a column are not significantly different at $P \leq 0.05$.
[x]Not measured.

in vitro rooting. These rootstock-specific requirements make the determination of rooting protocols tedious and time-consuming, especially as new rootstocks are being released. Optimized ex vitro rooting of stage II microshoots might eliminate the need for in vitro rooting as practiced by some commercial laboratories (21, 31), even though rooting success can approach 100% under the right conditions.

In contrast to rooting in vitro, all rootstocks appeared to be easy-to-root ex vitro under the same conditions (Table 2). Microshoots rooted ex vitro were superior to those rooted in vitro. Also, microshoots rooted ex vitro established well in the potting mix. Simmonds (21) and Webster and Jones (26) reported similar responses in difficult-to-root M.26 and B.9. Welander and Snygg (27) associated enhanced ex vitro rooting with absence of profuse callusing. We observed that roots facilitated shoot growth as evidenced by rapid new leaf initiation soon after rooting. Roots were also crucial in nutrient and water uptake that alleviated deterioration, because leaves of microshoots that failed to root developed chlorosis and abscised (10).

Based on the best quality root system (visually the largest and most branched), HL enhanced acclimatization; microshoots rooted under LL were inferior. And based on survival, HL enhanced acclimatization of G.65 and G.30 (Table 2). When averaged across CO_2 treatments, the effects of HL and ML on survival of G.11 were similar (Table 2). All microshoots of G.65 and G.30 died under LL with low CO_2, and at ML with high CO_2 (Table 2). Thus, the effect of CO_2 on survival varied. Other researchers also found that light significantly affected rooting compared to CO_2 (6, 25). Results of this study indicate that G.65, G.30 and G.11 can be rooted in the fog chamber under high light ($\approx$ 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with ambient CO_2.

With ex vitro rooting in the fog chamber, problems of nutrient deficiency, heat stress, basal rotting, and condensation affected our plants. Nutrient deficiency symptoms were observed, although fertilizer was applied biweekly. The uniform total amount of fertilizer applied to all plantlets regardless of size caused symptoms of nutrient deficiency in the largest plantlets, and of fertilizer leaf burn in the smallest plantlets. Dunstan (7) also observed leaf burn in apple microplants and recommended that a slow release fertilizer be used. So, nutritional requirements for apple microplants must be adjusted to plant size and age.

Heat in the fog chamber affected plants because survival was high when tempera-

tures were moderate (24 ± 2 °C) in winter, but low when temperatures were warm (> 28 °C) in summer. Microshoots did not adapt well in the fog chamber (turned necrotic, chlorotic and died) at these warmer temperatures. Nevertheless, some microshoots that survived an initial phase of high mortality adapted and grew vigorously under high temperatures. Dunstan (7) obtained vigorous growth for four apple rootstocks at 25 °C. In general, the optimal acclimatization temperature for apple microplants appears to be 24 ± 2 °C.

Basal rotting caused by *Pythium* and *Fusarium* (Karasevicz, D., personal communication) in the late summer (humid and warm) was eradicated using Banrot [5-ethoxy-3-trichloro-methyl-1,2,4-thiadiazole and dimethyl 4,4'-o-phenylenebis (3-thioallophanate)]. Dunstan (7) also observed damping-off and rotting in apple microplants transferred ex vitro and recommended incorporation of fungicides in acclimatization protocols.

When the fog chamber cooled to below the dew point at night, waterlogging occurred in the rooting medium close to the ventilator. Plant growth was partially impaired by wet conditions as reported in 'Northern Spy' (12) and M.7 (8). Thus, plant growth may be improved by draining stagnant water and gradually reducing the humidity cycle to minimize wet conditions.

Various hypotheses have been proposed to explain the induction of dormancy in microplants. In our investigation, micropropagated G.65 had fluctuating relative growth rates, characterized by the setting of a terminal dormant bud occasionally broken after fertilizing. Under optimal propagation practices, production of field transplants takes one season, but some dwarfing rootstocks take longer, suggesting that endogenous mechanisms are slowing growth (11). Murashige (15) and Borkowska (2) postulated that micropropagation advances dormancy in woody plant dwarfing rootstocks. Thus, this slow growth may be intrinsic in G.65, or may be due to some other factor such as a prolonged rooting period as reported in several experiments with other species (25).

Chilling is associated with physiological and metabolic processes that release dormancy (1, 2, 17). Among our experiments, chilling promoted the most consistent and uniform vigor in stunted G.65 plants. It was the most beneficial long-term treatment, and required only a cold room. In contrast, GA_3 sprays, ineffective when applied once, could increase production costs through repeated sprays such as those recommended by Howard and Oehl (11) and Borkowska (2).

Summary

Ex vitro rooting appeared to be superior to in vitro rooting at producing healthy, large plants, and was effective and rapid for all apple rootstocks tested. About 530 plantlets per square meter per six weeks could be produced if the fog chamber was used to full capacity under high light. The recommended protocol begins with excising stage II microshoots from culture, inserting them in a suitable rooting medium in a suitable growth unit receiving ≈ 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for ≈ six weeks, then transferring them to the greenhouse where light and RH are gradually adjusted to ambient levels. Chilling for four weeks could be imposed on rooted, intact microplants to break bud dormancy. Early growth in the greenhouse is maximized by fertilization to achieve 30 to 60 cm height, usually in less than six months from rooting stage. Desired high rootstock numbers can be obtained by repeated use and enlargement of the rooting unit. Ultimately, adoption of this alternative protocol could save time, but the exact amount of savings will depend on the rootstock.

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