

Factors affecting induction and development of *in vitro* rooting in apple rootstocks

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Shoots of apple rootstocks raised *in vitro* were transferred to various rooting media to study the effect of different factors on root initiation and development. Various concentrations of indole-3-butyric acid (IBA) initiated rooting but maximum rooting percentage was found with 2.0 and 2.5 mg l⁻¹ of IBA in M7 and with 1.0 mg l⁻¹ of IBA in MM106. The drawback was that the roots were thick, short and with profuse callus. The presence of activated charcoal (AC) in the rooting medium improved the rooting quality but reduced the rooting percentage in both the rootstocks. In high auxin dip of 70, 80 and 90 mg l⁻¹ IBA for 2, 2 and 1 hr showed 75-85 per cent rooting in M7, but lacked reproducibility of the results. Whereas in MM106, 66 – 70 % rooting was achieved with 70 mg l⁻¹ of IBA dip for 3 h. Root induction in shoots in IBA containing liquid medium (LM) in dark for few days and root elongation in IBA - free medium in light proved most effective. On the other hand, continuous light treatment showed reduced rooting. Reduction of MS salts and sucrose in root elongation medium showed decreased rooting. Plantlets from two - stage rooting procedure showed more rapid growth and satisfactory survival during hardening of plants and on transfer to field.

Keywords: Apple rootstocks, *In vitro* rooting, Propagation

Adventitious root formation is a key step in micropropagation, which are induced by an auxin^{1,2}. The most commonly used auxin for root formation is IBA. Rooting remains one of the critical steps of *in vitro* multiplication of fruit tree species especially the replication of high rooting percentage and optimal root quality. Consistent high frequency rooting of apple has been more difficult to achieve than shoot multiplication. An efficient rooting treatment yields a high percentage of rooted shoots and a high quality root system in tissue culture-raised plants, which is necessary for acclimatization also.

Poor quality of shoots at the time of planting out affects growth, which may be caused by auxin supplied during the rooting treatment³. Research on the factors involved in the development of effective rooting technique has resulted variable success⁴. Yepes and Aldwinckle⁵ studied the effect of indole-3 butyric acid (IBA) on various apple rootstocks and cultivars and have found that lower concentrations are necessary to induce rooting in liquid rather than in solid medium. Magyar *et al*⁶. studied the effect of activated charcoal (AC) and naphthalene acetic acid (NAA) on root elongation of *in vitro* apple cv. 'Royal Gala',. Magyar *et al*⁷. further investigated the rooting

response of apple rootstocks JTE-H, M26 and MM106 to activated charcoal and to various concentrations of IBA in root induction medium (RIM). There are many reports in apple cultivars and rootstocks^{1, 8-11} showing that roots initiated readily when excised shoots have been placed for a few days in dark in IBA containing medium and then transferred to a medium devoid of auxin. Modgil *et al*¹². induced maximum rooting in apple cultivars in sucrose (1.5%) and IBA (0.3 mg l⁻¹) in dark for 7 days. In our laboratory, varying rooting frequencies with each subculture, and sometimes poor shoot quality were found in clonal apple rootstocks which led us to evaluate several factors affecting root initiation and development as well as shoot quality. Using the information from earlier studies, in this communication, we evaluated several factors affecting rooting.

Materials and Methods

Stage II micro shoots (proliferating shoot cultures) of apple rootstocks M7 (2 years old) and MM106 (6-7 years old), maintained in the Department, were used for rooting experiments. Techniques for explant establishment and proliferation of shoots of M7 and MM106 have been previously described^{13,14}. Multiplication of shoots was obtained on Murashige & Skoog basal medium¹⁵ supplemented with growth

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regulators, [(benzyl adenine (0.5 mg l⁻¹), gibberellic acid (0.5 mg l⁻¹), IBA (0.01 mg l⁻¹) for rootstock M7, and BA (1 mg l⁻¹), GA₃ (0.5 mg l⁻¹), IBA (0.01 mg l⁻¹) for MM106). pH of media was adjusted to 5.6 and they were gelled with 0.7 per cent Difco bacto agar. After mixing agar, 25 ml of medium was dispensed in 100 ml capacity Erlenmeyer flasks. The media were sterilized at 121°C at 1.1 kg/cm² pressure for 15 min. Root induction medium (RIM) consisted of ½ MS salts with (mg l⁻¹), thiamine HCl-1, sucrose-20000. Media were solidified with 7 g l⁻¹ Difco bacto agar and dispensed in culture tubes.

For *in vitro* rooting, axillary shoot cuttings, 1.5 to 2.0 cm in length, were excised from 4-5 weeks old proliferating shoot cultures of M7 and MM106 growing on multiplication medium and treated as follows:

Experiment I—Shoots were placed in RIM with six IBA concentrations (1.0-3.5mg l⁻¹) without or with activated charcoal (AC) 0.02%. After two weeks, half of the shoots from RIM (without AC) were transferred to same medium devoid of IBA for root elongation and half remained as such. Shoots in RIM with activated charcoal were not transferred.

Experiment II—Microshoots were first dipped in high concentrations of IBA solution (30, 40, 50, 60, 70, 80, 90, 100, 200, 500 and 1000 mg l⁻¹) for different periods (10, 20 and 30 min, and 1, 2, 3, 4 and 5 h) for root induction and then transferred to auxin free medium.

Experiment III—For root induction, microshoots were grown in dark for 6 to 13 days in liquid RIM containing 0.5mg/l IBA. These shoots were then transferred to same solidified medium lacking IBA and kept in light to allow root development.

Experiment IV—The above experiment was carried out in continuous light.

Experiment V—In experiments III and IV, after root induction, shoots were transferred to the root elongation medium consisting of reduced concentrations of salts (1/3, 1/4) and sucrose (10 and 15 g/l) in different combinations.

Each experiment consisted of three replicates, and 24 cuttings were used per treatment. Rooting response was expressed in terms of rooting percentage (Number of rooted shoots × 100/total number of shoots kept for rooting), average root number per plantlet and average length of the roots were recorded after 4 weeks of incubation.

All the cultures were incubated at 26°±2°C with 16h photoperiod under cool white fluorescent light at an irradiance of 54mm m⁻² s⁻¹ at bench height. For dark treatment of shoots, culture tubes were wrapped with carbon paper or tin foil and placed in shelf without light. Shoots of M7 and MM106, rooted by either method, were transferred to disposable cups containing peat for hardening. The plantlets were covered with plastic bags to maintain high humidity and kept in glasshouse at 18°-20° C. Humidity was gradually reduced by making larger holes in the bags. These plants were later transferred to earthen pots and then planted in nursery.

Results

Effect of IBA concentration—All the IBA concentrations had significant effect on the rooting of M7 and MM106. The maximum rooting (89.63%) was observed with 2.5 mg l⁻¹ of IBA (Table 1) which differed significantly from others. IBA at 2.0 and 3.0 mg l⁻¹ conc. also resulted in good rooting. However, these treatments showed sufficient callusing at basal part of the stem. Moreover, they induced more root initials (Fig.1A), and inhibited root and shoot development, which resulted in delayed resumption of growth after transplanting, thus leading to poor plant survival. Lower IBA levels initiated less rooting

Table 1—Effect of different IBA concentrations with (+) and without (-) activated charcoal on rooting of M7 and MM106 after 4 weeks

IBA concentration (mg l ⁻¹)	Activated charcoal	Rooting in M7 (%)	% Rooting in MM106 (%)
0.5	+	8.20 (16.64)	0.00 (0.00)
0.5	-	25.83 (30.55)	50.00 (45.00)
1.0	+	16.10 (23.66)	0.00 (0.00)
1.0	-	43.50 (41.27)	62.00 (51.95)
1.5	+	21.40 (27.56)	0.00 (0.00)
1.5	-	30.60 (33.59)	56.00 (48.45)
2.0	+	25.43 (30.29)	0.00 (0.00)
2.0	-	86.63 (68.56)	42.00 (40.40)
2.5	+	23.43 (28.95)	8.00 (16.41)
2.5	-	89.63 (71.22)	25.00 (30.00)
3.0	+	20.70 (27.06)	0.00 (0.00)
3.0	-	79.00 (62.73)	16.00 (23.57)
3.5	+	21.73 (27.79)	8.00 (16.41)
3.5	-	31.90 (34.39)	24.00 (29.33)
4.0	+	13.60 (21.64)	6.00 (14.15)
4.0	-	20.43 (26.87)	22.00 (27.97)
CD _{0.05}		0.60	1.10

Figures in parentheses are arc sine transformed values
CD – Critical difference



Fig. 1-(A)—Callused roots in M7 with 2.5 mg l⁻¹ IBA; (B)—Rooting of shoots after high auxin dip in MM106; (C)—Development of root primordia after dark treatment in M7; (D)—Shoot growth in M7 and MM106 after light and dark treatment; (E)—Rooting in MM106 after 14 days of dark treatment; (F)—Hardened plants from 2 step rooting; and (G)—Three months old plants of M7 in field

(below 50%), whereas, IBA levels greater than 3 mg l⁻¹ were found ineffective and with profuse callus formation. In case of MM106, best rooting (62%) was observed in 1.0 mg l⁻¹ IBA showing slight callus at cut end of shoots (Table 1). Reduced rooting with callused roots was obtained with higher levels of IBA.

Activated charcoal (AC) suppressed callusing in both the rootstocks. Also, its presence resulted in decreased rooting (20-25%) in M7, but root length (upto 5.50 cm) was more as compared to treatments without AC. In MM106, no rooting was observed in most of the treatments, except at 2.5, 3.5 and 4.0 mg l⁻¹ of IBA. Generally, the roots were thin and often had secondary roots.

Effect of high auxin dip of explants—It is evident from Table 2 that in M7, the maximum rooting (86.00%) and root number (10.00) was achieved when

shoots were dipped in AC supplemented medium in 90mg/l IBA for 1h followed by 80mg l⁻¹ for 2h. Shoot length did not increase much, while expansion of leaf and increase in leaf number was observed (Fig. 1B). IBA levels above 200 mg l⁻¹ and below 60 mg l⁻¹ showed decreased rate of rooting with more callus. In MM106, all the treatments showed negligible rooting (Table 2), except 70 mg/l of IBA which resulted in 30 % rooting after 3 h dip.

Effect of dark vs light treatment- Dark treatment had significantly beneficial effect on rooting of M7. Root initials were visible at the cut ends of micro shoots between 7th and 10th day from the rooting treatment in liquid medium (Fig. 1C). After three weeks in root elongation medium (REM), roots became well developed. 82.10% rooting was found after dark treatment for 12 day. Shoots kept in dark

Table 2—Effect of high auxin dip on rooting of M7 and MM106 after 4 weeks

IBA concentration (mg l ⁻¹)	Time of treatment (h)	AC concentration	Rooting in M7 (%)	Time of treatment (h)	Rooting in MM106 (%)
30	3	+	42.00 (40.40)	3	6.57 (2.56)
30	3	-	40.00 (39.23)	4	6.00 (2.44)
30	-	-	-	5	3.73 (1.93)
40	3	+	43.00 (40.98)	-	-
40	3	-	32.00 (34.45)	-	-
50	2.30	+	40.67 (39.62)	3	7.93 (2.81)
50	2.30	-	35.00 (36.27)	4	6.97 (2.63)
50	-	-	-	5	7.07 (2.65)
60	2.30	+	46.00 (42.71)	-	-
60	2.30	-	60.00 (50.77)	-	-
70	2	+	76.00 (60.67)	3	29.87 (5.46)
70	2	-	66.00 (54.33)	4	26.27 (5.12)
70	-	-	-	5	29.07 (5.39)
80	2	+	80.00 (63.44)	2	11.73 (3.42)
80	2	-	67.00 (54.94)	-	-
90	1	+	86.00 (68.04)	1	20.97 (4.57)
90	1	-	60.00 (50.77)	-	-
100	1	+	66.00 (54.33)	1	13.37 (3.65)
100	1	-	50.00 (45.00)	-	-
200	30 min	+	60.00 (50.77)	30 min	26.00 (5.09)
200	30 min	-	44.00 (41.55)	-	-
500	20 min	+	28.00 (31.95)	-	-
500	20 min	-	33.00 (35.06)	-	-
1000	10 min	+	16.00 (23.57)	-	-
1000	10 min	-	20.00 (26.56)	-	-
CD _{0.05}			1.60		0.12

(+) – With and, (-) – Without activated charcoal

Figures in parentheses are arc sine transformed values

CD - Critical difference

were slightly pale, thin and lengthy with narrow leaves (Fig. 1D). However, they attained good growth on transfer to light. Minimum callus was formed at shoot bases. Root induction in continuous light was less as compared to dark in M7. The maximum rooting (54.00%) without intervening callusing was achieved in 12-day auxin- treated shoots in light. In MM106, light treatment induced negligible rooting, whereas in dark treated shoots, root primordia were visible on 8th day onwards. The highest (41.10 %) rooting was found after dark treatment for 14 day (Fig. 1E). Number of roots was 1-2 in these treatments.

Effect of low MS salts and sucrose concentration—Seventy one per cent rooting was achieved in 1/3 MS with 15 g l⁻¹ sucrose after dark treatment for 13 day followed by 12 days. In reduced salts and sucrose, the rooting percentage decreased in comparison to 1/2 MS with 20 g l⁻¹ of sucrose. In addition to this, roots were observed without laterals

and poor shoot growth with little leaf expansion. However, there were not much differences in root number and length of roots within the treatments. When 1/4 MS was used in place of 1/3 MS for 12 and 13 day dark treatments, the rooting response was at par in both the treatments. On the other hand, when sucrose was reduced, rooting was adversely affected.

During hardening, growth of plants raised from two-step rooting method was rapid (Fig. 1F) as compared to other treatments. The plants of these rootstocks were established in University nursery, with 90-95% survival producing vigorously growing and healthy plants. They attained height of more than 1 m in five months (Fig. 1G).

Discussion

In experiment 1, the rooting percentage decreased in MM106 and M7, when activated charcoal (AC) and IBA were present in rooting medium. On the other hand, in experiment II, after higher auxin dip, rooting

increased in M7 when AC was present in IBA free root elongation medium. Similar observations were found by a few workers^{7,16} in apple rootstocks. The favourable effect of AC on rooting was mainly due to adsorption of auxin. The differences observed may be due to the concentration of AC in root elongation medium (REM) and its presence in REM with or without IBA, depending upon the cultivar.

High auxin dip treatment yielded maximum percentage of rooted shoots of M7 but variable results were achieved while rooting was induced from successive *in vitro* culturing. Puente and Marin¹⁷ have concluded that the differences in rooting behaviour of individual shoots are only due to a short term physiological state, and it is unpredictable that which shoots will be the competent ones in the next subculture. It has further been found that in place of high auxin dip, few days dark incubation to shoots in low concentration of auxin followed by their transfer to light in auxin-free medium was better. It resulted in increased rooting in M7, which is even more when compared to continuous incubation in light. It was observed that continuous darkness during the rooting inductive phase, increases peroxidase activity which resulted in higher rooting rate¹⁸. Twelve days of auxin treatment in M7 seemed sufficient for rooting almost all the shoots and ensured reproducible high rooting rates. This period varies from one species to another and even from one shoot to another, all the shoots being physiologically different at the onset of rooting¹⁹. Improved rooting of shoots by dark treatment in apple cultivars has been suggested by many workers^{8,9,11,20}. In case of MM106, per cent rooting was not optimum. The possible reason may be that the cultures of MM 106 have been maintained *in vitro* for six years. It has been observed that the duration of dark treatment depends upon IBA concentration and the type of rootstock.

MS salts (1/2 strength) yielded better results than dilution to 1/3 or 1/4 strength when combined with low sucrose concentration tested. However, Simmonds²¹ has observed enhanced rooting in low conc of sucrose and 1/4 MS, but found its adverse effect on the plant establishment. The same effect was also seen in our experiments. This may be attributed to the excessive development of anaerobic roots (devoid of root hair) in agar which initially were not sufficient in an aerobic substrate²¹. A number of factors have been shown to be important for rooting in various apple cultivars including reduced salt

concentration^{16,22} and use of separate media for root initiation and root elongation^{16,23,24}.

Our results emphasize that a number of factors are important for rooting in apple rootstocks. In conclusion, it has been found necessary to have a short root induction period of a few days in auxin containing medium, followed by their transfer in auxin free medium.

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