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Successful propagation *in vitro* of apple rootstock MM106 and influence of phloroglucinol

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Successful *in vitro* propagation of clonal apple rootstock MM106 was achieved by culturing axillary buds on MS basal medium with BAP (1 mg/L), GA_3 (0.5 mg/L) and IBA (0.1 mg/L). Use of liquid medium (LM) in initial cultures reduced phenol exudation to a greater extent and gave maximum sprouting percentage when transferred to solid MS medium. Phloroglucinol (PG) did not enhance sprouting of buds but increased the rate of multiplication when added in the medium. Maximum number of shoots were obtained when MS medium was supplemented with BAP (0.5 mg/L), GA_3 (1 mg/L), IBA (0.1 mg/L) and PG (100 mg/L). For rooting, *in vitro* regenerated shoots were placed in IBA (30 mg/L) for 3 hr and transferred to solidified auxin free medium. Rooting was recorded in about 80% of shoots. Inclusion of PG in rooting medium was not beneficial but shoot cultures grown in its presence gave higher rooting percentage. Rooted plantlets showed about 70% survival rate in potting mixture of sand : soil : perlite (1:1:1).

Clonal rootstocks are desirable not only to produce uniformity but to preserve special characteristics and specific influences on scion varieties such as disease resistance, adaptability to different climates, growth, flowering habit and quality of fruit, and thus are better than seedling rootstocks. It is impossible to increase the production of apple trees by using conventional methods, so micropropagation technique is the only method which is more rapid and could be of major economic advantage especially in relation to modern high density orchards.

Previously, micropropagation of several apple cultivars and rootstocks was successfully developed[1,2]. Influence of phloroglucinol (PG) on shoot multiplication and rooting of a number of apple scion cultivars and rootstocks *in vitro* has been evaluated[3-6]. However, the response of apple cultivars to PG is not consistent. Inclusion of PG in tissue culture medium provided little, no benefit or was deterimental.

Because of varied climatic conditions in Himachal Pradesh, India, different rootstocks are needed to be planted at various sites. Malling Merton 106 (MM106) is the most widely used rootstock outside India. Trees of MM106 are semi-dwarf, well anchored due to vigorous root system, starts bearing early in life, resistant to woolly aphids but sensitive to excessive cold, heat and drought. Due to these characteristics, it is in great demand. Therefore, aim of the present study is to multiply MM106 *in vitro* so that it could be distributed to farmers after multiplying on large scale. It can perform well in mid-hill valleys like Kullu (5000-7000 ft) India, having flat and irrigated land and also some parts of Shimla, India.

Materials and Methods

Explant culture—Cultures were initiated from actively growing axillary buds of orchard trees (virus free), grown in the Department of Pomology, UHF, Nauni, Solan, India. Collection and surface-sterilization of explants were based on previously described by us[7]. The explants were cultured for 1-2 days in continuously shaked (100 rpm) liquid medium (LM) having Murashige and Skoog[8] salts and vitamins with (mg/L) : 1 benzylamino purine (BAP), 0.5 gibberellic acid (GA_3), 0.1 indole butyric acid (IBA), 3% sucrose and 0.1% polyvinylpyrollidone (PVP) and then transferred to solid medium (LM+0.8% agar). In other treatments, kinetin (Kn) and napthalene acetic acid (NAA) alongwith BAP were also tested.

Shoot culture—Shoots were multiplied by the method of enhanced release of axillary buds. Different combinations and concentrations of growth regulators (BAP, NAA, IBA, GA_3 and Kn) were tested to see their effect. Multiplication rate, average length of shoots, number of leaves per shoot were calculated on the basis of usable shoots per propagule from a total

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of six cultures from three subcultures. Subculturing into fresh medium was performed after 4-6 weeks.

Root culture—Vigorously growing shoots (1.5-2.0 cm) from the proliferating cultures were excised and given a dip for 1-3 hr in different concentrations of IBA (30, 50, 70 and 100 mg/L) and transferred to auxin-free solidified rooting medium. The rooting medium was modified from that used for shoot proliferation by halving MS salts, sucrose, by eliminating all the hormones and adding 0.1% activated charcoal. In a few experiments, the medium was supplemented with phloroglucinol (PG; 100 mg/L). Selection for other treatments were based on previous study[7,14]—(i) microshoots were directly kept in a solidified auxin containing rooting medium i.e. NAA and IBA (0.4 to 5.0 mg/L of each); (ii) micro-shoots were dipped in IBA (0.5 mg/L) and sucrose (1.5% mg/L) solution for 4 days in dark at 25°±2°C and then transferred to solidified auxin-free rooting medium. Number of roots per rooted plantlet and average length of roots were calculated. All the experiments were done in the presence or absence of phloroglucinol (100 mg/L).

Culture in polythene bags—Autoclavable polythene bags for multiplication and rooting of shoots were also used in place of culture vessels and tubes. The medium used were same as mentioned above. For comparison, the shoots were transferred to polybags and culture vessels simultaneously at the same stage of development under similar cultural conditions.

The *p*H of all the medium was adjusted to 5.7 before autoclaving at 1.1 kg/cm^2 and 121°C for 15 min. The cultures were grown at 25°±2°C with 16 hr photoperiods at a light intensity of 2-4 Klux using cool white or warm white fluorescent lights.

Pot culture—Four to six weeks old rooted plantlets were transferred to pots containing pre-sterilized potting mixture of sand, soil and compost (1:1:1) and covered with glass jars to maintain high humidity. When new leaves develop, jars were removed for 2-3 hr daily for 1 week, increasing the time gradually.

Results and Discussion

The present results clearly show that a number of factors are important for establishment, proliferation and rooting of rootstock MM106.

Explant culture—Initially, shaking of explants with LM reduced the browning intensity which helped to establish them successfully. The addition of antioxidants and adsorbents to the initiation medium reduced browning and improved establishment of explants of apple cv. fuji and pyrus cv. jinhua[9].

MS medium supplemented with BAP (1 mg/L); GA_3 (0.5 mg/L) and IBA(0.1 mg/L) showed maximum bud break (42%), while addition of PG showed only 20% bud break (Table 1). A few buds (7-18%) established in BAP alongwith NAA and Kn and no bud break was observed with PG. Thus, PG had no stimulatory effect on shoot initiation. Shoots were subcultured after 7-8 weeks when a few leaves developed (Fig. 1). But after subculturing, most of the established buds had gradual decaying of leaves and stem and died within 10 days. However, pouring of liquid medium over solid medium enhanced the growth of these buds by elongation of shoots and developed more axillary buds (Figs 1-3). Thus, instead of subculturing in any other medium, overlaying of LM saved the cultures.

Shoot culture—Maximum shoot number (6.66), shoot length (2.73 cm) and number of leaves/shoot (16.00) were achieved in a medium containing BAP (0.5 mg/L), GA_3 (1 mg/L) and IBA (0.1 mg/L) supplemented with PG (Table 2, Fig.4) as compared to other hormonal combinations and concentrations. BAP with Kn gave shoot length of 2.1 cm when supplemented with PG. BAP used singly or at low concentration of 0.1 mg/L did not give good results.

In the present study, BAP was the most effective cytokinin but response was dependent upon concentration. By reducing BAP from 1 mg to 0.5 mg/L, shoots formed were longer and displayed longer internodes and larger leaves. Inclusion of phloroglucinol (PG) in the medium improved multiplication rate, leaf size and length of shoots. BAP at 0.1 and 2 mg/L did not improve proliferation. High concentration (above 1.5 mg/L) caused vitrified shoots in apple cv.

Table 1—Effect of different concentrations and combinations of hormones in presence (+) or absence (-) of phloroglucinol (PG) on explant establishment

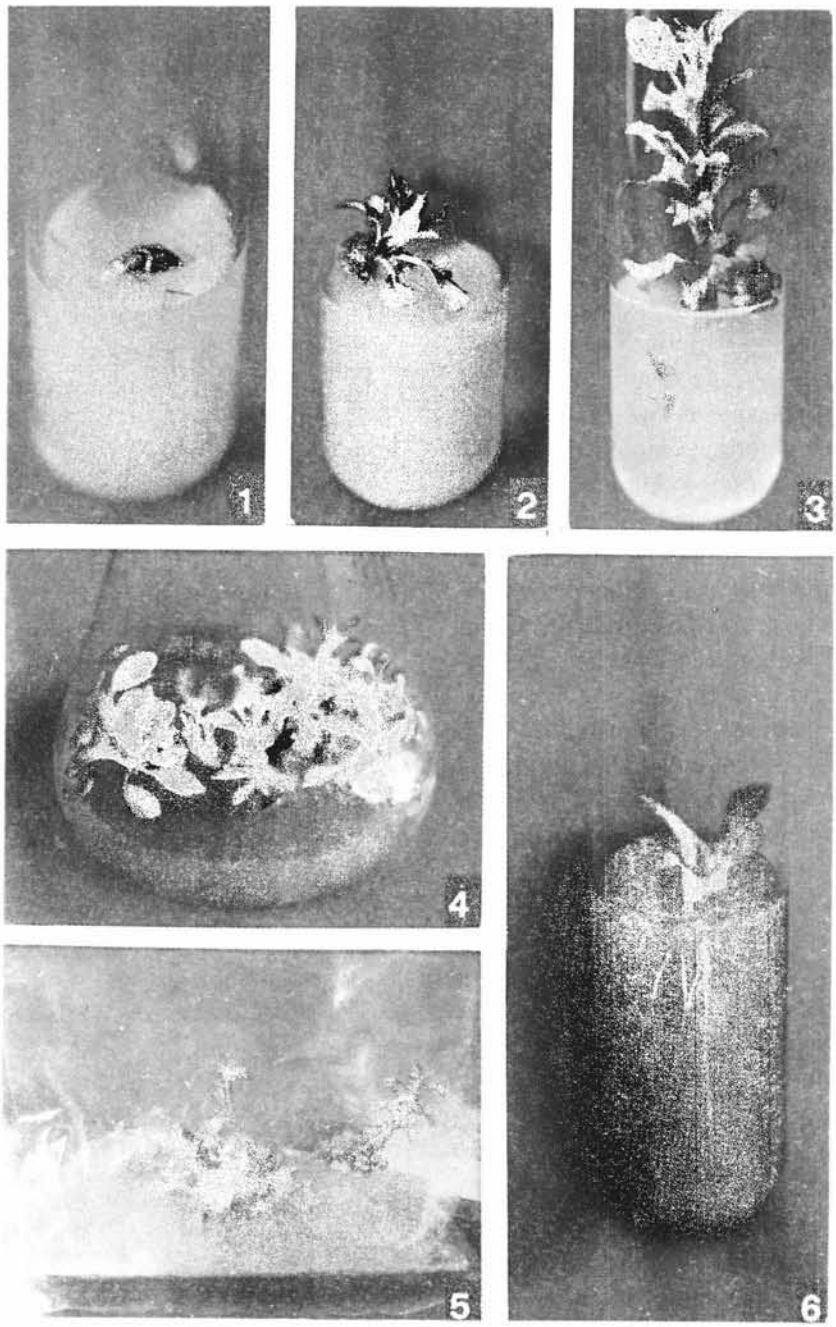
[Values are mean of 25 explants used for treatment]

Establishment medium	Per cent explants sprouted
A-PG	42.59 (40.73)
A+PG	20.37 (26.78)
B-PG	18.51 (25.44)
B+PG)	0.00 (0.00)
C-PG	7.403 (15.57)
C+PG)	0.00 (0.00)
CD (0.05)	(3.6833)

Figures within parentheses are arcsine transformed values.
(A)—MS + BAP (1 mg/L) + GA_3 (0.5 mg/L) + IBA (0.1 mg/L); (B)—MS + BAP (2 mg/L) + NAA (0.1 mg/L); and (C)—MS + BAP (0.5 mg/L)+ Kn (0.5 mg/L).

fuji[10]. However, BAP (1 mg/L) stimulated production of multiple shoots in apple rootstocks MM 106 and M26 (Ref. 6). Arello *et al.*[11] have observed that increasing BA concentration up to 2.0 mg/L resulted in better multiplication of shoots in MM111. Multiplication of shoots in polythene bags also showed better growth (Fig. 5) as compared to shoots in culture vessels. Multiplication rate remains almost the same (1:3-6) but elongated shoots with more number of nodes were produced in bags. Their use reduces the cost of labour and breakage of glassware.

Root culture—When shoots were directly transferred to rooting medium containing auxin, rooting (up to 10%) was achieved with NAA and 20-25% with IBA, both in the absence or presence of PG -



Figs 1—6—Micropropagation of apple rootstock MM106; (1)—Sprouted bud (20 days old culture); (2) — development of axillary buds after overlaying liquid medium (LM); (45 days old culture); (3)—elongation of shoot showing axillary buds and leaves after further overlaying LM (two and half months old culture); (4)—proliferated shoots in MS medium supplemented with BAP (0.5 mg/L), IBA (0.1 mg/L) and GA_3 (1.0/L); (5)—multiplication of shoots in polythene bag; and (6)—rooted shoot (2 weeks after culturing).

(Table 3). Higher concentrations (2-5 mg/L) were also used which developed callus. Highest rooting percentage (80.25%) was found when shoots were pretreated for 3 hr in IBA solution (30 mg/L). Dip in IBA (100 mg/L), resulted in lowest rooting percentage (Table 4). Maximum average number of roots/shoot and root length was observed with IBA (70 mg/L). In these experiments, roots started appearing after 9 days (Fig. 6).

IBA is more effective as compared to NAA for rooting as suggested previously[12]. A short period pretreatment with high concentration of IBA (auxin dip method) improved rooting. Dipping in high IAA concentration improves rooting in apple and pear rootstocks[13].

There was increased rooting (80-90%) when PG was supplemented in the shoot culture medium - (Table 5), while 53% rooting was observed when PG was present only in rooting medium. Presence of PG both in shoot culture and rooting medium showed 60.25% of rooting. It indicated that PG played an important role in shoot multiplication although it had no stimulatory effect on axillary bud initiation. Hence, the presence of this compound during multiplication stage was beneficial in disposing shoot cultures to root. To some extent, our results agree with Webster and Jones[1] and Alkan *et al.*[6] who have indicated that shoot cultures of M9 and MM106 grown in presence of PG regenerate higher rooting percentages than cultures grown in its absence. Preconditioning effect of PG on shoots for subsequent rooting suggested that raised levels of endogenous PG in the shoot favoured root development. However, inclusion of PG (100 mg/L) in rooting medium has proved beneficial for early and better root development in cv. Tydeman' Early Worcester[14].

High survival rate (70%) was observed in pots. Apple plants regenerated *in vitro* were not easy to adapt to green house conditions as plants of other fruit crops, because the leaves desiccate rapidly. It might be due to high humidity required by the newly transferred plantlets to adapt to outside environment.

Table 3—Effect of auxins (NAA and IBA) in the presence or absence of PG on rooting
[Values are mean of 30 explants used for treatment]

Auxin used	Conc (mg/L)	PG (100mg/L)	Rooting (%)
NAA	0.5	+	0.00 (0.00)
NAA	1.0	+	11.00 (3.31)
NAA	0.5	—	7.00 (2.62)
NAA	1.0	—	10.67 (3.26)
IBA	0.5	+	25.00 (4.99)
IBA	1.0	+	20.33 (4.50)
IBA	0.5	—	20.33 (4.50)
IBA	1.0	—	22.67 (4.76)
CD (0.05)			(0.3097)

Figures within parentheses are arcsine transformed values.
(—) without; (+) with PG.

Table 2—Effect of various concentrations and combinations of growth regulators on shoot proliferation
[Values are mean of 25 explants used for treatment]

Growth regulators number/shoot	Conc. (mg/L)	PG (100 mg/L)	No. of usable shoots propagule	Average length of shoots (cm)	Average Leaf no./shoot
BAP+	0.5	—	2.66	1.63	12.67
IBA +	0.1				
GA_3	1.0	+	6.66	2.73	16.00
BAP+	0.5	—	1.33	0.667	6.00
Kn	0.5	+	2.33	2.10	12.67
BAP+	0.1	—	1.33	0.933	6.66
IBA +	0.1	+	1.66	1.06	6.66
GA_3	1.0				
BAP +	0.1	—	1.00	1.20	7.00
IBA +	0.1	+	2.00	1.83	12.00
GA_3	0.5				
BAP +	0.1	—	1.33	0.96	8.00
GA_3	5.0	+	1.66	1.50	8.00
BAP +	0.1	—	1.00	1.00	4.66
NAA	0.1	+	1.66	1.56	8.66
BAP	2.0	—	1.00	0.633	6.00
		+	1.33	1.00	7.66
CD (0.05)			0.6169	0.1723	1.7666

(—) without; (+) with PG.

Table 4—Effect of dipping in different concentrations of IBA on per cent rooting
[Values are mean of 30 explants used for treatment]

IBA (mg/L)	Time (hr)	Average number of roots/shoot	Average length of roots (cm)	Rooting (%)
30	3	7.00	3.95	80.25 (63.63)
50	3	8.50	5.35	74.75 (59.85)
70	3	9.50	6.15	50.50 (45.29)
100	1	3.50	4.10	40.25 (39.38)
0.5+1.5% sucrose	4 days	3.25	2.12	51.00 (45.57)
CD (0.05)	(dark)			(2.1105)

Figures within parentheses are arcsine transformed values.

Table 5—Effect of phloroglucinol (PG) on per cent rooting of shoots
[Values are mean of 25 explants used for treatment]

Shoot culture medium	Solidified medium (hormone-free)	% rooting after 4th subculture
PG(-)	PG(—)	65.75 (54.19)
PG(+)	PG(—)	80.50 (63.86)
PG(-)	PG(+)	53.25 (46.87)
PG(+)	PG(+)	60.25 (50.92)
CD (0.05)		(2.5866)

Figures within parentheses are arcsine transformed values. (—) without; (+) With PG.

Present protocol is recommended for micropropagation of other rootstocks of apple. Polythene bag culture can be a better option for commercialization of *in vitro* culture of apple plants.

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