Indian Journal of Experimental Biology
Vol. 43, June 2005, pp. 561-565

In vitro clonal multiplication of an apple rootstock by culture of shoot apices and axillary buds

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Received 10 June 2004; revised 7 February 2005

In vitro clonal multiplication of apple rootstock MM 111 using axillary buds and shoot apices were carried out. Vegetative axillary buds of the size of 0.2-2.0 cm and shoot apices measuring 4 mm in length were initiated to shoot proliferation on MS medium supplemented with BA (0.5-1.0 mgl^{-1}), GA_3(0.5 mgl^{-1}), with or without IBA(0.05-0.1 mgl^{-1}). Small size explants showed less phenol exudation and less contamination. Following establishment phase, the small shoots emerged from explants were subcultured on MS medium supplemented with different combinations and concentrations of growth regulators. BA (1.0 mgl^{-1}) and GA_3 (0.5 mgl^{-1}) combination showed highest multiplication rate (1:5), and also produced longer shoots. Two step rooting was done by transferring microcuttings to auxin free solid medium after root initiation in dark on ½ strength MS liquid medium containing IBA (0.5 mgl^{-1}). Rooted plantlets were transferred to peat containing paper cups and resulting plants of MM 111 acclimated successfully for transfer to field.

Keywords: Apple rootstocks, Axillary bud, Clonal multiplication, Shoot apex
IPC Code: Int. Cl^7 A01H

Inspite of a great potential for apple in Himachal Pradesh, its productivity is still low. Main causes for low productivity are non-availability of high quality and healthy planting material as clonal rootstocks and inadequate knowledge of orchard management. Now, most of the commercial plantations are becoming old and diseased which need to be replaced. For many years, seedling rootstocks have been used for propagation in the major apple producing areas. A highly drought tolerant and semi-dwarf rootstock, MM 111 which is a virus-free clone of EMLA (East Malling Long Ashton), is the most suitable for high density apple plantations in Himachal Pradesh, India. It is vigorous and adaptable to a wide range of soil and climatic conditions. It is resistant to collar rot but susceptible to powdery mildew.

The traditional method of propagation and to maintain disease free rootstocks require elaborate nursery facilities which are time consuming as it will take many years to reach sizable numbers for commercial purposes. Micropropagation techniques have paved way for rapid multiplication of plants and is useful where the quality of planting material is required on large scale and in a short period of time.It is therefore, desirable to undertake clonal multiplication of improved rootstocks suitable to agro-climatic conditions of apple growing areas in India. In recent years, reliable methods of *in vitro* propagation of apple have been developed for both rootstocks and scions [1-9]. Accordingly, in view of the importance of rootstock MM 111 and its demand in Himachal Pradesh, India, the present investigation was aimed at *in vitro* clonal multiplication through axillary buds and shoot apices culture.

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Materials and Methods

Actively growing as well as dormant shoots of virus tested apple rootstock MM 111 were collected from the fields and kept in plastic bags to prevent wilting till their use in laboratory. Leaves were cut off alongwith the stipules, ensuring not to damage the buds. Axillary buds, as explants, (0.2-2.0 cm) were excised with sharp blade and collected in distilled water. Some of the shoots were kept in water at 25°-27°C to force lateral buds to grow and these growing tips were used as explants. All the explants were surface-sterilized with 1% NaOCl and 2-3 drops of tween 20, for 20 min and rinsed three times with sterile distilled water for 2, 5 and 15 min, to remove traces of the sterilant. Once the sterilant has been rinsed completely from the explants, these were transferred to Petri dishes lined with filter paper for water absorption and cultured in nutrient medium.

Shoot apices measuring *ca* 0.4 mm in length were dissected by removing the bud scales followed by leaf primordia under a stereomicroscope and inoculated on the filter paper bridges kept on liquid medium.

Establishment medium consisted of MS^{10} basal supplemented with benzyladenine (BA, 0.5-2mgl^{-1}), gibberelic acid (GA_3, 0.1-1mg^{-1}), indole butyric acid(IBA) or napthalene acetic acid (NAA, 0.05-0.1mgl^{-1}), 1% polyvinylpyrrolidone (PVP), 200 mgl^{-1} casein hydrolysate. After six weeks, buds with small shoots as well as growing shoot apices were transferred to MS basal or MS low salts supplemented with reduced concentration of BA (0.1 mgl^{-1}) to facilitate further growth and elongation. Effect of size of the explants on their establishment were taken into consideration. For multiplication of shoots, various concentrations and combinations of BA (0.5-1.0 mgl^{-1}), Kn (0.25-0.5 mgl^{-1}), GA_3 (0.1-1.0 mgl^{-1}) and IBA (0.01-0.1 mgl^{-1}) were used. The shoots were cut into 0.5-1 cm length and multiplied by the method of enhanced release of axillary buds. The multiplication rate and length of shoots were calculated on the basis of usable shoots per propagule from a total of 5 cultures during the fifth subculture.

To achieve rooting, two-step procedure was used. Microcuttings (4-8) of approximately 3-4 cm long were excised from the shoot clumps and cultured in 4 ml of liquid root induction medium consisting of ½ strength MS, thiamine (0.5 mgl^{-1}), sucrose (20 mgl^{-1}) and IBA (0.5 mgl^{-1}). They were incubated in dark for a few days to initiate roots after which transferred to same medium devoid of IBA and solidified with agar (4 gl^{-1}) for elongation of roots. Rooted cuttings were transferred to paper cups containing peat, which included biological plant protectant (commercial name Ecoderma or Defense SF) and kept under high humidity in glasshouse. The plantlets were irrigated with knop's nutrient solution whenever required. After 1-1½ months, the hardened plants were transplanted to field/ nursery for further evaluation. For each medium preparation, tap water, in place of distilled water, was used.

Results

There was great influence of the size of explants on browning intensity and per cent survival. It was observed that explants of the size 0.2 to 0.4 cm and 0.4 to 0.6 cm (cf. Material and Methods) showed less browning intensity and about 60% explant survival after 6 weeks. They did not exude pigmented substances on transfer from liquid to solid medium. On the other hand, large size (1-2 cm) explants had to be subcultured in fresh medium 3-4 times to overcome pigment exudation. Even then, the maximum explants died due to browning. Only a few buds of the size of 0.6-1.0 cm survived and established initially. When position of explants on the shoot was taken into consideration for their survival and bud break, it was observed that maximum number of the explants from distal part of dormant shoot showed bud break in comparison to basal buds, while in case of actively growing shoots, explants from both distal and basal parts showed almost equal bud break rates.

Growth of shoot apices was seen after 15-20 days in all the 4 combinations used (Table 1, Fig. 1). MS medium supplemented with BAP (1.0 mgl^{-1}), NAA (0.02 mgl^{-1}) and GA_3 (0.1 mgl^{-1}) resulted in 78 per cent survival. On subculture, most of the growing shoot apices either callused or became brown. However, the survived explants reached the size of 0.3-0.5 cm. After 6 weeks of growth, leaf rosettes were developed from shoot apices which were subcultured for their elongation and further growth. The axillary/terminal buds showed growth (Fig.2) within 15 days of culture in all the eleven combinations used, but III and IV combinations (Table 2) induced the maximum percentage of shoot proliferation (80-85%). Very few proliferated shoots showed elongation and development of leaves upon subculture onto basal medium or medium with low salts and low BA (Fig. 3). Between 16 to 84% of the explants either failed to grow further, turned brown and died or were contaminated with some bacteria which were sometimes visible on the micropropagation medium. It was also observed that the growing tips from shoots of MM 111 survived more and established earlier than lateral/terminal buds(Fig.4).

Table 1 —Survival of shoot apices of MM 111 in different concentrations and combinations of growth regulators.

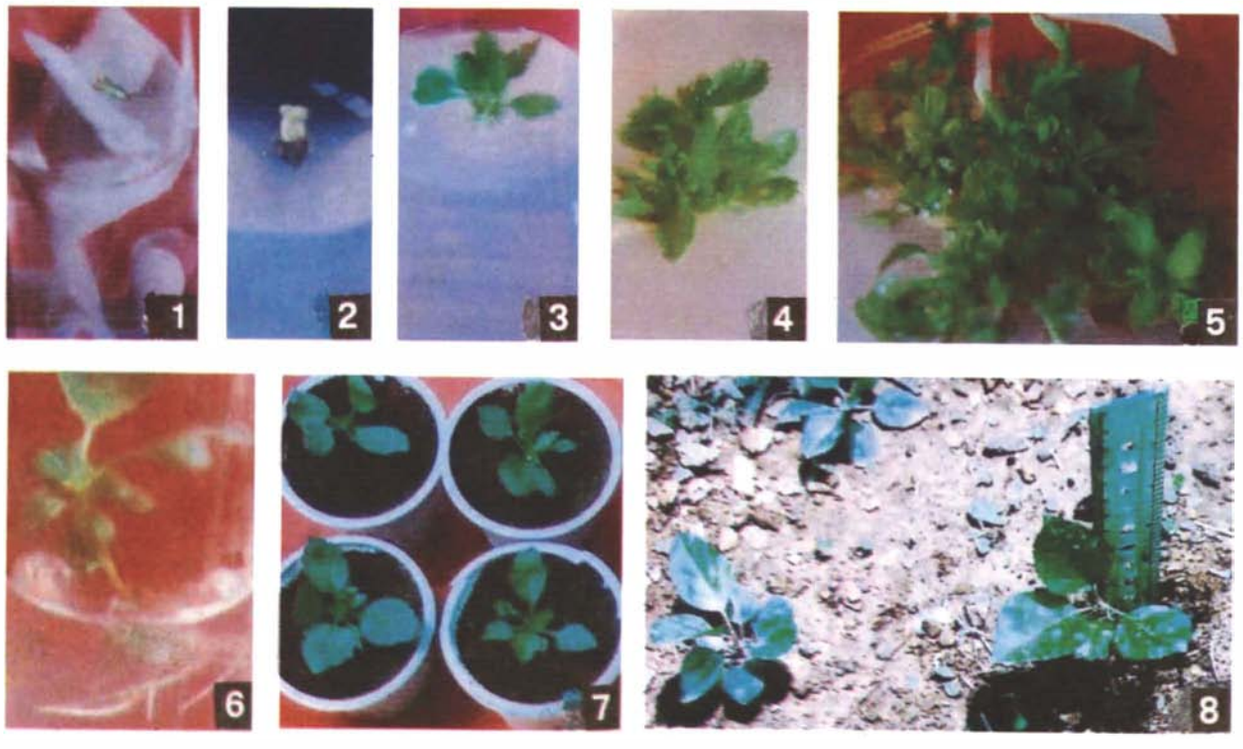
Initiation medium ($mg\ l^{-1}$)	Number of explants cultured	Survival (%)
BA - 1 NAA - 0.02 GA_3 - 0.1	77	78.00 (62.03)
BA - 0.5 IBA - 0.1	14	28.00 (31.95)
BA - 0.2 IBA - 0.02	20	50.00 (45.00)
BA - 1 GA_3 - 0.5 IBA - 0.1	18	35.00 (36.27)

The surviving bud/meristem cultures of MM 111 grew further and were multiplied by monthly subculture. The multiplication rate was lower for the first than for the second and third consecutive subcultures in the same medium. The maximum shoot length i.e. upto 4 cm, showing five fold multiplication

Table 2 —Effects of different combinations and concentrations of growth regulators on bud break

S.No.	Combination and concentration of growth regulators (mg l^{-1})				Total No.of explants cultured	Percent buds showed bud break after 6 weeks
	BAP	NAA	IBA	GA_3		
I	0.5	0.05	-	-	24	16.00 (23.57)
II	0.5	0.01	-	-	90	49.00 (44.43)
III	0.5	-	0.1	-	55	80.00 (63.44)
IV	0.5	-	0.1	0.5	24	41.00 (39.82)
V	1.0	0.1	-	0.5	25	28.00 (31.95)
VI	1.0	-	0.05	0.5	12	84.00 (66.43)
VII	1.0	-	0.1	0.5	24	28.00 (31.95)
VIII	1.0	-	0.5	0.5	24	70.00 (56.79)
IX	1.5	-	0.1	0.5	48	39.00 (38.65)
X	2.0	-	0.1	0.5	24	62.00 (51.95)
XI	2.0	-	0.1	0.5	48	18.00 (25.10)
S.E.					0.81	0.74
$C.D_{0.05}$					1.69	1.62

Values in parentheses are arc sine transformed values



Figs.1-8 —(1)-Shoot apices of MM 111 grown on filter paper bridges in liquid medium after six weeks culture;.(2) —Initiation of shoot growth from cultured explants; (3) —Elongation and development of leaves on medium with low salts & low BA; (4) —Growth of forced axillary shoots of MM 111; (5) —Multiple axillary shoots of MM 111; (6) —Rooted plantlets of MM 111; (7) —Acclimatized plants in peat containing paper cups before transplanting them to field; and (8)—Tissue culture raised plants of MM 111 growing in field.

(5 shoots/ propagule) were obtained in combination of BA (1.0) and GA_3 (0.5 mgl^{-1}) i.e. IX (Table 3, Fig.5) among a total of nine combinations. The addition of kinetin to a combination of BA, GA_3 and IBA neither favoured growth nor multiplication.

About 90% shoots developed root primordia with good shoot growth in liquid medium when kept in dark for 9 days. Roots elongated after transfer to solid medium but callus was found at the cut ends (Fig.6). Rooting percentage and amount of callus decreased with the decrease in treatment time in LM i.e. 8, 7, 6, 5, 4 days (Table 4). However, 80-85% rooting was achieved in 7 and 8 days dark treatment. Callus along with roots did not create much problem because roots emerged directly from the shoot cut ends. It was also observed that shoots with several roots developed *in vitro* established successfully in pots. A success rate of 80% was achieved after transplanting rooted plantlets to small paper cups (Fig.7) and 90-95% in the field (Fig.8). The plantlets attained a height up to 5 cm above soil within one month of transfer.

Discussion

The smaller size explants showed less contamination and more survival as compared to the bigger ones. Moreover, the effect of phenolics can be reduced because small explants produced less phenolics which diffused away quickly to avoid toxic concentrations at the location of the explant. Bigger explants excreted more polyphenolic compounds which prevented their growth. Tissue browning caused failure during micropropagation[11] of the apple rootstock M9. The problem of phenolic exudation has also been faced by other workers [7,12] during the first 2-3 days of culture. In the present studies, establishment of *in vitro* cultures of MM 111 posed considerable problems with high incidence of fungal and bacterial contamination in addition to more intensity of browning, particularly with large s explants.

The explants varied in their pre-disposition to growth, depending on the growth stage of the shoots from which they were collected. Buds which were in active growth, whether distal or basal can b successfully established, while in case of dormant shoots, distal buds were a more favourable source of explants. In this case, BA combined with GA_3 and IBA in the medium was found essential for meristem and axillary bud cultures. These results are in confirmity with those of Arello *et al.*[6] who propagated explants of MM 111 in the presence of GA_3 (0.1 mgl^{-1}), NAA (0.001 - 0.1 mgl^{-1}) and BA (0.5 - 4.0 mgl^{-1}). However, Lane and McDougald[13] have produced shoots of MM111 (5 µm) on BA (1.0 mg/l) only. Slow deterioration of some of the surviving cultures due to internal bacterial contamination even after 4-5 months of subculturing also remained a problem. Similar observation has been made[14] in shoot tip cultures of 'Granny Smith', MM 111, M 7, M 26, M 27, Ottawa 3, Ottawa 7.

Table 4— Effects of dark treatment on rooting of shoots

Days in dark	Number of cuttings/treatment	Rooting percentage	Number of roots/shoot
4	24	40	1-3
5	28	46	1-3
6	40	55	1-5
7	50	80+	1-3
8	52	84+	2-7
9	24	90++	2-12

+ Basal callusing at the cut end.

Table 3 —Effects of different concentrations and combinations of growth regulators on the multiplication of shoots (after 4 weeks of culture)

Sr.No.	MS medium supplemented with ($mg\ l^{-1}$)				Multiplication rate (no.of shoots per shoot)	Length of shoots (cm)
	BAP	GA_3	IBA	Kin		
I	0.5	0.5	0.01	-	1:3	0.5-0.7
II	1.0	1.0	0.05	0.25	1:3	0.5-1.0
III	1.0	1.0	0.05	-	1:3	1.0-2.5
IV		0.5	0.5	0.25	1:2	1.0-1.5
V		0.1	0.5	1.0	1:2	1.0-1.5
VI	1.0	1.0	-	0.5	1:2	0.5-1.0
VII	1.0	0.5	-	0.5	1:2	1.0-2.0
VIII	0.5	0.5	-	-	1:4	1.5-2.0
IX	1.0	0.5	-	-	1:5	1.0-4.0

Better multiplication and growth of shoots were found with BA (1.0 mg l^{-1}) and GA_3 (0.5-1.0 mg l^{-1}) without auxin. It has been found that increasing BA concentration up to 2.0 mgl^{-1} with NAA (0.01 mgl^{-1}) resulted in better multiplication of shoots[6]. For both axillary buds and shoot apices, BA was the most effective cytokinin, but the response was dependent upon the concentrations and combinations used. BA (0.5 and 1.0 mgl^{-1}) found to be optimum along with GA_3 (0.5-1.0 mg/l) in the present study was almost the same that have been found most effective in apple micropropagation by others[2,8,9,15]. Elliot[16] has reported abnormal and elongated leaves with high GA_3 (5-10 mgl^{-1}), while this effect was reduced in low GA_3. Effect of GA_3 can be expected to vary with the composition and type of other growth regulators or with the genotype.

Liquid medium containing IBA for root initation was found better as compared to solid medium used previously in our laboratory. For other rootstocks, it has been reported [17] that by simplifying the medium composition and using liquid rather than agar solidified form, the response of the apple cultivars is improved. Presence of auxin throughout the rooting phase did not prove useful in the present experiment and shoots with root initials were transferred to solidified medium without IBA for elongation of roots. The inhibitory effect of IBA on root elongation is well known[18]. For many of the cultivars, *in vitro* rooting is equally successful in agar solidified and liquid medium alongwith the use of peat plug system for root development[19]. However, Pereira *et al.* [20] have rooted MM 111 shoots in ½ MS and IBA (0.2 mgl^{-1}) and has found good survival rates after transplantation. In the present study also, the plants grew well in peat containing paper cups within 3-4 weeks of transfer. Hardened plantlets (1-1½ months-old) of MM 111 in peat can be successfully established in a single step in the field without much transplanting shock. The acclimatized plants exhibited normal development with no morphological variation. In conclusion, a micropropagation method has been accomplished in MM 111 by induction of axillary shoot proliferation excised from shoot apices/lateral buds by manipulation of growth regulators.

Acknowledgement

The authors wish to thank the Department of Biotechnology, Govt. of India, New Delhi, for financial assistance.

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