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Fast and Efficient *In-vitro* Multiplication of Apple Clonal Root Stock MM-106

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Apple is the major fruit crop of the world and and is vegetatively propagated. Since vegetative propagation of apple through grafting or budding depends on availability of quality root stocks. Generally apple rootstocks are carriers of many viruses which have no control measures after they are infecting the plant. Hence in present study production of virus free clonal root stocks in apple through meristem culture was done. Apple faces many biotic stresses majorly viral diseases. In the present study protocol for micropropagation of apple root stock MM-106 was standardized using meristem as an explant. Different phytohormone combinations were tried to optimize the best combination for development of fast and efficient micropropagation protocol. Meristem was cultured on MS medium supplemented with different concentrations of BAP alone and in combination with IBA, phloroglucinol and GA_3 for initial establishment, multiplication and rooting. Best initial establishment was observed on MS media supplemented with BAP (2 mg/l) + IBA (0.5 mg/l) + Phloroglucinol (100mg/l). Maximum number of shoots (13.0), length of shoots (9.67 cm), leaf length (3.47cm) and leaf number (15.3) was observed on MS media supplemented with BAP (1mg/l) + GA_3 (0.5 mg/l). Maximum number (16.3) and length of roots (10.20 cm) was observed on MS media supplemented with IBA (3 mg/l). Acclimatization of rooted plants was done on moist cotton for 10 -12 days followed by vermiculite:cocopeat for four weeks. About 80% of plants survive after 6 months of transfer to the soil under polyhouse house conditions.

Key words: Apple, micro-propagation, root stocks, meristem, MM-106

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INTRODUCTION

Apple is an important economic fruit crop widely cultivated in temperate and sub-tropical climate. It belongs to the rose family (Rosaceae) of order Rosales and class Agnoliopsida. Trees on MM106 are well anchored, do not sucker, are semidwarfing (60-75% the size of trees on apple seedlings), and very productive. MM106 has many attributes, i.e. good induction of cropping, resistance to woolly apple aphid and intermediate vigor. Apple is conventionally propagated by vegetative methods, such as budding or grafting. These traditional propagation methods do not ensure disease-free and healthy plants, they depend on the season; moreover, they typically result in low multiplication rates. Further if scion wood is taken from healthy disease free mother plants but virus infected rootstocks may lead to disease development. Thus production of virus free quality planting material through in-vitro meristem culture is the best alternative. Large scale production of disease free clonal root stocks will suffice the growing need of planting material in apple. Tissue culture has been extensively used for raising multiple clones of apple rootstocks and raising of virus-free planting material in apple (Dobra and Teixeira 2010). But the multiplication and survival rate of micropropagated plants was low (Sharma *et*

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al. 2000, Amiri *et al.* 2011). Therefore present study focused on the development of efficient and better quality micropropagation protocol with respect to higher multiplication and survival rates of micropropagated plants in apple clonal root stock MM-106.

The objective of the present study was to develop an efficient micropropagation protocol for production of quality planting material in apple root stock MM-106.

MATERIALS AND METHODS

Planting material, media and explants

Planting material for meristem isolation was obtained from green-house of Central Institute of Temperate Horticulture, Rangreth, Srinagar, Jammu and Kashmir. Twigs were taken in the month of Feb. 2010 and kept for sprouting in water treated with 0.1% carbendazim at 25°C in BOD incubator. After 3-4 weeks sprouted buds were used for isolation of meristem. Actively growing shoot tips (5 to 10 mm in length) were cut from shoots. Excised shoot tips were used for isolation of meristem under stereozoom microscope (model-SZX16, make-Olympus). The nutrient media contained inorganic and organic constituents according to (Murashige and Skoog 1962). Phytohormones were added to the Murashige and Skoog medium in different concentrations and combinations. Shoot tips were washed with Tween 80 and then rinsed with distilled water 3-4 times. About 1000 meristem explants were isolated from these shoot tips and surface disinfested with a 0.01% solution of mercuric chloride for 1 minute followed by rinsing with sterilized water for 4-5 times.

***In vitro* shoot multiplication and elongation**

After initial establishment on MS media supplemented with BAP (2 mg/l) + IBA (0.5 mg/l) and PG (100 mg/l), microshoots (>1 cm long) were subcultured in 500 ml jam bottles containing 100 ml MS medium supplemented with different combinations of BAP and GA_3 (Table 1). After 4 weeks observations on shoot and leaf multiplication parameters were recorded. Multiplied shoots were sub-cultured individually on elongation medium and observation on elongation was recorded after 3 weeks.

***In vitro* rooting**

Enlarged micrshoots about 5 cm long, were used for rooting experiment onMS formed after subculturing explants 5 times after every 15 days interval, were cultured in 500 ml jam bottles containing 100 ml MS medium supplemented with different combinations of IBA (Table 2). After 6 weeks observations on root number and length were recorded.

Acclimatization and hardening

Rooted plantlets were removed from media and the agar was washed gently with sterile water. The plantlets were transferred to moist cotton for 10 -12 days followed by vermiculite for four weeks. About 100 plantlets were acclimatized in the laboratory under 16 h light/8 h dark for 3 weeks. The polythene cover was removed after 1 week to reduce relative humidity. Acclimatized plants were transferred to polyhouse and survival percentage under polyhouse conditions was recorded 6 months after transfer.

Statistical analysis

Each treatment was replicated 5 times and observations in stages of development were recorded periodically. The data was analyzed by comparing means using one way ANOVA and the significance was determined by Duncan's Multiple Range Test using SAS (v. 9.2).

RESULTS AND DISCUSSION

Micropropagation of woody and semi-woody trees is reported as problematic and to obtain a material without viruses is a long-term, complex process using traditional methods. Many *in vitro* experiments have been conducted for these economically important fruit trees to overcome the problems of virus and large scale planting material production (Sedlak and Paprstein 2008, Adıyaman *et al.* 2004, Mir *et al.* 2010, Amiri *et al.* 2011).Multiplied shoots were sub-cultured individually on elongation medium and observation on elongation was recorded after 3 weeks).

Culture initiation and shoot multiplication

Meristem cultures were successfully established on MS media supplemented with MS media supplemented with BAP (2 mg/l) + IBA (0.5 mg/l) + Phloroglucinol (100 mg/l). Sharma *et al.* also observed similar media combinations suitable for shoot proliferation in MM106. Very good shoot multiplication rate was observed just after 4 weeks of culture. Shoot multiplication rate (13 shoots) in present study is much higher than reported earlier by Dalal *et al.* (2006) (5 shoots), Mert and Soylu (2010) (8 shoots) and Amiri *et al.* (2011) (5.7 shoots). Thus our results provide much refined and efficient micropropagation

Table 1. Influence of different phytohormone combinations on shoot and leaf development in MM-106 clonal root stock of apple

GA_3 (mg/l)	BA (mg/l)	Shoot length (cm)	Number of shoots	Leaf length	Number of leaves
0.5	0.5	2.73^{g}±0.37	4.33^{de}±0.71	1.53^{ef}±0.18	6.0^{e}±0.5
0.5	0.75	3.30^{egf}±0.21	6.67^{dc}±0.97	1.80^{ed}±0.12	7.0^{ed}±0.5
0.5	1	4.10^{edf}±0.21	10.33^{b}±0.71	2.43^{cb}±0.10	8.3^{ed}±0.3
0.5	1.5	5.87^{cb}±0.46	10.0^{b}±0.47	2.83^{b}±0.07	11.7^{b}±0.7
0.5	**2.0**	**9.67^{a}±0.60**	**13.0^{a}±0.47**	**3.47^{a}±0.12**	**15.3^{a}±0.7**
1	0.1	6.77^{b}±1.03	9.33^{b}±0.71	2.20^{cd}±0.12	11.3^{cb}±0.7
0.1	0.2	5.27^{cd}±0.50	7.00^{c}±0.47	1.73^{e}±0.12	12.0^{b}±0.5
1	0.5	4.30^{ed}±0.25	5.00^{cde}±0.47	1.53^{ef}±0.12	9.3^{cd}±0.7
1	1	3.17^{eg}±0.16	4.67^{cde}±0.71	1.23^{f}±0.12	7.7^{ed}±0.7
1	1.5	2.23^{g}±0.12	3.00^{e}±0.46	1.10^{f}±0.05	7.0^{ed}±0.5

Means followed by the same letter within the columns are not significantly different (P=0.05) using Duncan's multiple range test

protocol for multiplication of MM-106 clonal root stock. Results on shoot elongation were also promising, average shoot length of 9.67 cm was observed which is much better than previous findings of Dalal *et al.* (2006) (1.6 cm). Maximum leaf length (3.47 cm) and leaf number (15.3) was observed on MS media supplemented with BAP (1mg/L) and GA3 (0.5mg/L). Highest number of shoots and maximum length of shoots was also observed on MS media supplemented with BAP (1mg/L) and GA3 (0.5mg/L) (Table 1, Fig. 1). Treatments with high BAP concentrations produced significantly higher multiplication rates than treatments with low BAP concentrations. However, when increasing the concentration of BAP up to 3.0 mg/l (data not shown), shoot number was not significantly enhanced, moreover shoots did not elongate, leaves were very short.

Table 2. Influence of IBA concentration on rooting in MM-106 clonal root stock of apple.

IBA (mg/ l)	Root length (cm)	Number of roots
0.5	2.56^{f}±0.12	3.0d±0.5
1	3.43^{ef}±0.18	4.3d±0.7
1.5	4.10^{e}±0.17	7.0c±0.9
2	5.86^{d}±0.42	9.0c±0.5
2.5	6.93^{cb}±0.42	12.3b±0.7
3.0	**10.20^{a}±0.21**	**16.3a±0.7**
3.5	7.73^{b}±0.42	14.0b±0.5
4	7.83^{b}±0.10	12.0b±0.5
4.5	6.50^{cd}±0.20	9.0c±0.5
5	5.90^{d}±0.05	8.0c±0.5

Means followed by the same letter within the columns are not significantly different (P=0.05) using Duncan's multiple range test

Rooting

Elongated microshoots about 9 cm in length were transferred to test tubes with MS media supplemented with different concentrations of IBA. Rooting ability of the shootlets was about 80% which was parallel to the findings of some other researchers (Aklan *et al.* 1997, Öztürk 2004, Sharma *et al.* 2007, Mert and Soylu 2010). Maximum average number (16.3) and length (10.20 cm) of roots was obtained on MS media containing 3 mg/l of IBA (Table 2, Fig. 1). IBA increased the root length and number when used at low concentrations (0.5-3.0 mg/l), decrease in root length and root number was observed with increase in concentration of IBA from 3.0 to 5.0 mg/l (Table 2). Similar results with respect to root number and length was observed by (Karakurt *et al.* 2009) also observed that high IBA concentration (1000 ppm) was negatively correlated with rooting parameters. Our results on rooting parameters are much superior than reported earlier (Dal *et al*. 2006, Amiri *et al.* 2011). Since both number and length of roots play an important role in final hardening and survival of micropropagated plantlets therefore small and lesser number of roots become limiting factor for final survival of *in-vitro* raised plantlets.

Hardening

Significantly higher survival rate (80%) was observed when rooted plants were hardened in sterilized moist cotton for 10-12 days, transferred to steri-

Fig 1. *In vitro* multiplication of apple clonal root stock MM-106: A. Initial establishment of meristem explants B. Shoot multiplication C. Shoot elongation D. Rooting E. *In-vitro* hardening and F. transfer to polyhouse.

lized vermiculite: cocopeat in plastic pots for four weeks and then to polyhouse for about six months (Fig 1). Modgil *et al.* (2009) observed coco-peat the best medium for hardening of MM-106 rootstock, but in our study survival rate on cocopeat alone was very low (30%). Higher survival rate in present study may be due to long and large number of roots plus modified hardening conditions. Exposure of rooted plants to moist soft cotton during initial stages of hardening helps in initial establishment required for final survival. This study on *in vitro* multiplication of apple root stock MM-106 revealed that large number of high quality planting material of this root stock can be achieved in a very short period of time. The planting material derived through tissue culture techniques using meristem as explant, show uniformity with respect to different quality parameters of the plant. Also the planting material will have the least chances of viral and other disease infestation.

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