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Callus Induction and Plant Regeneration from Leaf Explants of Apple (*Pyrus malus* L.) cv. Golden Delicious

Ravi Shekhar Kumar*, Charu Joshi and Tapan Kumar Nailwal

Plant Tissue Culture and Molecular Biology Laboratory, Department of Biotechnology, Bhimtal-263136, Kumaun University, Nainital, Uttrakhand, India
**Corresponding author*

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A B S T R A C T

The present investigation was conducted to develop a protocol for rapid plant regeneration of apple. The leaf explants of apple (*Pyrus malus* L.) cultivar *viz.* Gold Delicious was cultured for plant regeneration. The explants were cultured on MS media supplemented with different concentrations and combinations of BA with IBA and NAA as well as Kinetin with NAA. Maximum induction (80%) of leaf explants of Golden Delicious was observed in medium containing BA + NAA (1.0+0.5 mg/L). MS medium supplemented with different concentrations and combinations of BA, NAA and KIN were also employed for shoot regeneration. MS medium comprises BA (2.0 mg/L) and NAA (0.02 mg/L) in a particular concentration was best for maximum shoot regeneration from leaf calli whereas MS medium containing BA (2.0 mg/L) + NAA (0.02 mg/L) + GA_3 (0.4 mg/L) was best for maximum shoot elongation. The micro shoots were successfully rooted on half strength MS medium supplemented with IBA (1.0 mg/L) alone and 96.66% rooted plantlets were obtained using the medium. The *in vitro* plantlets were acclimatized in jiffy pots after rooting and established in the soil. The protocol of *in vitro* propagation, presented here is suitable and efficient for cost effective as well as timely production of plantlets for apple (*Pyrus malus*).

Introduction

Apple is a woody plant belonging to the family, *Rosaceae* (Brown, 1992). Apple is very nutritious, aromatic and delicious fruit and a rich source of vitamins A, B and C. It contains about 11% sugar besides essential minerals in amounts. Apples purify the blood by transforming toxic chemicals into less harmful compounds and thereby lower blood cholesterol and different free-radicals as well as beneficial to the lymphatic system (Sabir and Shah, 2004).

It can reduce the risk of prostate, colon and lung cancer and may protect the brain from neurodegenerative diseases (Dobrzanski *et al.*, 2006). Apple fruit is very good source of important phytochemicals like antioxidants, flavonoids and other free phenolics which are not bound to other compounds (Boyer and Liu, 2004). These days, apples are growing all over the world. Apples are the second most important temperate fruit crop next to grapes and fourth among all fruit

species following oranges, bananas and grapes regarding world fruit production (Jackson and Palmer, 1999).

Apple is conventionally propagated by vegetative methods, such as budding or grafting. Although 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. Micropropagation of apple rootstocks has opened up new areas of research and fruit tree propagation allowing the problems of conventional methods to be overcome and enabling rapid multiplication of disease-free fruit plants at a commercial scale **(**Bahmani *et al.*, 2009; Zimmerman and Debergh, 1991).

Tissue culture propagation is a method to produce own rooted plants because it has the potential for large scale increase of plants in short time. Clonal propagation through tissue culture called micropropagation can be achieved in a short time and space. Thus, it is possible to produce plants in large numbers starting from single individual (Razdan, 1993). Micropropagation is a widely known method for plant multiplication and has been extensively used for the rapid multiplication of many plant species along with *Pyrus* species (Lane, 1992). Mature woody plants are more difficult to propagate vegetatively (Druart, 2003) but different techniques were developed for their *in vitro* micropropagation (Sharma *et al.*, 2000). In the present study, authors have developed an efficient plant regeneration protocol of Apple (*Pyrus malus* L.) cv. Gold Delicious from green leaf explants.

Materials and Methods

Plant Materials and Explants Preparation

Fresh young leaves of Apple (*Pyrus malus* L.) cv. Gold delicious were obtained from CISH (Central institute of Subtropical Horticulture) Mukteshwar, Uttarakhand, India. The explants were washed thoroughly under running tap water for 5 min, then immersed in water containing 3-5 drop of surfactant (Tween-20) for 5-10 min in gentle agitating condition and rinsed thoroughly. Leaves were treated with 0.5% fungicide (bavistine) solution for 20 min and were thoroughly washed with sterilized double distilled water under aseptic condition followed by 0.1% surface disinfectant (mercuric chloride, $HgCl_2$) treatment for 5 min. Finally, leaves were washed with sterile double distilled water for 4-6 times. Water present on the surface of the leaves was dried out with the help of autoclaved filter papers.

Culture Medium and Conditions

The MS cultured medium (Murashige and Skoog, 1962) was used with 3% (w/v) Sucrose (Hi-media, Mumbai, IN). The medium was supplemented with different concentrations and combinations of N^{6-} benzyladenine (BA), Kinetin (Kn), Indole butyric acid (IBA) and α-napthelene acetic acid (NAA) (Hi-media, Mumbai, IN). The pH of medium was adjusted to 5.8 prior to gelling it with 0.3% (w/v) CleriGel (Hi-media, Mumbai, IN). All the media culture bottles containing 50 ml MS media were autoclaved at 15 lbs for 20 min at liquid cycles.

Shoot Induction from Leaf Derived Callus

Sterile leaves were cut into 5×5 mm pieces and inoculated aseptically in culture bottles containing 50 ml MS media supplemented with various PGRs like BA (0.5-1.25 mg/L), NAA (0.4-1.25 mg/L), IBA (0.0-0.5 mg/L), and Kinetin (1.0-2.0 mg/L) in combinations (Table 1) for callus induction. Sub-culturing

was done in fresh medium at regular intervals for continuous growth. Data were recorded after 40 days of inoculation. For shoot induction from leaf derived callus, 45 days old callus was transferred to MS medium containing BA (0.75-3.0 mg/L), NAA (0.01-0.03 mg/L) and Kinetin (1.0-3.0 mg/L) in different combinations and concentrations (Table 2).

Shoot Multiplication and Elongation

The microshoots derived from callus were further used for multiplication and elongation so they were inoculated on MS medium having GA_3 (0.03-0.04 mg/L), BA (1.0-3.0 mg/L), NAA (0.01-0.04 mg/L) and Kinetin (1.0-3.0 mg/L) in different combination and concentrations (Table 3).

Root Induction and Acclimatization

Elongated shoots with 6-7 leaves (>5 cm in length) were excised *in vitro* from all treatments and were transferred to half-strength MS medium with 2% sucrose (pH 5.8) and various concentrations of IBA (0.5-2.5 mg/ L) (Table 4) in combination and incubated for overnight. Later, the treated shoots were grown on PGR free half-strength MS medium. Each treatment was replicated 5 times using single plantlet for each treatment. After 6 weeks of root induction plantlets were taken out from culture bottle and gently washed under running water. They were potted in thermacol cups (8×12 cm) containing sterile soil and farmyard manure (3:1), covered with plastic bags having small holes to maintain humidity and finally placed inside culture room at 25 $\pm$ 2 °C (16 hours photoperiod). After 15 days, covers were removed, maintained as such for 3 weeks and then transferred into earthen pots (15 × 20 cm) and kept inside greenhouse for further growth (Figure 1). Well hardened plants were taken out to the direct sunlight. Data for per cent rooting, average number of roots per shoot, average root length and average length of longest root were recorded after 6 weeks of inoculation in PGR-free half strength MS medium.

Results and Discussion

Shoot Induction from Callus

The response of leaf explants to various concentrations and combination of BA, IBA, NAA and Kinetin in MS culture medium was studied. The regeneration frequency of callus was the highest (86.66 %) on MS solid culture medium supplemented with 1.0 mg/L BA and 0.5 mg/L NAA (Table 1). The highest frequency was observed when leaves were cut-wounded and placed with the abaxial surface in contact with the medium. The results were nearly 3-fold better when leaves were cut instead of crushing.

The effect of the callus on MS solid culture medium supplemented with BA (2.0 mg/ L) and NAA (0.02 mg/L) was also studied. The results showed that the highest regeneration frequency of shoot induction (85.90 %) and the maximum number of shoots (8.0) were obtained when callus were placed horizontally in contact with the medium (Table 2).

Shoot Multiplication and Elongation

When shoots, excised from multiple shoot cultures, were placed vertically, multiplication was seen in all the combinations tested (Table 3). Highest multiplication and elongation rate (20-fold) with 9-10 cm long shoots was obtained on medium having 0.04 mg/L GA_3, 2.0 mg/L BA, and 0.02 mg/L NAA (Figure 1). From each explant 12-13 shoots were obtained on 1.5 mg/L BA, 0.01 mg/L NAA, and 0.03

mg/L GA_3, where some shoots was vitrified. Multiplication rate of 15-fold and longer shoots were achieved when 2.5 mg/L BA with 0.02 mg/L NAA, and 0.04 mg/L GA_3. The combinations of 0.03 mg/L GA_3, 1.0 mg/L Kn, and 0.01 mg/L NAA didn't showed any increase in multiplication and elongation of shoots.

Root Induction and Hardening

Roots were observed from regenerated shoots after 5 weeks of *in vitro* culture. The percentage of *in vitro* shoots that rooted varied significantly according to the IBA concentration of the medium, with the best rooting (96.66 %) obtained with 1.0 mg/L IBA (Table 4). In the present study IBA (1.0 mg/L) was effective for induction of roots. In this concentration, the roots reached maximum length of 10.0±0.57 cm within three weeks of culture (Figure 1). The increased root length leads to increase in the survival percentage of hardened and field grown plants. As the concentration of IBA increased the percentage of rooting was decreasing. The highest numbers of roots (Figure 1) were obtained on the MS half medium supplemented with 1.0 mg/L IBA. After three weeks, rooted plantlets were transferred into plastic pots containing autoclaved sand and soil (3:1) mixture and were maintained in the culture room for two weeks (Figure 1). They were then transferred to shade and ultimately to field conditions. The survival rate of plantlets was 80±5 % in the field.

Table.1 Effect of Different Combinations of Ba, Naa And Kinetin on Callus Induction of Apple (Golden Delicious) on Ms Solid Medium

PGR conc. (mg/L)	Days required for response	% of Response
BA+NAA +(0.0+0.0)	0.000±0.000 [a]	0.000±0.000 [a]
BA+NAA+ (0.5+0.4)	0.000±0.000 [a]	0.000±0.000 [a]
BA+NAA+ (1.0+0.5)	**6.000±0.577 [b]**	**86.667±3.333 [e]**
BA+NAA+(1.0+0.75)	10.333±1.453 [cde]	70.000±5.774 [cd]
BA+NAA+(1.5+1.0)	11.000±2.082 [def]	43.333±8.819 [b]
BA+IBA(0.75+0.5)	21.667±0.882 [h]	73.333±3.333 [cde]
BA+IBA+(1.0+0.5)	6.333±0.333 [b]	76.667±3.333 [de]
BA+IBA (1.25+0.5)	8.000±0.577 [bc]	66.667±8.819 [cd]
Kn+NAA+ (1.0+0.75)	13.667±0.882 [fg]	63.333±6.667 [cd]
Kn+NAA+(1.25+0.75)	9.333±0.333 [cd]	70.000±5.774 [cd]
Kn+NAA+(1.5+0.75)	11.333±0.882 [def]	60.000±0.000 [c]
Kn+NAA+(1.5+1.0)	12.667±0.882 [efg]	66.667±3.333 [cd]
Kn+NAA+(2.0+1.0)	15.000±0.577 [g]	63.333±3.333 [cd]
Kn+NAA+(2.0+1.25)	24.000±0.577 [h]	43.333±3.333 [b]

Note: The values represent the means (±SE) of three independent experiments. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P< 0.05$).

Table.2 Effect of Different Combinations of Ba, Naa And Kinetin on Shoot Induction from Leaf Derived Calli of Apple (Golden Delicious) on Ms Solid Medium

PGR conc. (mg/L)	Average no. of shoots	Mean Average shoot length (cm)	Average number of leaves
MS Full BA+NAA (0.0+0.0)	0.000±0.000 [a]	0.000±0.000 [a]	0.000±0.000 [a]
MS Full BA+NAA (0.75+0.01)	1.000±0.577 [a]	2.333±1.202 [b]	5.667±3.480 [a]
MS Full BA+NAA (1.0+0.01)	2.667±0.333 [b]	4.333±0.333 [bc]	18.333±2.667 [b]
MS Full BA+NAA (1.5+0.01)	6.333±0.333 [de]	7.000±0.577 [de]	40.000±4.509 [d]
MS Full BA+NAA (2.0.+0.02)	**8.000±0.577 [e]**	**8.667±0.882 [e]**	**57.333±5.239 [e]**
MS Full BA+NAA (2.5+0.03)	3.667±1.202 [bc]	6.000±0.577 [cd]	18.000±5.508 [b]
MS Full BA+NAA (3.0+0.03)	3.667±0.667 [bc]	5.667±0.882 [cd]	16.333±2.028 [b]
MS Full Kn+NAA (1.0+0.01)	5.333±0.333 [cd]	6.667±0.667 [cde]	24.333±2.848 [bc]
MS Full Kn+NAA (1.5+0.02)	6.000±0.577 [d]	5.000±0.577 [cd]	29.667±2.848 [c]
MS Full Kn+NAA (2.0+0.02)	6.333±0.333 [de]	5.333±0.882 [cd]	25.333±1.764 [bc]
MS Full Kn+NAA (2.5+0.02)	6.333±0.667 [de]	4.333±0.882 [bc]	24.333±1.667 [bc]
MS Full Kn+NAA (3.0+0.02)	5.333±0.333 [cd]	5.667±0.667 [cd]	25.333±1.333 [bc]
Note: The values represent the means (±SE) of three independent experiments. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (P< 0.05).			

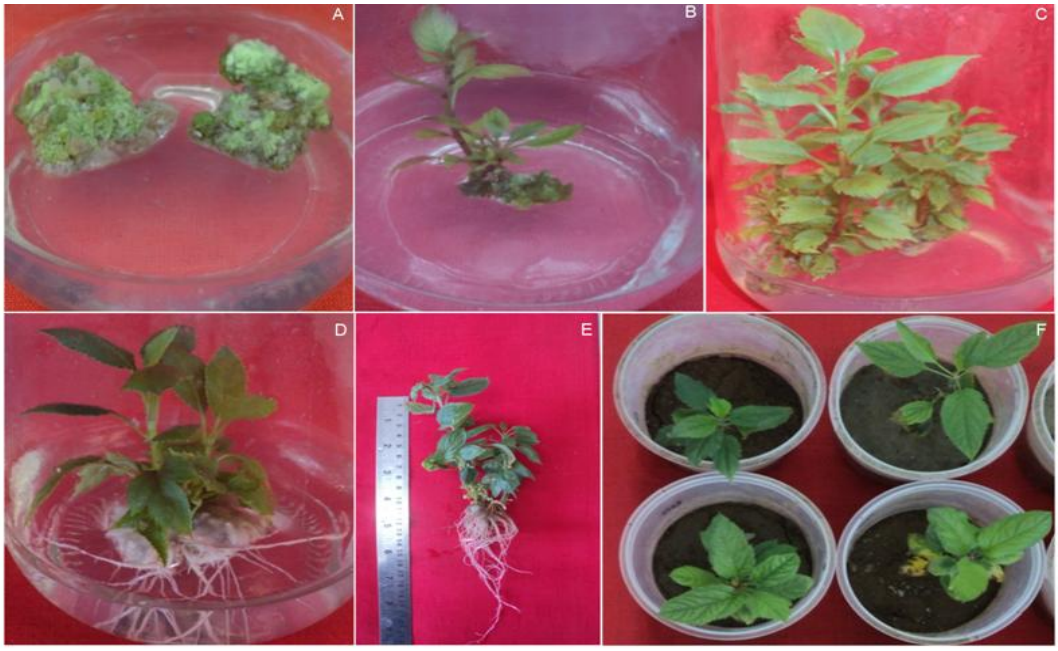
Table.3 Effect of Different Combinations of Ba, Naa And Ga3 on Shoot Multiplication and Elongation of Apple (Golden Delicious) on Ms Solid Medium

PGR conc. (mg/L)	Average no. of shoots	Average shoot length (cm)	Average number of leaves
BA+NAA++GA_3(0.0+0.0+0.0)	0.000±0.000 [a]	0.000±0.000 [a]	0.000±0.000 [a]
BA+NAA+GA_3(1.0+0.01+0.03)	6.667±0.333 [b]	6.333±0.333 [bc]	60.000±1.732 [b]
BA+NAA+GA_3(1.5+0.01+0.03)	14.333±1.202 [d]	7.333±0.333 [c]	106.667±8.819 [d]
BA+NAA+GA_3(2.0+0.02+0.04)	**19.667±0.882 [e]**	**9.000±0.577 [d]**	**139.333±1.764 [e]**
BA+NAA+GA_3(2.5+0.02+0.04)	14.667±0.882 [d]	6.333±0.333 [bc]	116.667±9.955 [d]
BA+NAA+GA_3(3.0+0.03+0.04)	8.333±0.882 [bc]	5.333±0.333 [b]	70.667±4.702 [bc]
Kn+NAA+GA3(1.0+0.01+0.03)	10.000±1.155 [c]	5.333±0.667 [b]	84.000±3.055 [c]
Kn+NAA+GA3(1.5.0+0.01+0.03)	8.667±0.882 [bc]	5.000±0.577 [b]	68.667±2.963 [bc]
Kn+NAA+GA3(2.0+0.02+0.03)	9.667±0.333 [c]	6.667±0.333 [bc]	65.000±2.887 [b]
Kn+NAA+GA3(3.0+0.04+0.04)	7.667±0.333 [bc]	6.000±1.155 [bc]	69.667±2.603 [bc]
Note: The values represent the means (±SE) of three independent experiments. At least 24 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (P< 0.05).			

Table.4 Effect of Iba on Root Regeneration of Apple (Golden Delicious) on Ms Solid Medium.

PGR conc. (mg/L)	Average no. of root	Average root length (cm)	% of Response
MS Full IBA(0.0)	0.000±0.000 [a]	0.000±0.000 [a]	0.000±0.000 [a]
MS Full IBA (0.5)	4.667±0.333 [bcd]	5.000±0.577 [bc]	43.333±3.333 [c]
MS Full IBA(1.0)	6.000±0.577 [de]	4.333±0.333 [b]	66.667±3.333 [de]
MS Full IBA (1.5)	3.667±0.333 [b]	5.333±0.667 [bcd]	66.667±3.333 [e]
MS Full IBA(2.0)	5.667±0.333 [cde]	4.667±0.667 [b]	33.333±3.333 [b]
MS Half IBA(0.5)	8.333±0.882 [g]	6.667±0.333 [d]	83.333±3.333 [f]
MS Half IBA(1.0)	**11.000±0.577 [h]**	**10.000±0.577 [e]**	**96.667±3.333 [g]**
MS Half IBA(1.5)	8.000±0.577 [fg]	6.333±0.333 [cd]	83.333±3.333 [f]
MS Half IBA(2.0)	6.667±0.333 [ef]	4.333±0.333 [b]	53.333±3.333 [d]
MS Half IBA(2.5)	4.333±0.333 [bc]	5.000±0.577 [bc]	46.667±3.333 [cd]
Note: The values represent the means (±SE) of three independent experiments. At least 24 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (P< 0.05).			

Figure.1 A. Initiation of Callus with 1.0 mg/l ba+0.5 mg/l naa; b. Shoot Proliferation from 45 Days Old Callus in ms Medium Supplemented with 2.0 mg/l ba+ 0.02 mg/l naa; c. Shoot Elongation and Multiplication 2.0 mg/l ba+0.02 mg/l naa+ 0.4 mg/l ga3;d. Rooted Plant in ms Half Medium Supplemented with 1 mg/l Indole-3 Butyric Acid (iba); e. Rooted Plant (35 days old);f. Hardened Plants in Greenhouse



For callus induction, NAA (0.4-1.0 mg/L) in combination with BA (0.5-1.25 mg/L) was employed. NAA and 2, 4-D are commonly used with BA for callus induction in various systems (Dhar and Joshi, 2005; Maheshwari and Kumar, 2006). Well-developed shoots were rooted on half MS medium fortified with two auxins (IBA and NAA; 2-8 mg/L). IBA supplemented medium exhibited superior rooting than NAA. IBA is considered as the most effective growth regulator for induction of roots in legumes (Ozean *et al.*, 1992). IBA stimulated rooting was observed in *Vigna radiata* (Husan and Siddquai, 2006), *Aegle marmelos* (Nayak *et al.*, 2007), *Clitoria ternatea* (Barik *et al.*, 2007) and *Cotinus coggygria* (Metivier *et al.*, 2007). During root induction, IBA treatment alone showed superior response for induction of roots. Similar to our reports, influence of IBA in root induction has been reported for several plants including *Hemidesmus indicus* (Sreekumar *et al.*, 2000) and *Cunila galiodes* (Fracaro and Echeverrigary, 2001).

Various studies were conducted to develop protocols for efficient micropropagation and regeneration of apple cultivars. Al-Maarri et al (1994) indicated that *Pyrus commuunis* cultivars established best on medium containing 2.0 mg/L BAP, 0.1 mg/L BA and 0.2 mg/L GA_3. Somatic embryos were produced from leaf explants of Golden Delicious cultured on MS media with B_5 10.0 mg/L BA and 3.0 mg/L NAA in the dark (Liu *et al.*, 1983). Compton and Gray (1993) observed that addition of IAA to the medium with BA increased callus formation. Lane (1982) reported that BA at concentration of 0.87 mg /L was required for establishment of shoot meristems of Macspur and Harrold and Red apple. During studies Belaizi et al (1989) depicted that 0.085 mg/L IBA and 0.035 mg/L BA gave better results for establishment during preliminary growth phase for apical buds. Regeneration of adventitious shoots has been obtained from *in vitro* leaves of apple cultivars with vitamins, BA and IBA (Fasolo *et al.*, 1990). However, BAP 0.77 mg/L in combination with the TIBA 0.17 mg/L gave the highest percentage of regeneration (Belaizi *et al.*, 1990).

In conclusion, the present protocol is efficient and simple for *in vitro* adventitious shoot multiplication from callus cultures, and whole plant regeneration of Apple (*Pyrus malus* L.). Plant regeneration protocol can be potentially utilized for *ex-situ* conservation and mass propagation of Apple (*Pyrus malus* L.) The protocol was standardised by some manipulations of different PGRs, amino acids and carbohydrates for enhanced multiplication. Clones to meet the growing demand of energy plantations as well as need of food industry for food purpose. Future studies will focus on comparison between *in vitro* grown and field-grown plants in terms of their seed. Protocol explained in this research paper provides a rapid plant regeneration system which could be used for the soma clonal variation induction.

Abbreviation

Kn, Kinetin; MS, Murashige T & Skoog F (1962); BA, 6-Benzyladenine; NAA, α-Naphthalene-acetic acid; GA_3, Gibberilic acid.

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