

Direct Organogenesis and Plantlet Multiplication from Leaf Explants of *in Vitro*-Grown Shoots of Apple (*Malus domestica* Borkh.) cv. 'Golden Delicious' and 'MM111' Rootstock

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ABSTRACT

The aim of the present study was to develop an efficient direct shoot formation system for apple (*Malus domestica* Borkh.) cv. 'Golden Delicious' and 'MM111' rootstock as a prerequisite for genetic transformation with antifungal genes and also as a method for rapid clonal multiplication. Adventitious shoot formation from leaf pieces of 'Golden Delicious' and 'MM111' was achieved using leaves from *in vitro*-grown shoots. Optimum conditions for 'direct' shoot organogenesis resulted in 92 and 90% of the explants producing one or more shoot per explant with high regeneration rate of 4 and 4.1 in 'Golden Delicious' and 'MM111', respectively on MS basal medium containing 1.0 g/l MES (morpholino ethanesulfonic acid), 2.0 mg/l TDZ, with 0.2 mg/l NAA. Organogenesis did not occur on media without cytokinins. The organogenic capacity of leaf pieces was dependent on the leaf maturity and the origin of the leaf piece with the youngest light green expanding leaves being more regenerative than the older ones. Middle leaf segments were more responsive than the upper or lower part of the leaf. Adventitious shoots originated from both cut areas and from surfaces of the wounded leaf explants. Shoot multiplication was achieved on media consisting of MS media supplemented with B5 vitamins, 1.0 g/l MES, 30 g/l sucrose, 1 mg/l BAP, 0.3 mg/l IBA, 0.2 mg/l GA₃ and 6 g/l agar and were subcultured every 4 weeks. *In vitro* rooting was achieved easily by transferring 2-3 cm long shoot tips to rooting ½ MS basal medium supplemented with 1.0 mg/l indole-3-butyric acid (IBA). Multiplied plants were successfully acclimatized and cultivated in the field under natural conditions to evaluate their phenotypic uniformity and field performance.

Keywords: adventitious, BAP, cytokinins, MES, shoot formation, TDZ

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, N⁶-benzylamino-purine; MES, 2-(N-morpholino) ethanesulfonic acid; MS, Murashige and Skoog medium (1962); NAA, α -naphthalene acetic acid; N6 medium (Chu *et al.* 1975); TDZ, thidiazuron (N-phenyl-N-1,2,3-thiadiazol-5-yl urea)

INTRODUCTION

Successful utilization of biotechnology for plant improvement through genetic transformation requires the development of an efficient and reliable *in vitro* shoot regeneration system from cultured cells or tissues. The availability of such systems for regeneration of plants through adventitious shoot formation is a prerequisite for the application of genetic engineering allowing the alteration of a few traits of existing superior fruit cultivars, can considerably accelerate the obtaining of genetically improved cultivars in comparison with traditional breeding in fruit trees.

Adventitious shoot proliferation from leaf blades has been demonstrated to be a highly productive regeneration method in apple cultivars and a few clonal rootstocks (Liu *et al.* 1983; Ancherani *et al.* 1990; Durfor 1990; Korban *et al.* 1992; Famiani *et al.* 1994; Yepes and Aldwinckle 1994; Caboni *et al.* 1996; Ferradini *et al.* 1996; Modgil *et al.* 1999; Boni *et al.* 2000; Sicurani *et al.* 2001; Wilson and James 2003). Also, transgenic apples have been regenerated from leaf discs (James *et al.* 1993; Maheswaren *et al.* 1992; Trifonova *et al.* 1994; Sriskandarajah *et al.* 1994; Yao *et al.* 1995; Puit and Schaart 1996; De Bondt *et al.* 1996; Norelli *et al.* 1996, 1999; Abdul Kader *et al.* 1999; Szankowski *et al.* 2001; McAdam-O Connell *et al.* 2004; Welander *et al.* 2004; Malony *et al.* 2008). However, it is a prerequisite for a successful transformation that efficient regeneration systems be worked out.

We set out to optimize the direct organogenesis system using leaf pieces as explants for the widely grown apple cv. 'Golden Delicious' and 'MM111' rootstock, so that tissue-cultured plantlets can be efficiently produced for rapid clonal multiplication and also to be used as a regeneration system for genetic transformation afterwards with *g2ps1* and chitinase genes to confer them fungal resistance.

MATERIALS AND METHODS

Plant material

Shoot cultures used in the present study were obtained from *in vitro* proliferating shoots of cv. 'Golden Delicious' and 'MM111' rootstock maintained at the Department of Biotechnology, GCSAR and subcultured every 4 weeks on proliferation media for three years (Alrihani *et al.* 2008; Altinawi *et al.* 2009). For induction of organogenesis, five shoots per 250 ml glass vessels containing 50 ml of MS medium supplemented with 1.0 mg/l BAP, 0.3 mg/l IBA and 0.2 mg/l GA₃ were subcultured every three weeks for three subcultures until having enough suitable leaf material for starting experiments for direct shoot formation using leaf explants. The shoot cultures were maintained in a growth room at 25 ± 1°C and a 16-h photoperiod provided by Philips fluorescent lamps giving average light intensity of ca. 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photon flux at the surface of culture vessels.

Table 1 Media used for adventitious shoot formation in apple using leaf pieces as explants.

Medium	Medium composition
MS=R0	MS + B5 Vit.+ 1 g/l MES + 2.5 g/l Gelrite + 30 g/l sucrose (control medium)
RO1	R0 + 2.0 mg/l TDZ + 0.2 mg/l NAA
RO2	R0+ 5.0 mg/l BAP + 0.2 mg/l NAA
RO2*	R0 with 30 g/l sorbitol instead of sucrose + 5.0 mg/l BAP + 0.2 mg/l NAA
RO2**	MS + B5 Vit.+ 1 g/l MES+ 5.0 mg/l BAP+0.2 mg/l NAA+0.625 g/l Gelrite + 5.25 g/l agar + 30 g/l sucrose
RO3	MS + 5.0 mg/l BAP + 0.2 mg/l 2,4-D + 2.5 g/l Gelrite + 30 g/l sucrose
RO4	N6 macroelements + MS microelements + B5 Vit. + 1 g/l MES + 5.0 mg/l BAP + 0.2 mg/l NAA +2.5 g/l Gelrite + 30 g/l sucrose
R05	MS + B5 Vit.+ 1 g/l MES + 0.5 mg/l TDZ + 0.5 mg/l BAP + 0.2 mg/l NAA + 2.5 g/l Gelrite + 30 g/l sucrose

Adventitious shoot formation

At the end of the third subculture, the first 3-4 actively expanding apical youngest leaves, that showed no signs of chlorosis with light green colour and strong vein pattern on back of the leaf on the shoot apex were harvested from 3-weeks old proliferating cultures, wounded with non-traumatic forceps (BDR No.157, Aesculap, Tuttingen, Germany), and then cut into three parts (upper, middle and lower). Leaf pieces were then placed in a moistened chamber until cultured on different regeneration media which consisted of Murashige and Skoog's (MS; 1962) inorganic salts, supplemented with 100 mg/l *myo*-inositol, B5 vitamins, 1 g/l MES (2-morpholino ethanesulfonic acid (Applichem GmbH, Germany) as buffering agent, 3% sucrose and solidified with 2.5 g/l gelrite (Gellan Gum, Sigma) and referred to as basal medium. Eight combinations of supplements to the basic medium for efficacy in induction of organogenesis were tested (Table 1). All media were adjusted to pH 5.7 with 1 N KOH or 1 N HCl prior to autoclaving at 121°C, 1.4 kg/cm² for 20 min.

Culture conditions

Eight leaf sections from each leaf part with five replications each, were cultured with the adaxial face in contact with the medium in 90 mm-diameter plastic Petri dishes containing 20 ml of different media (Table 1) for direct shoot formation (Fig. 1). Leaf explants were treated with non-traumatic forceps to induce light wounding. Cultures were incubated in full darkness for an initial 3 weeks. Cultures were then transferred to distributed fluorescent light for further one week, where after they were transferred to conditions of a growth room at 25 ± 1°C with a 16-h photoperiod at 50 µmol m⁻²s⁻¹ photon flux to assess shoot organogenic responses.

Shoot multiplication, rooting and acclimatization

Regenerated shoots were excised and transferred to proliferation media with 1 mg/l BAP, 0.3 mg/l IBA and 0.2 mg/l GA₃. Subcultures were done every 4 weeks.

For rooting, shoot tips of 2-3 cm long were transferred to rooting media with half-strength MS basal medium supplemented with 1 mg/l IBA.

Rooted plantlets were then acclimatized gradually to field conditions. Routine procedures for the multiplication, rooting and acclimatization were carried out as previously described (Alrihani *et al.* 2008; Altinawi *et al.* 2009).

Experimental design and scoring

After 8 weeks on culture media, effects on the percentage of responsive explants and the number of shoots/explant were evaluated.

Each Petri dish was a repetition in a randomized block experimental design, in which the three different explants were compared. For each treatment (explant type), 40 explants per each leaf part were used with 8 explants/plate and 5 replications. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple range test using MSTAT-C computer programme. All experiments were repeated three times.

RESULTS AND DISCUSSION

Effects of different leaf explants and growth regulators on direct organogenic ability

We have developed highly efficient systems for rapid clonal multiplication and direct shoot organogenesis from cultured leaf explants which can be used for the genetic transformation of apple cv. 'Golden Delicious' and 'MM111' rootstock.

Adventitious shoots originated directly along the cut basal edges of the explants and were clearly visible after four to eight weeks culture. Considering both percentage of explants producing shoots and the number of shoots/explant, the best shoot multiplication was achieved on media supplemented with 2.0 mg/l TDZ and 0.2 mg/l NAA, where high regeneration frequency (92 and 90%) was obtained with regeneration rate of 4 shoots/explant. Significant reductions in shoot regeneration were observed when TDZ levels were lowered to 0.5 mg/l, but with the addition of 2.5 mg/l BAP which resulted in a regeneration rate up to 82 and 79% for 'Golden Delicious' and 'MM111', respectively (Tables 2, 3; Fig. 1).

Mature leaf explants displayed low capacity for shoot organogenesis, while the youngest light green leaves, incu-

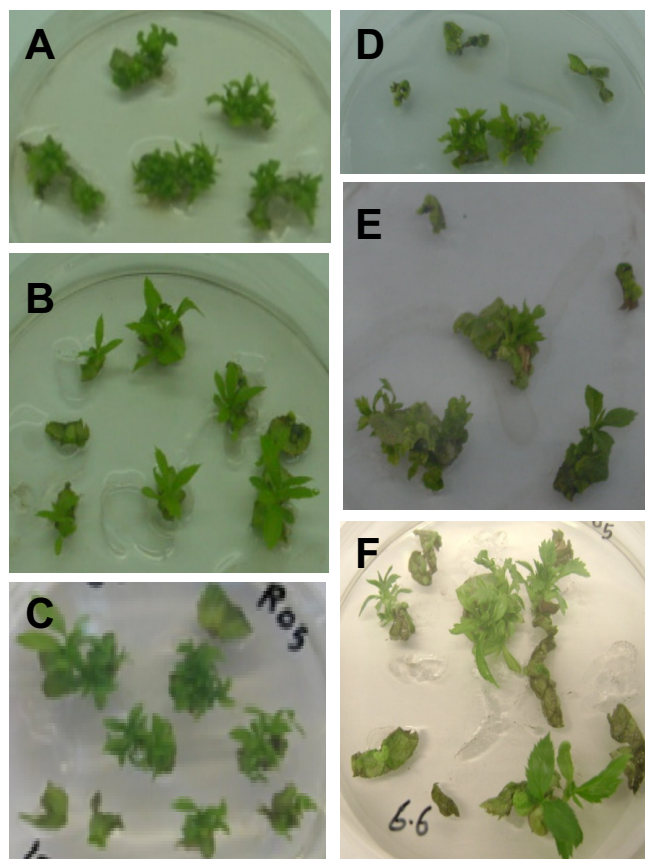


Fig. 1 Adventitious shoot formation from *in vitro* cultured leaves of 'Golden Delicious' (left) (G.D.) and 'MM111' (right) on different media containing TDZ or BAP, 8 weeks after culture initiation. (A, D) R01, (B, E) R02, (C, F) R05.

Table 2 Mean number of adventitious shoots formed *in vitro* from leaves of apple cv. ‘Golden Delicious’ (GD) and ‘MM111’ rootstock, in relation to the explant type on media used.

Explant	R01		R02		R04		R05	
	GD	MM111	GD	MM111	GD	MM111	GD	MM111
Lower part of leaf	2.275 ± 0.129 b	2.350 ± 0.092 b	1.825 ± 0.061 b	1.40 ± 0.078 b	1.00 ± 0.00 b	1.150 ± 0.057 b	2.125 ± 0.114 b	1.7875 ± 0.067 b
Middle part of leaf	4.025 ± 0.141 a	4.075 ± 0.173 a	2.225 ± 0.067 a	2.500 ± 0.080 a	1.52 ± 0.080 a	1.650 ± 0.076 a	2.525 ± 0.124 a	2.825 ± 0.101 a
Upper part of leaf	1.60 ± 0.10 c	1.775 ± 0.06 c	1.00 ± 0.00 c	1.00 ± 0.00 c	1.00 ± 0.00 c	1.0 ± 0.00 c	1.40 ± 0.086 c	1.350 ± 0.081 c
LSD 0.05	0.366	0.280	0.175	0.226	0.130	0.130	0.321	0.248

Data represent mean of 40 replicates ± SE after 2 months on experimental media.

Values within each column followed by different letters are significantly different at the 0.05 probability level ($\alpha < 0.05\%$) using Duncan’s multiple range test.

Table 3 Organogenesis (Regeneration %) *in vitro* from leaves of apple cv. ‘Golden Delicious’ (G.D.) and ‘MM111’ rootstock, in relation to the explant type on media used.

Explant	R01		R02		R04		R05		R0	
	GD	MM111	GD	MM111	GD	MM111	GD	MM111	GD	MM111
Lower part of leaf	84 ± 0.138 b	80 ± 0.082 b	8 ± 0.07 b	9 ± 0.08 b	15 ± 0.10 b	7 ± 0.057 b	20 ± 0.114 b	20 ± 0.066 b	0	0
Middle part of leaf	92 ± 0.143 a	90 ± 0.21 a	20 ± 0.02 a	18 ± 0.08 b	36 ± 0.09 a	10 ± 0.080 a	82 ± 0.123 a	79 ± 0.012 a	0	0
Upper part of leaf	40 ± 0.20 c	58 ± 0.07 c	2 ± 0.10 c	2 ± 0.00 c	10 ± 0.00 c	1 ± 0.08 c	18 ± 0.09 c	10 ± 0.09 c	0	0
LSD 0.05	0.430	0.280	0.92	0.169	0.22	0.420	0.332	0.21		

Values within each column followed by different letters are significantly different at the 0.05 probability level ($\alpha < 0.05\%$) using Duncan’s multiple range test.

Values are means of 300 explants each.

bated on organogenic induction medium, regenerated with an efficiency of up to 92%. Under these conditions, leaf explants showed an overall expansion with an about 4-fold increase within 4-8 weeks of culture (Table 2, 3).

On control medium (RO) free of plant growth regulators (PGRs), shoot organogenesis was not observed.

Dufour (1990) obtained improved yield in *in vitro* adventitious regeneration in apple cultivars ‘Granny Smith’, ‘Mark’, ‘Novole’, ‘Lancep’ and ‘Cepiland’ with a significant increase in the number of regenerated shoots from ‘Gala’ and ‘Golden Delicious’, where plants could be regenerated from callus or directly from leaves of micropropagated plants, with 100% regenerating leaves and an average of 14.2 shoots per leaf in ‘Gala’.

Observations of different leaf parts allowed the identification of more organogenic leaf areas. Meristemoids formed on the cut margins, and also on the adaxial surface of leaves, possibly due to the closer contact with the regeneration medium. Furthermore, leaf parts that were closer to the petiole were more regenerative, confirming previous observations carried out in experiment on the adventitious shoot proliferation from leaves of ‘M26’ apple rootstock (Ferradini *et al.* 1996).

The results presented here confirm earlier observations (Ferradini *et al.* 1996; Sicurani *et al.* 2001) showing that leaves serve as good explants for adventitious shoot formation. However, it should be pointed out that selection, excision, wounding, cutting and arrangement on the medium was time-consuming and labor-intensive.

Cytokinins such as TDZ and BAP have considerable effects in inducing regeneration in most woody plants, where it was shown that TDZ is more effective than BAP (Korban *et al.* 1992; DeBondt *et al.* 1996).

TDZ, a substituted phenylurea compound with cytokinin activity, was used to induce adventitious shoot formation in apple (Van Nieuwkerk *et al.* 1986; Fasolo *et al.* 1990; Theiler-Hedtrich and Theiler-Hedtrich 1990; Sarwar and Skirvin 1997; McAdam-O’Connell *et al.* 2004).

For shoot regeneration from leaf discs, a range of BAP and TDZ concentrations was examined. McAdam-O’Connell *et al.* (2004) developed a leaf disc regeneration system for ‘Bramley’s’ seedling apple (*Malus × domestica* Borkh.); while ‘Greensleeves’ responded in line with published data, ‘Bramley’ produced significantly fewer shoots. ‘Bramley’ shoots were obtained from 5 mg/l BAP and 1 mg/l NAA, while TDZ did not increase regeneration significantly (McAdam-O’Connell *et al.* 2004). Our results, however, are in contrast to such findings, where TDZ proved to be more efficient in inducing regeneration than BAP; in addition, high regeneration was also attained on media with just 0.2 mg/l NAA compared to 1 mg/l, which other authors used (Table 4).

The differences among different parts of the same plant may be attributed to the various levels of endogenous PGRs of explants from different positions (Magyarné *et al.* 2001; Jámborné and Dobránszki 2005).

In the present study, however, although induction of shoots was observed in most media tested, TDZ proved to be more efficient than BAP in induction of shoots (Table 2).

Our results demonstrate that developmental stage and quality of donor leaves combined with optimized combinations of PGRs play a key role in the successful induction of shoot organogenesis *in vitro*. Best results were attained using 21 days-old light green expanded donor leaves with a strong vein pattern, while explants from older leaves were unresponsive. The percentage of explants producing shoots and the number of shoots/explant were influenced by explant type and quality as well as type and concentrations of PGRs used. The percentage of regenerated shoots varied between 7 and 92% (Table 3).

In the present study, the frequency of shoot organogenesis could be increased with combinations of TDZ and NAA. TDZ-NAA combinations in the media revealed an efficient pathway for adventitious shoot formation in leaves of apples studied. No abnormality, necrosis or chlorosis was observed during culture. Most explants produced shoots and green shoot meristems were seen on a range of media containing BAP or TDZ and NAA (Table 2). A high percentage of regenerated shoots was achieved on a range of media supplemented with 5.0 mg/l BAP or 2 mg/l TDZ + 0.2 mg/l NAA.

The use of 2,4-D induced callus formation and inhibited adventitious shoot regeneration, and was therefore excluded afterwards.

Multiple shoot induction rate and organogenic response significantly varied to a greater extent according to the explant type and concentrations of PGRs used (Sarwar and Skirvin 1997; D’Angeli *et al.* 2001; Magyarné *et al.* 2001; Jámborné and Dobránszki 2005). Caboni *et al.* (2000) developed a protocol for the induction of adventitious shoot formation and plant regeneration from apple callus using MS media without glycine and supplemented with 17.8 µM BA, 2.7 µM NAA and 250 mg/l cefotaxime. They found that the degree of adventitious shoot regeneration from shoot tips were significantly higher than that from leaves. In our study, however, direct shoot formation could be developed without an intervening callus phase; moreover, the type of explant and culture medium with specific PGR concentrations influenced organogenesis considerably. We demonstrated that leaf explants can be used for rapid clonal propagation with optimized culture medium, and also for recovering transgenic shoots in genetic transformation studies, which are in progress with promising results (data not yet published).

Table 4 Comparison between conditions of the present study with some similar previous studies.

Apple cv./rootstock	Explant/method of regeneration	Basal medium	Growth regulators	Carbon source	Culture conditions	Reference
Greensleeves	Leaf discs or strips, adventitious buds	MS	2 µM BAP 0.5 µM NAA	2% sucrose	25 C, 3 days in dark	James <i>et al.</i> 1989
Delicious	Fully expanded leaves cut in half to the midrib; Callus followed by shoot formation	MS + Staba vit.	10-60 µM TDZ IAA, NAA, or IBA: 0.1-100 µM	3% sucrose	2-4 weeks at 4 or 28°C, then 25-28°C under cool white fluorescent tubes (80 µmol m ⁻² s ⁻¹) with continuous light.	Sriskandarajah <i>et al.</i> 1994
Royal Gala	Young expanding leaves cut transversely (2-3 mm wide)	MS +B5 vit.	1 mg/l BAP 0.2 NAA	3% sucrose	16-h photoperiod (30 µmol m ⁻² s ⁻¹)	Yao <i>et al.</i> 1995
McIntosh	Leaves without axillary buds cut into 3 segments	MS	2-3 µM TDZ 12-20 µM BAP + 1.5-6.5 NAA	3% sucrose	16-h photoperiod (131 µmol m ⁻² s ⁻¹)	Sarwar and Skirvin 1996
Jonagold	Whole leaves Callus induction	MS macro, Druart 1980 micro, 40 mg/l FeNaEDTA, 100 mg/l <i>myo</i> -inositol, 250 mg/l casein hydrolysate	0.3 mg/l IBA, 8 mg/l 2-iP, or TDZ, 2-ip, 0.1 mg/l GA ₃	2% sucrose	16-h photoperiod	De Bondt <i>et al.</i> 1994, 1996
Delicious, Pink Lady	Leaves 3-5 cm long	MS + Staba vit., 2 mg/l glycine, 50 mg/l ascorbic acid	15 µM TDZ 2.5 µM NAA	3% sucrose	8 x 2 L at 60 µmol m ⁻² s ⁻¹ 4 x 2 D, 25-28°C	Sriskandarajah <i>et al.</i> 1998
Delicious, Golden delicious, Royal gala, Greensleeves	Top 3 fully unfolded leaves of rooted shoots cut with a scalpel blade perpendicular to the mid vein into 2-3 mm	MS 3 g/l phytigel	5 mg/l BA + 1 mg/l TDZ + 1 mg/l NAA	3% sorbitol	10-20 days in dark, then at 25°C with 16-h photoperiod (110 µmol m ⁻² s ⁻¹)	Maximova <i>et al.</i> 1998
Marshal McIntosh	Youngest unfolded leaf cut transversely through the midrib resulting three leaf segments (2-3 mm wide)	MS/modified N6 medium	1 mg/l BA, 2.2 mg/l TDZ, 1 mg/l NAA	3% sucrose	25 ± 2°C in the dark for 2 weeks, then 16-h light (photon flux of 5-10 µmol m ⁻² s ⁻¹), 1 week, then 16/8 hrs. at 20-30 µmol m ⁻² s ⁻¹	Bolar <i>et al.</i> 1999
Jork, M26, Gala, McIntosh	Vegetative shoot apieces, callus induction, shoot regeneration	MS without glycine, 250 mg/l cefotaxime	17.8 µM BA, 2.7 µM NAA	29.2 mM sucrose, 109.8 mM sorbitol	20 days dark, then 16-h photoperiod (25 µmol m ⁻² s ⁻¹)	Caboni <i>et al.</i> 2000
Jork 9	Vegetative shoot apices, adventitious shoots from callus	MS, LP (Quoirin and Lepoivre 1977)	17.8 µM BA, 2.7 µM NAA	3% sucrose	20 days dark, then 16-h photoperiod	D'Angeli <i>et al.</i> 2001
Queen Cox	Leaves; callus induction	DKW MS	1 mg/l BAP 1 mg/l TDZ 0.1 mg/l NAA	3% sucrose, 40 g sorbitol	16-h photoperiod	Wilson and James 2003
Elstar, Holsteiner Cox	4 youngest unfolded leaves cut in strips, using only middle part	MS + MS vit. 3% gelrite	2.0 µM TDZ 0.5 µNAA	3% sorbitol	16-h photoperiod 25°C	Szankowski <i>et al.</i> 2003
Fuji, Gala	Shoot apices, adventitious shoot regeneration	MS with LS vitamins	5 mg/l TDZ, 0.3 mg/l IBA, 0, 10, 20, 40 or 80 µM AgNO ₃ or 0, 10, 35 or 70 µM AVG	3% sorbitol	Darkness at 25°C for 4 weeks	Seong <i>et al.</i> 2005
MM106	Leaves cut around the edges	MS/ WPM (Lloyd and McCown 1980)	8.8, 22 µM BA, 5.4 µM NAA or 0.5 and 1 µM IBA	3% sucrose	1-2 weeks darkness, then 16-h photoperiod	Modgil <i>et al.</i> 2005
<i>Malus domestica</i> cv. Gami Almasi	Unfurled leaf 15 ± 3 mm)	MS/ N6	7.5 mg/l BA + 2.0 mg/ 1 NAA	3% sucrose	10 days dark at 25 ± 2°C, then to 16-h photoperiod (500 lux)	Rustae <i>et al.</i> 2007
M9 (clone T337), M26	Cut, unfolded leaf segments	MS and vitamins with Van der Salm modification	20 µM TDZ, 1.1 µM NAA	3% sorbitol or 2% glucose	24°C with 16-h photoperiod	Höhnle and Weber 2007

Table 4 (Cont.)

Apple cv./rootstock	Explant/method of regeneration	Basal medium	Growth regulators	Carbon source	Culture conditions	Reference
Apple cv. Chadel	Leaf segments, somatic regeneration	MS	7.5 µM TDZ 5, 10, 15, 20 µM IAA	1% sucrose, 3% sorbitol	15 days in darkness, then 16-h photo-period (40 µmol m ⁻² s ⁻¹ PAR at 22 ± 2°C for 25 days	Gercheva <i>et al.</i> 2009
Golden Delicious, MM111	21 days-old leaves cut into three segments	MS /or N6 macro + MS micro + B5 vit. + 1 g/l MES	2 mg/l TDZ or 5 mg/l BAP + 0.2 mg/l NAA	3% sucrose	3 weeks darkness, 1 week low light intensity, 4 weeks full light	Present study

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; AVG, Aminoethoxyvinylglycine; BA, 6-benzyladenine; BAP, N⁶-benzylamino-purine; B5, vitamins (Gamborg 1968); DKW, *Juglans* medium (Driver and Kuniyuki 1984); IBA, indole-3-butyric acid; MES, 2-(*N*-morpholino) ethanesulfonic acid; MS, Murashige and Skoog medium (1962); N6 medium (Chu *et al.* 1975); NAA, α -naphthalene acetic acid; Staba vitamins (Staba 1969), TDZ, thidiazuron (*N*-phenyl-*N*-1,2,3-thiadiazol-5-yl urea); WPM, McCown Woody Plant Medium (1980).

Effects of carbon source, MES and gelling agent on organogenic response

Karhu (1997) tested three carbon sources (glucose, sorbitol and sucrose) for their regeneration efficacy in different apple (*Malus domestica*) explants and found that sorbitol and sucrose has similar effects in induction of regeneration. Jámborné and Dobránszki (2005) also reported different effects of carbon sources (sucrose, sorbitol and glucose) on regeneration in apple.

In the present study, however, two energy sources were tested for regeneration efficiency, sucrose and sorbitol. The former was more efficient in inducing regeneration, while replacing sucrose with sorbitol negatively affected the regeneration ability of new shoots (data not shown).

Gelrite gellan gum is a self-gelling hydrocolloid that forms rigid, brittle, transparent gel in the presence of soluble salts. Chemically, it is a polysaccharide comprised of uronic acid, rhamnose and glucose. Gelrite is used in place of agar because it costs less per liter of medium and its clarity makes it easy to observe plant growth and bacterial contamination (Pasqualetto *et al.* 1986). In our preliminary experiments, it was shown that agar alone had inhibitory effects on regeneration and was therefore excluded or used in combination with gelrite. Gelrite was better than agar or a combination of both. Adding agar in combination with Gelrite had an inhibitory effect of regeneration (Table 3). Hyperhydricity was not observed in the present study because gelrite was used (data not shown), since low concentrations were used, while using higher concentrations (3 g/l) in our preliminary experiments resulted in shoot hyperhydricity, in accordance with the study of Szankowski *et al.* (2003) who experienced strong hyperhydricity accompanied

with abnormal development during the regeneration phase when they used 3% gelrite. Pasqualetto *et al.* (1986) found that shoot cultures of apple cv. ‘Gala’ grown on medium gelled with Gelrite become vitrified within 2-3 weeks at a concentrations of 1.5-2 g/l. However, hyperhydricity was reduced by using agar in combination with Gelrite.

On the other hand, MES was used in the present study as a pH buffering agent to stabilize pH of culture media, since it is well-known that pH can alter nutrient absorption and consequently affect on shoot formation efficiency. MES appears to be biologically inert and does not interact significantly with other ions (Bugbee and Salisbury 1985). The effect of MES and *myo*-inositol and different combinations of these with a number of plant growth regulators on somatic embryogenesis in black henbane was evaluated, where maximum frequency of direct somatic embryogenesis and germination was achieved on MS basal medium containing 1 mg/l NAA, 2 g/l *myo*-inositol and 0.5 g/l MES (Shanjun *et al.* 1996). MES strongly affected plant growth of cucumber (*Cucumis sativa* L. var. ‘Marketer’) in hydroponic culture with increasing concentration in nutrient solution. Tissue and nutrient solution analysis determined that MES affects Mn uptake. The suitability of MES as a pH buffer in hydroponic culture was discussed in terms of this effect (Stahl *et al.* 1999).

Rooting and acclimatization of proliferated shoots

As for the performance of the adventitious shoots formed, they were subcultured every 4 weeks for multiplication and rooted easily according to the protocols developed earlier in our laboratory by Altinawi *et al.* (2009) and Alrihani *et al.* (2008) for ‘Golden Delicious’ and ‘MM111’, respectively.

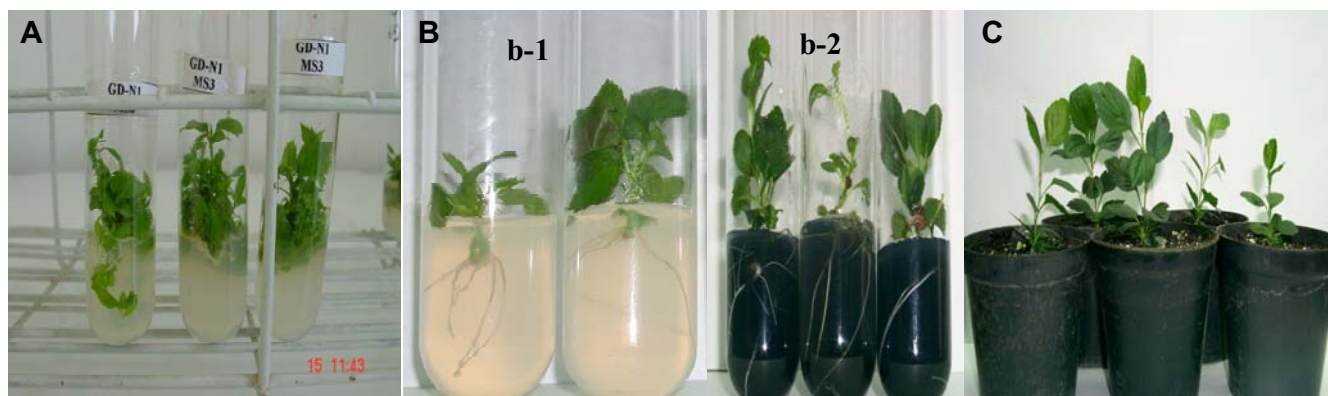


Fig. 2 Multiplication, rooting and acclimatization of *in vitro* regenerated ‘Golden Delicious’ (G.D.) and ‘MM111’ apple plantlets. (A) Shoot multiplication *in vitro* starting from leaf explants. (B) *In vitro* rooting of ‘MM111’ (b-1), ‘Golden Delicious’ (b-2). (C) 1-month old acclimatized plantlets in the greenhouse.

Proliferated shoot tips (20-30 mm length) were excised and rooted readily on half-strength MS medium supplemented with 1.0 mg/l IBA. Rooting was observed from the cut ends of the shoots within 30 days. All of the developing roots were physically vigorous and healthy.

Rooted plantlets were acclimatized to ambient conditions with 85% efficiency and later were established under greenhouse conditions and finally in the field under natural field conditions (Fig. 2).

CONCLUDING REMARKS

The results presented in the current investigation indicate that *in vitro* direct organogenesis in apple using leaf explants is a good pathway for rapid clonal propagation as alternative to the methods developed earlier in our laboratory (Alrihani *et al.* 2008; Altinawi *et al.* 2009) in which shoot tips and axillary buds were used as explants with better efficiency in the present study and also as a regeneration protocol for genetic transformation which is in progress using *g2ps1* and chitinase genes to confer fungal resistance to the studied apples. Cut leaves maintain their regenerative ability and can be multiplied and rooted. The youngest light green leaves obtained from 21 day-old proliferating cultures of apple are a very potent explant type for efficient adventitious shoot formation. Furthermore, the present study underlines the importance of explant age and characteristics as well as the combinations of TDZ and NAA or TDZ, BAP and NAA for high shoot formation from leaves *via* organogenesis and may be used easily in genetic transformation studies, currently in progress. The regeneration system described herein will be used to obtain transgenic shoots from leaf explants of these apple varieties using *Agrobacterium tumefaciens*-mediated transformation with constructs containing *g2ps1* or chitinase genes that will potentially lead to production of transgenic apple resistant to fungal diseases.

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