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

Nabeela Ali Bacha1 • Kinan Darkazanli2 • Ahmad M. Abdul-Kader1*

1 Biotechnology Department, General Commission for Scientific Agricultural Research (GCSAR), Damascus, Douma, P.O. Box 113, Syria
2 Aleppo University, Faculty of Agriculture, Biotechnology Research Center, Aleppo, Syria

Corresponding author: * ahmad59@gmx.de

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-butryic 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-naphthale 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; Maheswared 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 μmol m-2 s-1 photon flux at the surface of culture vessels.
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, Ascutap, Tuttlingen, 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.

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.

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

<table>
<thead>
<tr>
<th>Medium</th>
<th>Medium composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>R0</td>
<td>MS + B5 Vit.+ 1 g/l MES + 2.5 g/l Gelrite + 30 g/l sucrose (control medium)</td>
</tr>
<tr>
<td>R01</td>
<td>R0 + 2.0 mg/l TDZ + 0.2 mg/l NAA</td>
</tr>
<tr>
<td>R02</td>
<td>R0 + 5.0 mg/l BAP + 0.2 mg/l NAA</td>
</tr>
<tr>
<td>R02*</td>
<td>R0 with 30 g/l sorbitol instead of sucrose + 5.0 mg/l BAP + 0.2 mg/l NAA</td>
</tr>
<tr>
<td>R02**</td>
<td>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</td>
</tr>
<tr>
<td>R03</td>
<td>MS + 5.0 mg/l BAP + 0.2 mg/l 2,4-D + 2.5 g/l Gelrite + 30 g/l sucrose</td>
</tr>
<tr>
<td>R04</td>
<td>R0 with 30 g/l sorbitol instead of sucrose + 5.0 mg/l BAP + 0.2 mg/l NAA + 2.5 g/l Gelrite + 30 g/l sucrose</td>
</tr>
<tr>
<td>R04*</td>
<td>R0+ 5.0 mg/l BAP + 0.2 mg/l NAA</td>
</tr>
<tr>
<td>R05</td>
<td>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</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Effects of different leaf explants and growth regulators on direct organogenetic 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-
bated on organogenic induction medium, regeneration, 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; Scurani 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 Nieuwerkerk et al. 1986; Fasolo et al. 1990; Theiler-Hedrich and Theiler-Hedrich 1990; Sarwar and Skirvin 1997; McAdam-O’Connell et al. 2004).

For shoot regeneration from leaf discs, a range of media 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 ‘Greensleaves’ 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; Jamborné 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; Jamborné 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).

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**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.

<table>
<thead>
<tr>
<th>Explant</th>
<th>Media used</th>
<th>R01</th>
<th>R02</th>
<th>R04</th>
<th>R05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower part of leaf</td>
<td>GD</td>
<td>2.75 ± 0.129</td>
<td>2.350 ± 0.092</td>
<td>1.825 ± 0.061</td>
<td>1.40 ± 0.078</td>
</tr>
<tr>
<td>Middle part of leaf</td>
<td>GD</td>
<td>4.025 ± 0.141</td>
<td>4.075 ± 0.173</td>
<td>2.225 ± 0.067</td>
<td>2.50 ± 0.080</td>
</tr>
<tr>
<td>Upper part of leaf</td>
<td>GD</td>
<td>1.60 ± 0.10 c</td>
<td>1.775 ± 0.06 c</td>
<td>1.00 ± 0.00 c</td>
<td>1.00 ± 0.00 c</td>
</tr>
<tr>
<td>LSD 0.05</td>
<td></td>
<td>0.240 ± 0.366</td>
<td>0.175 ± 0.226</td>
<td>0.130 ± 0.321</td>
<td>0.321 ± 0.248</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Explant</th>
<th>Media used</th>
<th>R01</th>
<th>R02</th>
<th>R04</th>
<th>R05</th>
<th>R0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower part of leaf</td>
<td>GD</td>
<td>84 ± 0.138 b</td>
<td>80 ± 0.082 b</td>
<td>8 ± 0.07 b</td>
<td>9 ± 0.08 b</td>
<td>15 ± 0.10 b</td>
</tr>
<tr>
<td>Middle part of leaf</td>
<td>GD</td>
<td>92 ± 0.143 a</td>
<td>90 ± 0.21 a</td>
<td>20 ± 0.02 a</td>
<td>18 ± 0.08 b</td>
<td>36 ± 0.09 a</td>
</tr>
<tr>
<td>Upper part of leaf</td>
<td>GD</td>
<td>40 ± 0.20 c</td>
<td>58 ± 0.07 c</td>
<td>2 ± 0.10 c</td>
<td>2 ± 0.00 c</td>
<td>10 ± 0.00 c</td>
</tr>
<tr>
<td>LSD 0.05</td>
<td></td>
<td>0.430 ± 0.280</td>
<td>0.92 ± 0.169</td>
<td>0.22 ± 0.420</td>
<td>0.332 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

Values within each column followed by different letters are significantly different at the 0.05 probability level (α<0.05%) using Duncan’s multiple range test. Values are means of 300 explants each.
<table>
<thead>
<tr>
<th>Apple cv./rootstock</th>
<th>Explant/method of regeneration</th>
<th>Basal medium</th>
<th>Growth regulators</th>
<th>Carbon source</th>
<th>Culture conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greensleeves</td>
<td>Leaf discs or strips, adventurous buds</td>
<td>MS</td>
<td>2 μM BAP 0.5 μM NAA</td>
<td>2% sucrose</td>
<td>25°C, 3 days in dark</td>
<td>James et al. 1989</td>
</tr>
<tr>
<td>Delicious</td>
<td>Fully expanded leaves cut in half to the midrib; callus followed by shoot formation</td>
<td>MS + Staba vit.</td>
<td>10-60 μM TDZ IAA, NAA, or IBA: 0.1-100 μM</td>
<td>3% sucrose</td>
<td>2-4 weeks at 4 or 28°C, then 25-28°C under cool white fluorescent tubes (80 μmol m² s⁻¹) with continuous light</td>
<td>Sriskandarajah et al. 1994</td>
</tr>
<tr>
<td>Royal Gala</td>
<td>Young expanding leaves cut transversely (2-3 mm wide)</td>
<td>MS + B5 vit.</td>
<td>1 mg/l BAP 0.2 NAA</td>
<td>3% sucrose</td>
<td>16-h photoperiod (30 μmol m² s⁻¹)</td>
<td>Yao et al. 1995</td>
</tr>
<tr>
<td>McIntosh</td>
<td>Leaves without axillary buds cut into 3 segments</td>
<td>MS</td>
<td>2-3 μM TDZ 12-20 μM BAP + 1.5-6.5 μM NAA</td>
<td>3% sucrose</td>
<td>16-h photoperiod (131 μmol m² s⁻¹)</td>
<td>Sarwar and Skirvin 1996</td>
</tr>
<tr>
<td>Jonagold</td>
<td>Whole leaves Callus induction</td>
<td>MS, MS macro, Drurat 1980 micro, 40 mg/l FeNaEDTA, 100 mg/l myo-inositol, 250 mg/l casein hydrolysate</td>
<td>15 μM TDZ 2.5 μM NAA</td>
<td>3% sucrose</td>
<td>16-h photoperiod</td>
<td>De Bondt et al. 1994, 1996</td>
</tr>
<tr>
<td>Delicious, Pink Lady</td>
<td>Leaves 3-5 cm long</td>
<td>MS + Staba vit., 2 mg/l glycine, 50 mg/l ascorbic acid</td>
<td>5 mg/l BA + 1 mg/l TDZ + 1 mg/l NAA</td>
<td>3% sorbitol</td>
<td>10-20 days in dark, then at 25°C with 16-h photoperiod (110 μmol m² s⁻¹)</td>
<td>Maximova et al. 1998</td>
</tr>
<tr>
<td>Delicious, Golden delicious, Royal gala, Greensleeves</td>
<td>Top 3 fully unfolded leaves of rooted shoots cut with a scalpel blade perpendicular to the mid vein into 2-3 mm</td>
<td>MS 3 g/l phytagel</td>
<td>5 mg/l BA + 1 mg/l TDZ + 1 mg/l NAA</td>
<td>3% sorbitol</td>
<td>20 μmol m² s⁻¹</td>
<td>Sriskandarajah et al. 1998</td>
</tr>
<tr>
<td>Marshall McIntosh</td>
<td>Youngest unfolded leaf cut transversely through the midrib resulting three leaf segments (2-3 mm wide)</td>
<td>MS/modified N6 medium</td>
<td>1 mg/l BA, 2.2 mg/l TDZ, 1 mg/l NAA</td>
<td>3% sucrose</td>
<td>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 m² s⁻¹</td>
<td>Bolar et al. 1999</td>
</tr>
<tr>
<td>Jork, M26, Gala, McIntosh</td>
<td>Vegetative shoot apices, callus induction, shoot regeneration</td>
<td>MS without glycine, 250 mg/l cefotaxime</td>
<td>17.8 μM BA, 2.7 μM NAA 29.2 mM sucrose, 109.8 mM sorbitol</td>
<td>3% sucrose</td>
<td>20 days dark, then 16-h photoperiod (25 μmol m² s⁻¹)</td>
<td>Caboni et al. 2000</td>
</tr>
<tr>
<td>Jork 9</td>
<td>Vegetative shoot apices, adventitious shoots from callus</td>
<td>MS, LP (Quoirin and Lepoivre 1977)</td>
<td>17.8 μM BA, 2.7 μM NAA</td>
<td>3% sucrose</td>
<td>20 days dark, then 16-h photoperiod</td>
<td>D’Angeli et al. 2001</td>
</tr>
<tr>
<td>Queen Cox</td>
<td>Leaves; callus induction</td>
<td>DKW MS</td>
<td>1 mg/l BAP 1 mg/l TDZ 0.1 mg/l NAA</td>
<td>3% sucrose, 40 g sorbitol</td>
<td>16-h photoperiod</td>
<td>Wilson and James 2003</td>
</tr>
<tr>
<td>Elstar, Holsteiner Cox</td>
<td>4 youngest unfolded leaves cut in straps, using only middle part</td>
<td>MS + MS vit. 3% gelrite</td>
<td>2.0 μM TDZ 0.5 μM NAA</td>
<td>3% sorbitol</td>
<td>16-h photoperiod 25°C</td>
<td>Szankowski et al. 2003</td>
</tr>
<tr>
<td>Fuji, Gala</td>
<td>Shoot apices, adventitious shoot regeneration</td>
<td>MS with LS vitamins</td>
<td>5 mg/l TDZ, 0.3 mg/l IBA, 0, 10, 20, 40 or 80 μM AgNO₃ or 0, 10, 35 or 70 μM AVG</td>
<td>3% sorbitol</td>
<td>Darkness at 25°C for 4 weeks</td>
<td>Seong et al. 2005</td>
</tr>
<tr>
<td>MM106</td>
<td>Leaves cut around the edges</td>
<td>MS/ WPM (Lloyd and McCown 1980)</td>
<td>8.8, 22 μM BA, 5.4 μM NAA or 0.5 and 1 μM IBA</td>
<td>3% sucrose</td>
<td>1-2 weeks darkness, then 16-h photoperiod</td>
<td>Modgil et al. 2005</td>
</tr>
<tr>
<td>Malus domestica cv. Gami Almasi</td>
<td>Unfurled leaf 15 ± 3 mm)</td>
<td>MS/ N6</td>
<td>7.5 mg/l BA + 2.0 mg/l NAA</td>
<td>3% sucrose</td>
<td>10 days dark at 25 ± 2°C, then to 16-h photoperiod (500 lux)</td>
<td>Rustace et al. 2007</td>
</tr>
<tr>
<td>M9 (clone T337), M26</td>
<td>Cut, unfolded leaf segments</td>
<td>MS and vitamins with Van der Salm modification</td>
<td>20 μM TDZ, 1.1 μM NAA</td>
<td>3% sorbitol or 2% glucose</td>
<td>24°C with 16-h photoperiod</td>
<td>Hohmle and Weber 2007</td>
</tr>
</tbody>
</table>
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) for their regeneration efficacy in different apple cv. 'Gala' explants and found that sorbitol and sucrose have important effects in the induction of regeneration. 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 is a self-gelling hydrocolloid that forms rigid, brittle, transparent gel in the presence of solurates. 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 gelrule 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 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.

Table 4 (Cont.)

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

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; AVG, Aminoethoxyvinylglycine; BA, 6-benzyladenine; BAP, N⁶-benzylamino-purine; B5, vitamins (Gamborg 1968); DWK, 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).

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 cost pathway for rapid clonal propagation as an alternative to the methods developed earlier in our laboratory (Alrhihani 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 pGPTV and chitinase genes to confer fungal resistance to the studied apples. Cut leaves maintain their regenerative capacity and can be multiplied in vitro. 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 pGPTV or chitinase genes that will potentially lead to production of transgenic apple resistant to fungal diseases.

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