Adventitious Shoot Regeneration and Flow Cytometry of *Cydonia oblonga* Mill. (cv. Isfahan)

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**ABSTRACT**

Modern breeding methods such as genetic transformation or *in vitro* mutation are valuable tools to improve fruit trees and overcome traditional breeding problems such as long juvenile period, high level of self-incompatibility and heterozygosity which are usually associated with *Cydonia oblonga* (quince). Adventitious shoot regeneration is the prerequisite step in using modern breeding methods in horticultural crops. The aim of the present investigation was to optimize adventitious shoot regeneration in quince (cv. Isfahan, known as ‘KVD2’). Different explant types (whole leaf, leaf basal section, leaf apical section and petiole), different concentrations of naphthaleneacetic acid (NAA) (0, 1, 2 µM) in combination with thidiazuron (TDZ) (0, 2.5, 5, 10, 20, 40 µM) and two culture media [Nitsch and Nitsch (NN) and Murashige and Skoog (MS)] were compared. To detect genome size changes, ploidy level and 2C DNA amounts of the regenerated shoots and the original plants were measured by flow cytometry. The results indicated the maximum frequency of regeneration (46%) was obtained from the whole leaf explants cultured on NN medium containing 40 µM TDZ and 1 µM NAA. The highest mean number of shoots (7.46) was obtained on MS medium containing 5 µM TDZ and 1 µM NAA. Flow cytometry analysis showed that regenerated shoots and donor shoots both were diploids and their 2C DNA amounts had not changed throughout the regeneration process.

**Keywords:** 2C DNA amounts, leaf explant types, NAA, ploidy level, TDZ.

**Abbreviations:**
- BAP: 6-benzylaminopurine
- IBA: indole-3-butyric acid
- MS: Murashige and Skoog Medium
- NAA: Naphthaleneacetic Acid
- NN: Nitsch and Nitsch Medium
- TDZ: thidiazuron.

**INTRODUCTION**

*Cydonia oblonga* Mill. (quince), a small tree is cultivated in gardens under warm temperature and grows up to 8 m high and 4 m wide. The young branches are covered with pale grayish wool, leaves are elliptical, flowers are pink or white, fruits are bright yellow and usually pear shaped (Khoubsasabjafari and Jouyban, 2011). The name of the genus *Cydonia* has originated from the northwestern coast of Crete, Greece, the region of Kydonia, where the tree has been cultivated since the ancient times (Ganopoulos et al., 2011). The main origin of quince is unknown, but different regions such as Iran, Turkey, Anatoly, Middle East and Greece are introduced as the probable origin of this species (Bayazit et al., 2011; Sykes et al., 1972). Quince tree has been famous for a long time as the rootstock for many pear cultivars (Dehbashi et al., 2013). Although Iran, has different kinds of quince genotypes, but the research on genetic diversity of Iranian quince and breeding new varieties using traditional or modern techniques is very limited.

Breeding fruit trees through conventional procedures is difficult and expensive due to several reasons including heterozygosity, polyploidy, long breeding cycles, and long field trial procedures (Nagaty, 2012). Therefore, modern breeding methods such as genetic transformation or *in vitro* mutation are valuable tools to improve fruit trees and overcome traditional breeding problems such as long juvenile period, high level of self-incompatibility and heterozygosity which are usually associated with quince. Regeneration of adventitious shoots is an essential step for using modern breeding methods. Fruit tree species are usually recalcitrant to *in vitro* propagation (Bell et al., 2012). Various endogenous and exogenous
factors affect regeneration of adventitious shoots from plants, among which hormonal balance has a primary role. In particular, the auxin–cytokinin ratio appears to be the most important factor in channeling regeneration response towards a specific in vitro morphogenic process rather than another (D’Onofrio and Morini, 2005). Among the fruit tree species that have shown regenerating capacity, the quince has produced somatic embryos by a combination of kinetin and naphthaleneacetic acid (NAA) (D’Onofrio et al. 1998; D’Onofrio and Morini, 2002) or adventitious shoots by treatments with thidiazuron (TDZ) and NAA (Dolcet-Sanjuan et al. 1991; Baker and Bhatia, 1993) applied to in vitro cultured leaves. TDZ also stimulated regeneration from leaves of apple, pear and rose effectively (Chevreau et al., 1989; Fasolo et al., 1989; Swartz et al., 1990; Pourhosseini et al., 2013). TDZ, a substituted phenyl-urea, has proven to be a highly effective regulator of plant morphogenesis. Originally TDZ was considered as a cytokinin and induced many responses typical of natural cytokinins (Murthy et al., 1998). However, later research demonstrated that TDZ, unlike traditional cytokinins, was capable of fulfilling both cytokinin (bud formation) and auxin (somatic embryogenesis) functions involved in various morphogenetic responses of different plant species (Huetteman and Preece, 1993).

In the present investigation in order to study the adventitious shoot regeneration in quince (cv. Isfahan, known as ‘KVD2’) and use the results in future gene transformation breeding, different hormone (TDZ and NAA) concentrations, different explant types and two culture media were compared. To detect genome size changes, 2C DNA amounts of the regenerated shoots and the original plants were measured by flow cytometry.

MATERIALS AND METHODS

Plant Material and General Culture Conditions:

Cydona oblonga Mill. (quince), cv. Isfahan, known as ‘KVD2’, were supplied by the department of horticultural research, Seed and Plant Improvement Institute, Karaj, Iran. In vitro shoots were established by researchers at department of tissue culture and gene transformation at Agricultural Biotechnology Research Institute of Iran (ABRII). In vitro shoots were proliferated on MS medium (Murashige and Skoog, 1962), containing 3 µM BAP (6-benzylaminopurine), 0.5 µM IBA (indole-3-butyric acid), 1.66 µM GA₃, 100 mg l⁻¹ phloroglucinol and 3% (w/v) sucrose and gelled with 7 gr l⁻¹ plant agar. pH was adjusted to 5.8 using 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl) before adding plant agar.

All the media were autoclaved for 15 min at 1.2 kPa pressure and 121˚C. The cultures were grown at 24±2˚C in a 16-h photoperiod at light intensity of 60 µ mol m⁻² s⁻¹ provided by cool white fluorescent tubes and sub-cultured onto fresh medium every 40 days.

Interactive Effect of Explant Type and Hormone Concentrations on Adventitious Shoot Regeneration:

Four types of in vitro leaf explants were used as different explant types (Fig. 1); whole leaf that was wounded by three cuts transversely to the mid-rib, leaf basal section, leaf apical section and petiole.

The explants were treated with NN medium (Nitsch and Nitsch, 1969) supplemented with various concentrations (0, 1, 2 µM) of NAA (α-naphthaleneacetic acid) in combination with different concentrations (0, 2.5, 5, 10, 20, 40 µM) of TDZ (thidiazuron). All the explants were maintained for 30 days in darkness, then transferred to light conditions. Percent of explants forming shoots and number of shoots per explant were recorded 40 days after the beginning of the experiments.

Fig. 1. Representation of the procedure adopted for preparing the explants: right to left: the whole wounded leaf, leaf apical section, leaf basal section and the petiole.

Interactive Effect of Culture Media and TDZ Concentrations on Adventitious Shoot Regeneration:

Using the best explant type and the best concentration of NAA from the previous experiment on interactive effect of hormone concentrations and explant type, different concentrations (0, 2.5, 5, 10, 20, 40 µM) of TDZ and two culture media (NN and MS) were compared. All the explants were maintained for 30 days in darkness then transferred to light conditions. Percent of explants forming shoots and number of shoots per explant were collected 40 days after the beginning of the experiments.

Shoot Elongation and Rooting of the Regenerated Shoots:

Regenerated shoots were transferred to elongation MS culture medium containing
RESULTS AND DISCUSSION

Interactive Effect of Explant Type and Hormone Concentrations on Adventitious Shoot Regeneration:

The effect of growth regulator combinations and different explant types were investigated in order to optimize adventitious shoot formation from leaves of Cydonia oblonga ‘Isfahanknown’. Table 1 illustrates that there was a significant difference between different explant types and hormone concentrations on the percent of regeneration and number of shoots per explants.

The highest percent of regeneration (46%) and maximum number of shoot per explants (1.92) were observed on medium containing 40 μM TDZ + 1 μM NAA from the whole leaf explants (Table 1). Fig. 2A illustrates regeneration in whole leaf explants treated with 40 μM TDZ + 1 μM NAA. Dolcet-Sanjuan et al. (1991) reported that regeneration of shoots from leaves of quince required high concentrations of TDZ. TDZ also stimulated regeneration from leaf discs of apple (Fasolo et al., 1989) and pear (Chevreul et al., 1989) effectively. A high cytokinin: low auxin ratio was required for promoting regeneration in apple leaf explants (Fasolo et al., 1989; Welander, 1988). The ability of TDZ to stimulate cell division has been demonstrated in soybean callus (Thomas and Katterman, 1986). Apart from cell division, it was reported that TDZ induced auxiliary shoot proliferation in several plant species, i.e. apple (Virscek-Marn et al., 1999), pear (Javadi et al., 2013), Prunus (Liu and Pijut, 2008) peach (Soliman, 2013) and rose (Pourhosseini et al., 2013).

Callus production is frequently an essential step in the regeneration of adventitious organs. Although Huetteman and Preece (1993) stated that growth of callus inhibit axillary shoot proliferation in woody plant tissue culture. Nevertheless, Bassi and Cossio (1994) reported that in quince organogenesis was associated with callus formation. In the present investigation callus formation was necessary for induction of organogenesis. Calli appeared mainly on the wounded edges and midribs of the leaf explants (Fig 2B). Auxin-containing media, without cytokinins, induced adventitious roots at edge and midrib of leaf explants (Fig 2C). Pourhosseini et al. (2013) also reported that medium containing 1 μM NAA promoted only root initiation in rose.

Tang et al. (2008) reported that in pear cultivars, the highest rate of regeneration and highest number of shoots were observed on the basal section of the leaves. Whereas, higher regenerations were observed from mid leaf segments in apple (Sarwar and Skirvin, 1997). Bhagwat and Lane (2004) reported that

BAP (2.2 μM), IBA (0.5 μM), GA₃ (3.33 μM) for 6 weeks prior to transferring them to rooting stage. The elongated shoots were cultured on rooting medium containing MS culture medium supplemented with NAA (2.5 μM) and IBA (2.5 μM) for six weeks. The in vitro plantlets were transferred to plastic cups containing peat and perlite (1:1) and were acclimatized to the greenhouse conditions.

**Flow Cytometry Analysis of the Regenerated Shoots and their Donor Shoots:**

Since there was a possibility of change in genome size of regenerated shoots, the DNA amounts of regenerates shoots was measured by flow cytometry and was compared with the donor shoots. Leaf discs (total 50 mm²) of young leaves of regenerates or donor quince shoots were chopped with a razor blade together with a similar amount of young leaves of parsley (Petroselimum crispum 'Champion Moss Curled') 2n=2x=22; 2C DNA amount=4.46 pg), as an internal standard, according to Yokoya et al. (2000). Polyvinylpyrrolidione (PVP) was added to the nuclei isolation buffer (Partec, Germany) at a concentration of 10 g l⁻¹. Polyvinylpyrrolidone (PVP) was added to the nuclei isolation buffer (Partec, Germany) by means of laser beam excitation. Estimates of the ratio of fluorescence intensities of each quince to parsley (P₁/P₂) were based on the mean of three samples (from one plant), each with a minimum of 10,000 nuclei, giving peaks for the parsley shoot. The elongated shoots were cultured on rooting medium containing MS culture medium supplemented with NAA (2.5 μM) and IBA (2.5 μM) for six weeks prior to transferring them to rooting stage.

**Experimental Design and Statistical Analysis:**

Each treatment was performed in five replicates with 3 explants. The experiments were designed in a factorial based completely random design. Data were analyzed using statistical programs SAS and Excel. Statistically significant averages were compared using Duncan’s multiple range tests. Graphs were plotted with the Excel program. Differences were regarded as significant at P ≤ 0.05.
regeneration of shoots from sweet cherry (*Prunus avium*) leaf segments was much lower than from whole-leaf explants. However, in the present study the percent of regeneration and number of shoots per explant were higher in the whole leaf explants compared to the other leaf explants (Table 1). This could be attributed to the cut surfaces in mid segments of the whole leaf which increased the rate of absorption of components from media.

**Table 1.** Comparing the effect of different explant types and different concentrations of TDZ and NAA on adventitious shoot regeneration of *Cydonia oblonga* (cv. Isfahan).

<table>
<thead>
<tr>
<th>Growth Regulator (µM)</th>
<th>Explants producing shoot (%)</th>
<th>Number of shoots per explant</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Whole Leaf</td>
<td>Basal Section</td>
</tr>
<tr>
<td>NAA</td>
<td>TDZ</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>0%</td>
</tr>
<tr>
<td>-</td>
<td>2.5</td>
<td>6%</td>
</tr>
<tr>
<td>-</td>
<td>5</td>
<td>6%</td>
</tr>
<tr>
<td>-</td>
<td>10</td>
<td>26%</td>
</tr>
<tr>
<td>-</td>
<td>20</td>
<td>33%</td>
</tr>
<tr>
<td>-</td>
<td>40</td>
<td>46%</td>
</tr>
<tr>
<td>1</td>
<td>2.5</td>
<td>6%</td>
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<tr>
<td>1</td>
<td>5</td>
<td>8%</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>8%</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>6%</td>
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</tbody>
</table>

In the present investigation all the explants from all the treatments were cultured in the dark for 30 days and were transferred to light conditions. Explants which were not exposed to the dark period did not regenerate (data not shown). Many researchers have suggested that an optimum dark period varying between 1 and 4 weeks is necessary for shoot regeneration in *Cydonia* species (Dolcet-Sanjuan et al., 1991; Zalunskaité et al., 2007) *Pyrus* species (Leblay et al., 1991), *Prunus* species (Espinosa et al., 2006; Gentile et al., 2002; Perez-Tornero et al., 2000; Miguel et al., 1996) and apple (Welander, 1988). Dark period may be important to initiate regeneration, because of its...
possible influence on the levels of endogenous hormones and interaction with exogenously applied growth regulators (Zalunskaitė et al., 2007).

In the present investigation the adaxial surface of the leaf explants was in contact with culture media. Higher percent of regeneration from adaxial position in quince has been reported by Dolcet-Sanjuan et al. (1991) and D’onofrio and Morini (2005). Blanke and Belcher (1989) reported that increased oxygen exchange occurs on the abaxial side of the leaves, because more stomata are located on this side of the leaf. Therefore it is possible that when abaxial side is up, higher rates of gas exchange takes place. The ability of the palisade parenchyma on the adaxial surface to transport nutrients and growth regulators from the medium into the explants were also stated by Welander (1988). Perez-Tornero and colleagues (2000) reported that when adaxial side of the leaf was touching the culture medium, the regeneration in apricot was four- or five-fold higher than when the leaf abaxial side was in contact with the medium.

**Interactive Effect of Culture Media and TDZ Concentrations on Adventitious Shoot Regeneration:**

Previous experiment revealed that the highest percent of adventitious shoot regeneration (46%) was observed in the NN medium containing 40 μM TDZ+ 1 μM NAA, however, the highest number of regenerated shoots per explant in this medium was only 1.92. Therefore, in order to optimize number of regenerated shoots per explant, interactive effect of two culture media (NN and MS) with different concentrations (0, 2.5, 5, 10, 20, 40 μM) of TDZ, using whole leaf explants was studied. The results indicated that highest number of shoots per explant (7.65) was obtained in the MS medium containing 5 μM TDZ. MS culture medium has higher nitrogen content and more balanced ratio of nitrate to ammonium, compared to NN culture medium, which may have contributed to higher number of regenerated shoots (Table 2).

Hennayake et al. (2003) found that ammonium and total nitrogen play an essential role in regeneration. The balance between NH$_4^+$ and NO$_3^-$ has been reported to induce shoot regeneration in pear (Leblay et al., 1991). Abu-Qaoud et al. (1991) confirmed that nitrogen amount and its form and NH$_4^+$-N/NO$_3^-$-N ratios all influenced regeneration of shoots from leaves of Pyrus communis. NH$_4^+$ promoted the penetration of anions into the plant at the expense of cations, while NO$_3^-$ led to the reverse process. Similarly, the balance between the two types of nitrogen ions in the nutrient medium regulated the differential absorption of other ions by the leaf (Hennayake et al., 2003). Yepes and Aldwinckle (1994) showed that culture media effected regeneration of different apple genotypes. They reported that some genotypes such as 'Golden delicious' regenerated on MS medium better than N6 medium and suggested that it is likely concentrations of CaCl$_2$.2H$_2$O and MgSO$_4$.7H$_2$O were higher in MS medium than N6 medium, which results in higher rates of regeneration from leaf explants. Fisichella et al. (2000), also reported that combination of salt in MS medium successfully induced somatic embryogenesis in quince. However, Tang et al. (2008) used NN, MS and QL (Quoirin and Lepoivre, 1977) media. They reported that NN or QL media resulted in higher rates of regeneration in pear compared to MS medium. They also showed that TDZ was more efficient than BA in inducing shoot regeneration in Pyrus species.

**Table 2.** Comparing the effect of two culture media and different concentrations of TDZ on adventitious shoot regeneration from whole leaves of Cydonia oblonga (cv. Isfahan).

<table>
<thead>
<tr>
<th>TDZ (μM)</th>
<th>Explants Producing Shoot (%)</th>
<th>Number of Shoots per Explant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NN medium</td>
<td>MS medium</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>6%</td>
<td>33%</td>
</tr>
<tr>
<td>5</td>
<td>6%</td>
<td>26%</td>
</tr>
<tr>
<td>10</td>
<td>26%</td>
<td>20%</td>
</tr>
<tr>
<td>20</td>
<td>33%</td>
<td>40%</td>
</tr>
<tr>
<td>40</td>
<td>46%</td>
<td>40%</td>
</tr>
</tbody>
</table>

**In vitro Elongation and Rooting of Regenerated Shoots:**

After 10 weeks, explants enfolded in shoot tip clumps (Fig. 3A) were transferred to MS hormone-free medium for 6 weeks, to induce elongation. The elongated shoots (Fig. 3B) were transferred to rooting MS medium supplemented with NAA (2.5 μM) and IBA (2.5 μM). Fig. 3C shows induction of roots in the elongated shoots, where 70% rooting was achieved. Rooted plantlets were acclimatized in the greenhouse successfully. Dolcet-Sanjuan et al. (1991) rooted shoots by culturing them first on medium containing 5 μM NAA for one week and then on auxin free medium for four weeks. The
rooting regime consisting of a sequence of NAA-containing and auxin-free medium, developed for micropropagated shoots also induced rooting on shoots regenerated from leaves.

Hennayake et al. (2003) used NAA and IBA for inducing roots in pear. They reported higher percentages of rooting were obtained with higher IBA concentration (5 mg l\(^{-1}\)). In most of the treatments, there was direct association between high frequencies of root formation and callus production. Most of the roots developed from the base of calli, which were initially produced from the cutting surface of the shoots.

![Image](image_url)

**Fig. 3.** Elongation and rooting of *Cydonia oblonga*: A) explant enfolded in shoot tip clumps; B) elongated shoot in MS medium containing BAP (2.2 µM), IBA (0.5 µM), GA\(^3\) (3.33) µM; C) induction of roots in elongated shoots in rooting medium.

**Flow Cytometry Analysis:**

Flow cytometry has been used as a reliable tool in measuring ploidy level and 2C DNA amounts in many plants including roses (Yokoya et al., 2000; Jowkar et al., 2009) and quince (Stanys et al., 2003). In the present study the flow cytometry histograms showed two peaks which were relevant to the quince donor shoot or regenerated shoot (peak 1) and parsley (peak 2), respectively (Fig. 4). The relative ratio of quince’s peak to the parsley's peak was estimated between 0.36 to 0.38 in all the samples, indicating similar ploidy level in donor and regenerated shoots. The 2C DNA amounts of the regenerated shoots and the donor shoots were measured between 1.60 to 1.69 pg, signifying that the DNA amount was stable throughout the regeneration process.

![Image](image_url)

**Fig. 4.** Flow cytometry histogram of (A) a donor shoot and (B) a regenerated shoot of quince. Peak 1 is the quince shoot and peak 2 is the parsley shoot (calibration standard). To evaluate the 2C DNA amounts, the mode of peak 1 was divided by the mode of peak 2 (P\(_1\)/P\(_2\)) and was incorporated in the formula given by Yokoya et al. (2000).

**CONCLUSION**

Present investigation was designed in order to study the adventitious shoot regeneration in quince (cv. Isfahan, known as 'KVD2') and use the results in our future gene transformation program. The maximum frequency of regeneration (46%) was obtained from the whole leaf explants cultured on NN medium containing 40 µM TDZ and 1 µM NAA. The highest mean number of shoots (7.46) was also obtained from whole leaf
explants cultured on MS medium containing 5 µM TDZ and 1 µM NAA. Flow cytometry analysis showed that regenerated shoots and donor shoots both were diploids and their 2C DNA amounts had not changed throughout the regeneration process. The relatively high frequency of regeneration and high number of regenerated shoots per explant coupled with the stability of the genome size are very important findings for the future modern breeding of quince.

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