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# DEFINING JUVENILITY IN THE APOMICTIC SYSTEM OF *MALUS HUPEHENSIS* FOR BASIC STUDIES ON REGENERATION AND TRANSFORMATION

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**Abstract**

The apomictic system in *Malus* was used as a model to examine the competence of genetically identical mature and juvenile tissues for regeneration and to undergo genetic transformation using *Agrobacterium tumefaciens*. Thidiazuron (TDZ) 1.00 mg $l^{-1}$ added in the regeneration media enhanced the frequency of regeneration and the mean number of shoots per explant and was found to be more effective then Benzyladenine (BA). Explants of juvenile (J) origin and those with the adaxial side (A) in contact with the medium exhibited a higher regeneration frequency and mean number of shoots regenerating per explant than explants of mature (M) origin and explants with the abaxial side (B) in contact with the medium. It was possible to obtain transgenic callus lines, and shoot organogenesis from the transformed callus using the hyper virulent strain EHA101 although *M. hupehensis* is reported to be resistant to crown gall disease (Sax, 1949). However, higher regeneration frequency from the J clones did not correlate with ease of transformation.

## Introduction

Defining efficient regeneration systems from somatic tissues or cells *via* organogenesis is critical for the development of a transformation system. In many instances, the lack of an efficient regeneration system is the major limiting factor preventing the development of gene transfer technologies for perennial crops. Juvenile tissues are often more responsive in culture to organogenesis and embryo genesis (Mitchell *et al.*, 2004) and are preferred sources of tissue for regeneration and transformation work. For this reason the apomictic system in *Malus* was used in this study as a model to generate genetically identical tissue culture lines that had entirely different developmental origins: either apomictic embryo derived tissues (juvenile clones) or somatic tissue from the adult/mature tree (mature clones). These two lines were compared for regeneration from leaf discs and potential to undergo genetic transformation using *Agrobacterium tumefaciens*. Studies included the influence of plant growth regulators, explant orientation and comparison of mature (M) and juvenile (J) phase in competence for regeneration and gene transformation when cultured under identical conditions. Although regeneration of adventitious shoots in apple from micro propagated shoot cultures of more than 20 scion cultivars and rootstocks has been described by several authors however, no report exists of regeneration and gene transfer in apomictic crab-apple species or on the regeneration capacity of mature and juvenile clones of identical genetic make-up.

## Materials and Methods

### Regeneration

**Regeneration media (RGN):** For shoot regeneration from leaf discs four culture media (RGN1 to RGN4) were used. These media were based on MS medium (macro-, microelements and vitamins) plus sorbitol 30.0 g $l^{-1}$, Gelrite 2.5 g $l^{-1}$, pH adjusted to 5.2 before autoclaving and with the supplements as in Table 1.

**Plant material and culture conditions:** The juvenile (J) clones were raised *In vitro* from germination of excised embryonic axes and their clonal fidelity was assessed using RAPD. The method of DNA extraction and RAPD amplification is described elsewhere (Ur-Rahman *et al*., 1997). From apices (shoot-tips) of the maternal/adult tree mature (M) clones were established *In vitro* (Ur-Rahman, 1997). Leaf discs were excised from young, fully expanded leaves from 4-week-old *In vitro* rooted shoots of mature (M), and juvenile (J) clones of *M. hupehensis* for regeneration and transformation competence. Twelve leaf discs from the same clone were cultured per Petri dish (90 mm containing approximately 25ml of medium), six with the adaxial side (A) and six with the abaxial side (B) in contact with the medium. For each regeneration medium (RGN1 to RGN4) five Petri dishes of each mature and juvenile clone were cultured. The cultures were incubated for six weeks in the dark at 25 ± 0.5$^{o}$C. After six weeks the data were recorded for the number of regenerating leaf discs and the number of shoots per regenerating leaf disc for each of the treatments. Analysis of variance was followed to analyse the data.

**Antibiotic effect to assess inherent sensitivity:** Any antibiotic effect was tested separately using the regeneration media that gave the best results in the first set of experiment. Cefotaxime sodium (Claforan$^{R}$ Roussel Laboratories Ltd.) and kanamycin monosulfate (Sigma) were filter- sterilized (Sterile Acrodisc$^{R}$ Gelman sciences, 0.2µm pore size) and added to the autoclaved regeneration media. Cefotaxime was added at 200.0 mg $l^{-1}$, alone and in combination with kanamycin. Kanamycin levels of 0.0, 25.0, 50.0, 100.0, 150.0 mg $l^{-1}$ were used to determine the optimal concentration for selection of transformants.

### Gene transformation

**Bacterial strain:** The hyper virulent *Agrobacterium tumefaciens* strain EHA101 harbouring the plasmid pSCV1.6 (Dr. Glyn Edwards, Shell Forestry, unpublished) was used. The plasmid contained the *gus* (scorable marker) gene and the *nptII* (selectable marker) gene, both driven by CaMV 35S promoter.

The use of an intron-containing â-glucuronidase (GUS) reporter gene has greatly facilitated analysis of transformation events in many plant species, and false readings caused by *Agrobacterium* contamination are eliminated due to the presence of an intron in the GUS coding region.

**Culture conditions:** Following the establishment of high levels of regeneration from leaf disc explants of *M. hupehensis*, *Agrobacterium tumefaciens*-mediated transformation was carried out, using the protocol for the cultivar Greensleeves (James *et al*., 1993).

**Table 1. Composition of regeneration media.**

| Media codes | BA mg $l^{-1}$ | NAA mg $l^{-1}$ | TDZ mg $l^{-1}$ |
|---|---|---|---|
| RGN1 | 5.0 | 0.5 | 0.1 |
| RGN2 | 2.5 | 0.5 | 0.2 |
| RGN3 | 1.5 | 0.5 | 0.5 |
| RGN4 | 1.0 | 0.5 | 1.0 |

**Table 2. Code and sequence of GUS and NPTII primers used for the analysis.**

| Code | Sequence (5′-3′) |
|---|---|
| GUS-27 | CCTGTAGAAACCCCAACCCGTG |
| GUS-392 | CCCGGCAATAACATACGGCGTG |
| APH/S-951 | GCCCTGAATGAACTGCAGGACGAGGC |
| APH/S-952 | GCAGGCATCGCCATGGGTCACGACGA |

The data were recorded for number of leaf discs regenerating calli. Twenty-five leaf discs with regenerated calli were picked at random for histochemical GUS assay. Regenerated calli were excised and sub cultured onto selection medium. These calli were kept for 5 months onto selection medium in the dark at 22°C. After 5 months, sub culturing onto fresh selection medium they were placed under a 16-h photoperiod at 22°C.

## Confirmation of transformation

**GUS staining:** The histochemical localization of GUS expression was assayed using X-Gluc (5-bromo-4-chloro-3-indolyl-â-D-glucuronic acid). Leaf discs with regenerated calli were immersed in an X-Gluc solution consisting of 2mM X-Gluc, 100mM Tris-HCl pH 7.0, 50mM NaCl, 2mM potassium ferricyanide and 0.1% (v/v) Triton X 100. The leaf discs were incubated over night at 37°C. Assayed leaf discs were observed under a binocular microscope.

**DNA extraction and PCR analysis:** For PCR analysis, DNA was extracted (Ur-Rahman *et al.*, 1997) from young expanding leaves of plantlets regenerated from calli (putatively transformed) and a non-transgenic control. Two specific oligonucleotide primers (Genosys Europe, Cambridge, England) used for the *gus* and the *nptII* genes are given in Table 2.

## Results

**Regeneration:** Morphogenesis occurred mostly on the cut edges and mid ribs in association with vascular tissue. Adventitious shoots developed with no intermediate callus phase although the leaf discs developed some callus along the cut edge.

**Frequency of shoot regeneration:** A significantly higher regeneration frequency ($p<0.001$) was exhibited on regeneration media RGN4 compared with the rest. The physiological state and orientation of the explant also influenced significantly ($p=0.001$, $p<0.001$ respectively) the regeneration frequency. Juvenile (J) explants with their adaxial side (A) in contact with the medium exhibited 90% regeneration whereas the regeneration frequency was 66.7% with explants of mature origin with their adaxial side (A) in contact with medium. Explants with their abaxial side (B) in contact with the medium exhibited 30% and 50% regeneration for the mature (M) and the juvenile (J) clone respectively on RGN4 (Table 3).

**Table 3. The effect of regeneration media RGN1-4, physiological state (mature, juvenile), and orientation of the explant (adaxial, abaxial side of the leaf disc in contact with the medium) on frequency of shoot regeneration in *Malus hupehensis* (after 6 weeks in dark at 25°C).**

| Regeneration media | Frequency of shoot regeneration (%) | | | |
|---|---|---|---|---|
| | Mature (M) | | Juvenile (J) | |
| | Adaxial (A) | Abaxial (B) | Adaxial (A) | Abaxial (B) |
| RGN1 | 16.7 | 6.7 | 26.7 | 6.7 |
| RGN2 | 30.0 | 6.7 | 50.0 | 6.7 |
| RGN3 | 40.0 | 16.7 | 53.3 | 33.3 |
| RGN4 | 66.7 | 30.0 | 90.0 | 50.0 |
| **Statistical significance:** | F. prob. | (32 d.f.) | $LSD_{5\%}$ | Sample size |
| Media | < 0.001 | | 10.35 | 60 |
| Physiological state (M×J) | = 0.001 | | 7.31 | 60 |
| Side of the explant (A×B) | < 0.001 | | 8.84 | 30 |

**Table 4. The effect of regeneration media RGN1-4, physiological state (mature, juvenile), and orientation of the explant (adaxial, abaxial side of the leaf disc in contact with the medium) on mean number of shoots/regenerating explant in *Malus hupehensis* (after 6 weeks in dark at 25°C).**

| Regeneration media | Mean no. of shoots/regenerating leaf disc | | | |
|---|---|---|---|---|
| | Mature (M) | | Juvenile (J) | |
| | Adaxial (A) | Abaxial (B) | Adaxial (A) | Abaxial (B) |
| RGN1 | 0.99 | 1.00 | 1.58 | 1.09 |
| RGN2 | 1.06 | 1.07 | 1.22 | 2.22 |
| RGN3 | 1.46 | 1.30 | 2.55 | 2.10 |
| RGN4 | 2.94 | 2.45 | 3.16 | 2.06 |
| **Statistical significance:** | F. prob. | (d.f.) | $LSD_{5\%}$ | Sample size |
| Media | < 0.001 | (30) | 0.41 | 60 |
| Physiological state (M×J) | = 0.003 | (30) | 0.29 | 60 |
| Side of the explant (A×B) | = 0.295 | (14) | 0.41 | 30 |

**Mean number of shoots/regenerating explant:** Regeneration medium RGN4 yielded a significantly higher ($p<0.001$) mean number of shoots per regenerating explant. The physiological state and orientation of the explant also influenced significantly ($p=0.003$, $p=0.295$ respectively) the mean number of shoots per regenerating explant: when the adaxial side (A) of the explant was in contact with the medium the mean number of shoots per regenerating explant was 3.16 for the juvenile (J) clone compared with 2.94 for the mature (M) clone. Explants with the abaxial side (B) in contact with the medium yielded an average of 2.45 and 2.06 shoots per regenerating leaf disc for the mature (M) and the juvenile (J) clone respectively (Table 4).

**Antibiotic effect:** The effect of the antibiotics, kanamycin and cefotaxime either singly or in combination, on regeneration and callus induction is summarised in Table 5. No shoot regeneration was observed on kanamycin-containing medium at any of the doses studied. However, on kanamycin at 25.0 mg $l^{-1}$ some callus was observed which only survived until the sixth week, whereas at concentrations 50.0 mg $l^{-1}$ or over kanamycin completely inhibited regeneration and callus formation. Cefotaxime (200.0 mg $l^{-1}$) enhanced the rate of shoot regeneration (100%, compared with 93.33% for the control). No regeneration was observed when kanamycin at 50.0 mg $l^{-1}$ was tested in combination with cefotaxime (200.0 mg $l^{-1}$).

**Table 5. The effect of added antibiotics to RGN4 on frequency of shoot regeneration/ callus induction from *in vitro* leaf discs of *Malus hupehensis* (juvenile), 6 weeks after incubation in the dark at 25°C.**

| Antibiotics (mg $l^{-1}$) | Frequency of shoot regeneration (%) | Callus induction (+) |
|---|---|---|
| **Kanamycin** | | |
| 0.00 | 93.33 | ++ |
| 25.0 | 0.00 | + |
| 50.0 | 0.00 | 0.00 |
| 100.0 | 0.00 | 0.00 |
| 150.0 | 0.00 | 0.00 |
| **Cefotaxime** | | |
| 200.0 | 100.0 | +++ |
| **Cefotaxime+Kanamycin** | | |
| 200.0+50.0 (Respectively) | 0.00 | 0.00 |

**Table 6. The effect of growth phase of leaf disc explants (mature, juvenile J) on frequency of calli development on selection media (RGN4 plus cefotaxime @ 200.0 and kanamycin @ 50.0 mg $l^{-1}$) 3, 9 weeks after transformation with *Agrobacterium tumefaciens* EHA101 (pSCV1.6) (explants were subcultured after 3 weeks, then after every 4th week).**

| No. of weeks on selection media | Total number of leaf discs | | No. of leaf discs developing calli (%) | |
|---|---|---|---|---|
| | Mature (M) | Juvenile (J) | Mature (M) | Juvenile (J) |
| 3 Weeks | 100 | 300 | 36 | 45 |
| 9 Weeks | 100 | 300 | 28 | 34 |

## Gene transformation

**Development of kanamycin-resistant calli and calli-induced shoots:** Co-cultivated leaf discs developed callus after 3-4 weeks on selection medium. There was no direct adventitious shoot regeneration. Twenty-five leaf discs with calli were picked randomly and hstochemical GUS-assay showed that all the leaf discs had one GUS-positive region at the mid rib on apical cut end of the leaf disc. The surviving calli after 9 weeks were removed surgically and sub cultured to fresh selection medium of the same composition. Callus growth was enhanced when the pieces were excised from the leaf discs. The frequency of surviving calli on selection medium, 3 and 9 weeks after co-cultivation ranged from 36 to 28% (leaf discs from mature explants) and 45 to 34% (leaf discs from juvenile explants) (Table 6). These calli were kept in dark at 22°C on selection medium for 5 months but there was no shoot regeneration. Twenty-five of these kanamycin resistant calli (hard globular, yellowish white colour) were picked and after taking small pieces from each for GUS-assay, calli were sub cultured onto selection medium, and kept in light (16-h) at 22°C. All 25 calli were histochemically GUS-positive. The calli in the light after one week turned light green with red pigmentation, and after about 5 weeks one of the calli developed shoot primordia from the base. The regenerated shoot had a stunted and hyperhydric phenotype with red pigmentation. The histochemical GUS-assay showed GUS-positive result for this shoot. A leaf sample was taken from the shoot for (DNA extraction) PCR analysis, to show the presence of the *gus* and *nptII* genes in the plant genome. Predicted internal fragments of the *gus* and *nptII* of about 366bp and

411bp respectively were amplified from the DNA sample of the shoot. No amplification occurred from DNA isolated from a control non-transformed plant. The shoot became necrotic and died before further analysis could be carried out.

## Discussion

In this study, the main factors affecting shoot regeneration were the regeneration media, explant origin (mature, juvenile), and orientation of the explant (adaxial or abaxial side in contact with regeneration media).

Regeneration media RGN1 to 4 contained both BA and TDZ at varying concentrations. Decreasing levels (5.0, 2.5, 1.5, 1.0 mg $l^{-1}$) of BA and increasing levels (0.1, 0.2, 0.5, 1.0 mg $l^{-1}$) of TDZ were combined in regeneration media RGN1 to 4 respectively. TDZ enhanced the frequency of regeneration and the mean number of shoots and was found to be more effective than BA. The results reported here confirm those of the Karami & Kordestani (2007). Explants of juvenile (J) origin and those with the adaxial side (A) in contact with the medium exhibited a higher regeneration frequency and mean number of shoots regenerating per explant than explants of mature (M) origin and explants with the abaxial side (B) in contact with the medium. Durkovic, (2003) assayed juvenile and fully mature *Acer caudatifolium* Hayata explants for their organogenic capacity and reported that mature explants failed in shoot regeneration: juvenile tissue grew at a significantly faster rate than mature phase tissue. Orientation of the leaf explants proved to be an important factor in increasing the regeneration response in apple and a possible reason for this may be increased oxygen exchange since stomata are located abaxially, and/or ability of the palisade parenchyma on the adaxial surface to transport nutrients and growth regulators from the medium more efficiently into the explant.

Cefotaxime has been used successfully to promote regeneration and, in transformation experiments, to eliminate several strains of *Agrobacterium tumefaciens* allowing the recovery of transgenic plants (Yu *et al*., 2001, Silva & Fukai, 2001). Kanamycin is used routinely to select transformants although certain species, e.g., walnut, are quite resistant to kanamycin, possibly due to the presence of endogenous non-specific kanamycin phosphotransferase activity (Prakash & Gurumurthi, 2005). In this study, 25.0 mg $l^{-1}$ of kanamycin for *M. hupehensis* as selection of transformed cells is critical for recovering transgenic plants and this has been an obstacle in several crops that are sensitive to kanamycin. Typically the optimum concentration of the selective agent should be sub-lethal, preventing regeneration without being too toxic to the target explant.

Transgenic callus lines and shoot organogenesis from the transformed callus were obtained following *Agrobacterium*-mediated transformation of leaf discs of *M. hupehensis*. This species is reported to be resistant to crown gall disease (Sax, 1949). The hyper virulent *A. tumefaciens* strain EHA101 harbouring with the binary vector pSCV1.6, having *gus* and *nptII* gene constructs with in the T-DNA borders was able to infect leaf disc explants. The transgenic nature of the regenerated calli was confirmed by histochemical GUS-assay. *M. hupehensis* exhibited a high frequency of adventitious shoot regeneration from leaf discs in the absence of kanamycin but kanamycin had a strong inhibitory effect on the regeneration of adventitious shoots. Ability to regenerate at higher frequency does not necessarily correlate with ease of transformation. There are

other factors at play therefore; conditions have to be established to optimise the selection pressure for the efficient recovery of transgenic plants, as the selection pressure can severely inhibit the differentiation of transgenic plants. Putatively transformed shoots showed hyperhydry, stunted growth, necrosis and subsequent death on shoot proliferation media. Shoot proliferation media and culture conditions therefore need to be manipulated to prevent the hyperhydry and necrosis of putatively transformed shoots.

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