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Genetic engineering of apple (*Malus domestica* Borkh.) for resistance to fungal diseases using g2ps1 gene from *Gerbera hybrida* (Asteraceae)

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Summary: In the present study, g_2ps_1 gene from *Gerbera hybrida* coding for 2-pyrone synthase which contribute for fungal and insect resistance was used. The aim was to work out an efficient approach of genetic transformation for apple cvs. 'Golden Delicious', 'Royal Gala' and 'MM111', 'M26' rootstocks for improving their fungal resistance using genetic engineering techniques. Adventitious shoot formation from leaf pieces of apples studied was achieved using middle leaf segments taken from the youngest leaves from *in vitro*-grown plants. Optimum conditions for ,direct' shoot organogenesis resulted in high regeneration efficiency of 90%,95%,92%,94% in the studied apples respectively. Putative transgenic shoots could be obtained on MS media with B5 Vitamins, 5.0 mg l^{-1} BAP, or 2.0 mg l^{-1} TDZ with 0.2 mg l^{-1} NAA in the presence of the selection agent "PPT" at 3.0-5.0 mgl^{-1}. Shoot multiplication of transgenic shoots was achieved on: MS + B5 vitamins + 1.0 mg l^{-1} BAP + 0.3 mg l^{-1} IBA, 0.2 mg l^{-1} GA3+1.0 g/l MES+ 30 g/l sucrose + 7.0 g/l Agar, with the selection agent PPT at 5.0 mg l^{-1} and were subcultured every 4 weeks in order to get sufficient material to confirm transformation of the putative shoots obtained. Six, seven, one and six transgenic clones of the apples studied respectively have been obtained and confirmed by selection on the media containing the selection agent "PPT" and by PCR analysis using the suitable primers in all clones obtained for the presence of the selection" bar gene (447 bp) and the gene-of- interest *"g2PS1"* (1244 bp), with transformation efficiency of 0.4%, 0.6%, 0.1% and 0.3% respectively. These transgenic clones were multiplied further *in vitro* in the presence of the selection agent 'PPT' and rooted *in vitro*. Rooted transgenic plantlets were successfully acclimatized and are being kept under-containment conditions according to the biosafety by-law in Syria to evaluate their performance for fungal resistance .

Abbreviations: **BAP**, N6-benzylamino-purine; **IBA**, Indole-3-butyric acid; **GA3**, Gibberelic acid; **MES**, 2-(N-Morpholino) Ethanesulfonic acid; **MS**, Murashige and Skoog medium (1962); **NAA**, a-naphthalene acetic acid; **PPT**, Glufosinate-amonnium Pestanal ® (Riedel-de Haen); **TDZ**, Thidiazuron (N-phenyl-N-1,2,3-thiadiazol-5-yl urea), **GD**, apple cv. Golden Delicious; **RG**, apple cv. Royal Gala

Keywords: *Agrobacterium tumefaciens*, Genetic transformation, g_2ps_1 gene, *Gerbera hybrid*, *In vitro* culture, Organogenesis

Introduction

Apple (*Malus domestica* Borkh.) is one of the most important fruits in temperate zones with a total world production amounted to 76.3 million t/year in 2012 (FAO, 2012). Conventional breeding of apple is very long term and cannot reproduce the desirable qualities of our best commercial varieties and rootstocks. However, genetic transformation is a key process to sustain its demand by permitting the potential enhancement of existing cultivars as well as the development of new cultivars resistant to pests, diseases, and storage problems that occur in the major production areas (Polanco *et al.* 2010). It offers an attractive alternative to conventional breeding for the creation of resistant varieties since it is faster, can use genes from many sources, and will preserve the desirable qualities of the transformed variety or rootstock. (Aldwinckle *et al.* 2000). On the other hand, genetically modified (GM) crops have gained ground on their conventional counterparts. Biotech crop hectares increased by an unprecedented 100–fold from 1.7 million hectares in 1996, to over 170 million hectares in 2012. Of about 1.5 billion hectares of arable land worldwide, about 12% were used to plant GM crops in 2012 (James 2013).

Gene transfer manipulations are used for genetic modification of important characters in apple such as resistance to diseases. Some of these studies included the chosen variaties and rootstocks in the present work. These included genetic transformation of Royal Gala (Hyung *et al.* 1995, Liu *et al*. 1998, 2001; DeBondet *et al*. 1994, 1996; Norlli *et al*. 1999, Schaart *et al*. 1995, Puite and Schaart 1996; Yao *et al*. 1995; Faize *et al*. 2003, 2004; Liu *et al*. 1998, 2001), Golden Delicious (Schaart *et al*. 1995; Puite and Schaart 1996; Maximova *et al*.1998), M26 (Norlli *et al*. 1994;

Welander *et al*. 1998; Holefors *et al*. 1998, 2000), while no single study was published on genetic transformation of the apple rootstock MM111. However, the traiats expressd in transformation of these apples included: resistance to fire blight *Erwinina amylovora* in M26 (Norelli *et al* .1994, ko *et al*. 2000, Aldwinckle *et al*. 2003; Hanke *et al*. 2000; Abdul -Kader *et al*. 1999, Aldwinckle *et al*. 2003; Malony *et al.* 2007a); Royal Gala ((Liu *et al*. 199, 2001), insect resistance in RG (Markwic *et al*. 2003), fungal resistance in M26 (Markwic *et al*. 2003; Xue *et al*. 2008; Holfors *et al*. 2000), RG (Artlip *et al*. 2007), Color modification in RG (Espley *et al*. 2007), Modified metabolism in RG (Hrazdina *et al*. 2003), Cell adhesion in RG (Alkinson *et al.* 2002), Promoter studies in RG and M26 (Malony *et al*. 2006), M26 (Norelli *et al*. 2007), Selectable markerstudies in M26 (Zhu *et al*. 2004, Malony *et al*. 2007 b). On the other hand, development of an effective system for gene transfer in the different Rosaceae species depends largely on the availability of tissue culture techniques that permit regeneration of shoots, selection of transformants, and propagation of transgenic plants. Increasing leaf regeneration efficiency is critical for the development of a transformation system in the Rosaceae family using an *Agrobacterium tumefaciens* vector or by biolistic process (Aldwinckle and Malnoy 2009).

g2ps1 gene codes for 2- pyrone synthase (2ps) from *Gerbera hybrida* (Helariutta *et al*. 1995). The expression of this gene is suitable for the manipulation of the phytoalexin spectrum. The Chalcone Synthase -2 gene was previously considered as an unusual member of a chalcone synthase (CHS) gene family in the ornamental plant *Gerbera hybrida* (*Asteraceae*). GCHs gene utilizes acetyl-coA and 2-malonyl-co-A for the biosynthesis of two types of 6-Methyl-4-hydroxy-2-pyrone derivatives, 'gerberin' and 'parasorboside', which contribute for insect and fungal pathogen resistance as well as medical interest. Later, the GCHS2 gene was renamed as the *g2ps1* gene based on its function associated with 2-pyrone Synthesizing.

Because of the high susceptibility to fungal diseases of the most important commercial apple cultivars and rootstocks, genetic transformation has been one good method for the development of resistant cultivars. Therefore, the aim of the present study was to work out an efficient approach of regeneration system directly from leaf discs and genetic transformation of apple cvs. ‚Golden Delicious', ‚Royal Gala' and ‚MM111', ‚M26' rootstocks for improving their fungal resistance using the g2PS1 gene from *Gerbera hybrida.*

Materials and methods

This study has been carried out at the General Commission for Scientific Agricultural Research (GCSAR), Biotechnology Department, Genetic Engineering Division during the period 2010-2013. The cloning of the g_2ps_1 gene was carried out at the section of Plant Biotechnology, Institute of Plant Genetics, Leibniz Hannover University, Hannover, Germany.

Adventitious shoot regeneration: Shoot cultures were obtained from *in vitro* proliferating shoots of cvs. Golden Delicious, Royal Gala and MM111, M26 apple rootstocks maintained at the Department of Biotechnology which has been subcultured on storage and proliferation media for six years.

The first apical 3-4 youngest unfolded leaves but still in an active state of leaf expansion showing no signs of chlorosis with light green color and strong vein pattern on back of the leaf on the shoot apex were harvested from 3-weeks old proliferating cultures and cut to three parts (upper, middle and lower), but using only the middle part of leaf for regeneration and transformation based on our previous explorations (Ali Bacha *et al*. 2009). Explants were cultured in 90 mm-diameter Petri dishes with 20 ml of different media as shown in table 1 placing the abaxial face in contact with the medium with five leaf sections were cultured in each plate. All media were adjusted to pH 5.7 prior to autoclaving at 121ºC, 1.4 kg/cm^2 for 20 min.

For 'direct' adventitious regeneration, leaves were cultured on different media in darkness for an initial 3 weeks at 25 °C±1 and then transferred to a 16 h /8 h light/ darkness regime for further 4 weeks to assess morphogenetic responses.

For shoot multiplication of regenerated shoots, the concentrations of NAA, BAP, were investigated previously where it was found that the optimum shoot multiplication media consisted of MS salts, 30 g l^{-1} sucrose, 1 mg l^{-1} BAP, 0.3 mg l^{-1} IBA, 0.2 mg l^{-1} GA3 and 6 g l^{-1} Agar (pH 5.7) (Ali Bacha.,*et al*. 2009).

Table 1. Media used for regeneration of apple using leaf discs as explants

Media Composition	Code
MS+2.5 g/l Gelrite + 30 g/l Sucrose (control medium)	MS=R0
MS+2.0 mg/l **TDZ**+0.2 mg/l NAA+2.5 g/l Gelrite + 30 g/l Sucrose	R1
MS+ 5.0 mg/l **BAP**+0.2 mg/l NAA+2.5 g/l Gelrite + 30 g/l Sucrose	R2
MS+ 0.5 mg/l **TDZ** +0.5 mg/l **BAP** + 0.2 mg/l NAA+2.5 g/l Gelrite + 30 g/l Sucrose	R3
N6 macro + MS micro + B5 vitamin + 5.0 mg/l **BA**P + 0.2 mg/l NAA+ 2.5 g/l Gelrite + 30 g/l Sucrose	R4

Optimization of genetic transformation of apple studied: Several experiments of *Agrobacterium*–mediated genetic transformation of apples studied have been done to transfer *g2ps1* gene harbored on a pGreenII-35S-g2PS1 plasmid vector (Fig. 1) in order to evaluate its efficiency in conferring tolerance to fungal diseases. Young green leaves were used for transformation using several regeneration media. Young light green leaves were treated with the non-traumatic forcep as recommended by Norelli *et al.* 1996 to induce wounding and co-cultured for three days with *Agrobacterium* strain EHA105 harbouring pSoup-pGreenII-35S-g_2ps_1 vector for 3 days (Fig. 1). Cultures were incubated in full darkness for an

initial 3 weeks in the growth room with temperature of 25 °C±1 °C. Cultures were then transferred to exposed distributed light for further one week, where after they were transferred to conditions of a 16/8 h light/darkness regime with 50 µmol $m^{-2}s^{-1}$ photon flux to assess their morphogenetic responses.

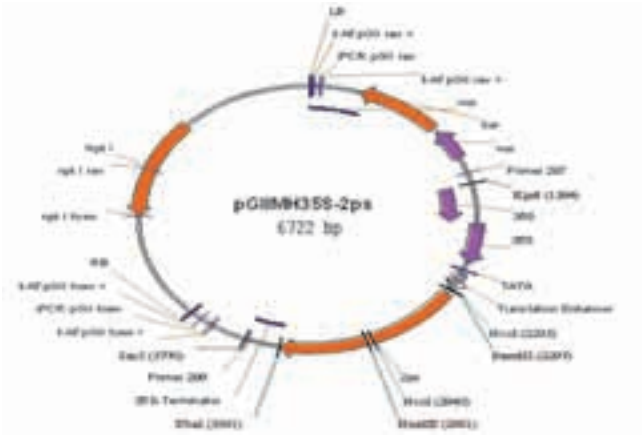

Fig. 1. Map of the pGreen II 35S-*g2PS1* harboring the *g2ps1* used in the present study

Regenerated shoots were sub-cultured for multiplication on media containing growth regulators in the presence of the selection agent „PPT" at 3.0-5.0 mg l^{-1} in order to get sufficient material to confirm transformation of the putative shoots obtained. Transformation was confirmed by PCR for the presence of „bar" and „*g2PS1*" genes using specific primers as follows:

For bar gene (447 bp):
bar for: 5'-GATTTCGGTGACGGGCAGGA -3'
bar rev: 5'-TGCGGCTCGGTACGGAAGTT -3'

For g2ps1 gene (1244 bp):
(g2ps1 for): 5'-CCG ACG GTA CCC CCC CTG CAG GTC GAC GG-3'
(g2ps1 rev.): 5'- GTC GGT CTA GAT CAG TTT CCA TTG GCA ACC GC-3'

The PCR program consisted of an intial incubation at 94 °C for 3 min. for bar gene and 1 min. for g2ps1 gene, followed by 30 cycles for both genes as follows: denatuation at 95 °C for 1 min., primer annealing at 60 °C for 1 min. and extension at 72 °C for 1 min., then final extension at 72 °C for 10 min. and hold at 4 °C.

Regenerated transgenic shoots were transferred to proliferation media with 1.0 mg/l BAP, 0.3 mg/l IBA and 0.2 mg/l GA3. The media used were adjusted to pH 5.7 prior to autoclaving at 121ºC, 1.4 kg/cm^2 for 20 min.

Shoots were then transferred to rooting media with half strength MS medium supplemented with 1.0 mg/l IBA under light intensity of 5.0 W.m^{-2} (16 h per day). Rooted transgenic clones were then acclimatized gradually under- containment conditions.

Experimental design

Scoring for adventitious shoot regeneration and transformation was done after 8 weeks of culture. The following criteria were evaluated: number of explants that produced adventitious shoots and number of shoots produced by each explant showing organogenesis. Each Petri dish was a repetition in a randomized block experimental design. For each treatment, 8 petri dishes (i.e. 40 explants were used). 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 (Michigan State University). All the experiments described here were repeated at least twice and results were pooled.

Results

Explants started regeneration after at least 4 weeks of beginning of the regeneration experiment and continued until the eighth week, where there no regeneration could be seen afterwards. Numerous shoots were produced in each Petri dish between 4-8 weeks.

A regeneration system from leaf discs of apples studied was established on MS based media and supplemented with 1.0 g/l MES, 5 mg/l BA or 2.0 mg/l TDZ , 0.2 mg/l NAA, 30 g/l Sucrose, 2.5 g/l Gerlite. Organogenesis did not occur on media without cytokinins.

The effects of combinations of BAP x NAA and explants on shoot multiplication were statistically significant. ($p<0.05$) *(Table 1).* Most of explants produced shoots and green shoot initials were seen on a range of media containing BAP or TDZ and NAA within eight weeks. The highest percentage of regenerated shoots (95%) was achieved on a range of media supplemented with 0.5 mg/l or 5.0 mg/l BAP + 0.2 mg/l NAA or 2.0 mg/l TDZ + 0.2 mg/l NAA in leaf explants. Whereas, the highest shoot multiplication capacity (82%) was obtained on a medium containing 0.5 mg/l BAP, 0.5 mg/l TDZ + 0. 2 mg/l NAA.

Explants were tested with special attention to the regeneration rate. A high regeneration frequency (92%, 95% and 90% , 94%) with good regeneration ability (4.0, 5.6, 4.1 and 4.5 new shoots/explants could be obtained in apples studied respectively on MS basal medium containing 2.0 mg/l TDZ and 0.2 mg/l NAA and 3% sucrose. Middle leaf segments from 21-days old leaves were used and showed higher regenerative responses (table 3). Reducing TDZ concentration to 0.5 mg/l, but with adding 0.5 mg/l BAP could result also in high regeneration frequency (82%, 90% and 79%, 85% respectively) *(Tables 2 & 3).* Adventurous shoots preferentially located along the cut basal edge of the explants were clearly visible after four to five weeks of culture.

Table 2. Mean shoot number regenerated *in vitro* (organogenesis ability) per explants in response to the media tested for the apple cvs. and rootstocks

	Apple cvs.		**Apple Rootstocks**	
Media	Golden Delicious	Royal Gala	MM111	M26
R01	4.025a ±0.141	5.615a ±0.121	4.100a ±0.171	4.500a ±0.160
R02	2.225c ± 0.067	2.505c ± 0.67	2.512d ± 0.80	2.100c ± 0.80
R03	2.525b ± 0.080	4.212b ± 0.080	2.800b ± 0.103	3.100b ± 0.103
R04	1.575d± 0.080	2.00b ±0.80	1.65c ±0.80	1.500c ±0.60
RO	0.00	0.00	0.00	0.00
LSD 0.05	0.290	0.310	0.293	0.301

Note: *Values within each column followed by different letters are significantly different at the 0.05 probability level (a<0.05%) using Duncan's multiple range test.

Table 3. Organogenesis/ Regeneration % *in vitro* in Apple cvs. and rootstocks on different media using middle part of the leaves

Media	Apple CVs.		Apple Rootstocks	
	Golden Delicious	Royal Gala	MM111	M26
R01	92	95	90	94
R02	20	20	18	20
R03	82	90	79	85
R04	36	30	10	15
RO	0	0	0	0

Overall organogenesis was satisfactory in all treatments both in terms of regeneration rate and of adventitious shoot production using mid-leaf explants *(Figure 2).* Further, no abnormality, necrosis or chlorosis was observed during the culture.

Putative transgenic shoots could be obtained on MS media with B5 Vitamins, 5.0 mg l^{-1} BAP, or 2.0 mg l^{-1} TDZ with 0.2 mg l^{-1} NAA in the presence of the selection agent „PPT" at 3.0-5.0 mg l^{-1}. Appearance of putative transgenic shoot initials on leaf explants could be seen after 4–8 weeks of culture. However, excessive wounding could be detrimental initially by slowing down regeneration, due to oxidation of the tissues.

Transformed explants regenerated on media containing the selection agent. They were divided and subcultured further on media with PPT at concentrations of 3–5 mg/l and could survive in the presence of the selection agent „PPT",while the non-transformed explants were died (brown tissues) *(Figure 3).*

These survivd putative transformants were further subcultured and proliferated further in vitro in the presence of the selection agent ‚PPT' and rooted also on media containing PPT *(Figure 4).* As for the performance of the adventitious shoots, they were multiplied and rooted easily according to the protocols developed by Altinawi *et al.* 2008 for the cv. Golden Delicious and Alrihani *et al.* 2008 for MM111 rootstock..

Molecular Confirmation of Transformation by PCR

Confirmation of putative transgenic regenerants was carried out by PCR. Specific primers for detection of the selection bar gene and also for the gene of interest „g2PS1'

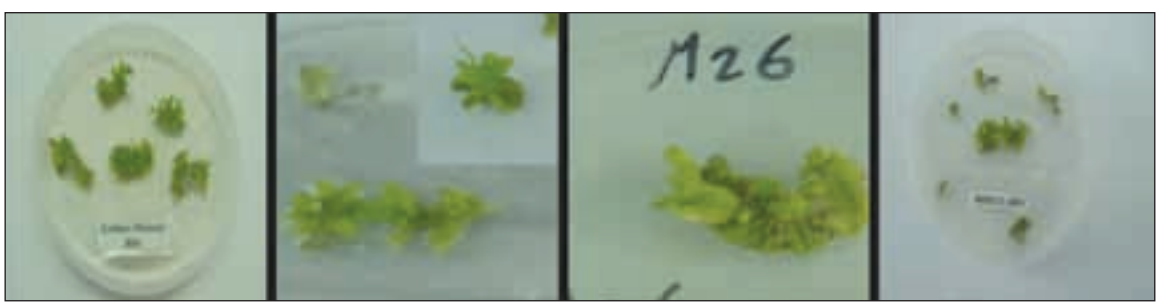

Figure 2

Figure 3

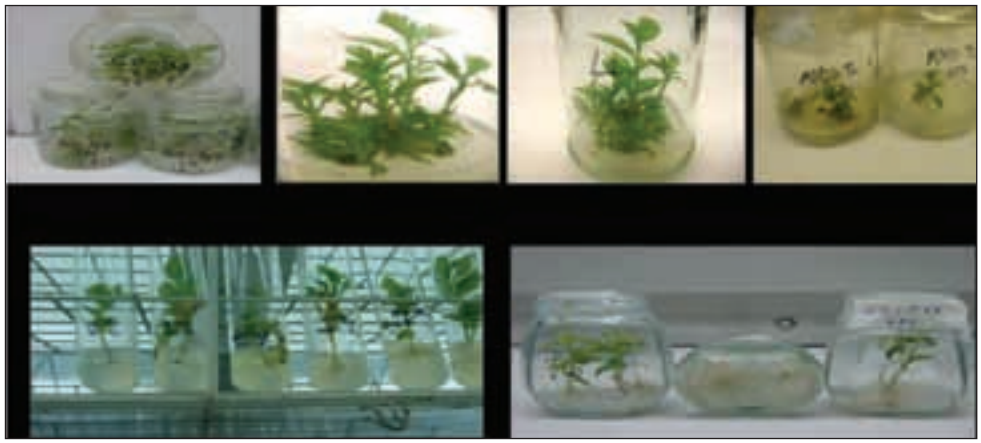

Figure 4

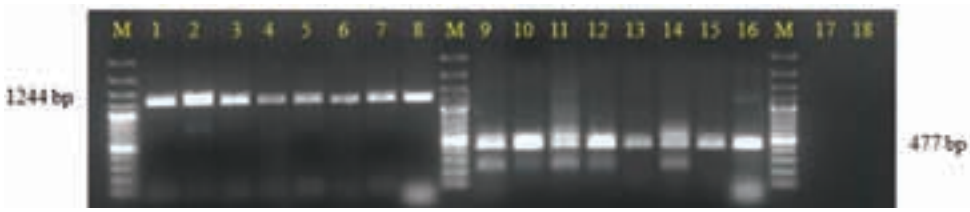

Figure 5. Molecular Confirmation of Transformation by PCR for the gene of interest g2ps1 and the selection agent bar gene.
Lanes: 1,2 GD; 3,4: M26; 5,6: RG, 7: MM111, 8: positive control.
Lanes: 9,10: GD; 11,12: M26; 13,14: RG; 15: MM111, 16: positive control; 17: water,18: negative control (DNA isolated from non-transformed apple)
M: 100 bp marker

showed the transfer of the gene according to the expected band size of 447 bp for the bar gene and 1244 bp for the gene-of-interest „g2PS1" gene, while no band was shown in the negative control -not transformed apple-, nor in the water containing lane as negative control (well, containing no DNA) *(Figure 5).* Six, seven, one and six transgenic clones of the apples studied respectively have been obtained and confirmed by selection on the media containing the selection agent „PPT" and by PCR analysis using the suitable primers in all clones obtained for the presence of the selection" bar gene (447 bp) and the gene-of- interest *"g2PS1"* (1244 bp), with transformation efficiency of 0.4%, 0.6%, 0.1% and 0.3% respectively.

In vitro rooting of transgenics

Roots were formed easily in the presence of the selection agent PPT within 2-4 weeks with about 85 % efficiency in apples studied (fig. 4)

Acclimatization of rooted transgenics

Rooted transgenic plantlets were successfully acclimatized to ambient conditions with about 70% efficiency and kept in the greenhouse under containment conditions according to the biosafety by-law in Syria to evaluate their performance for fungal resistance *(Figure 6).*

Figure 6

Discussion

Cytokinins such as TDZ and BAP have considerrable effects in inducing regeneration in most woody plants. For shoot regeneration from leaf discs, a range of BAP and TDZ concentrations was examined. Although induction of shoots was observed in most media tested in the present study, however, there was statistically significant difference between the TDZ and BA. However, other studies show that TDZ is more effective than BAP (Korban *et al.*,1992; DeBond *et al.*, 1996; Sarwar and Sirvin,1997; Hammatt and Grant,1998). Whilst ‚Greensleeves' apple

responded in line with published data, ‚Bramley' produced significantly fewer shoots. ‚Bramley' shoots were obtained from 5.0 mg l^{-1} BAP and 1 mg l^{-1} NAA. TDZ did not increase regeneration significantly**.** TDZ was also used to induce adventitious shoot regeneration in many other plants including *Phaseolus vulgaris* L (Malik and Saxena 1992) henbane *Hyoscyamus niger* L., (Uranbey 2005), mulberry (Thomas 2003), Lentil (*Lens culinaris* Medik.) (Khawar *et al*. 2004); peanut (*Arachis hypogaea*) (Kanyand et al. 1994). McAdam-O Connell *et al.* (2004) developed a leaf disk regeneration system for ‚Bramley's' seedling apple (*Malus* × *domestica* Borkh.) and obtained shoots using MS media with 5.0 mg l^{-1} BAP and 1 mg l^{-1} NAA, whereas TDZ did not increase regeneration significantly**.** Our results, however, are in contrary to such findings where TDZ proved to be more efficient in inducing regeneration in apples studied than BAP. A detailed review on apple micropropagation also showed using mostly both BAP and TDZ for inducing regeneration in many apples varities and rootstock with different responses (Dobranszki and Teixeira da Silva 2010). In the present study, the percentage of explants producing shoots and the number of shoots per explant were influenced by the type and concentrations of TDZ and BAP tested (p.0.05). The percentage of regenerated shoots fluctuated between 6- 95% while the highest number of shoots per explant ranged between 4 and 5.6 which occurred with 2.0 mg/l TDZ and 0.20 mg/l NAA. Considering both percentage of explants producing shoots and the number of shoots per explant, the best shoot multiplication was achieved on media supplemented with 2.0 mg/l TDZ and 0.20 mg/l NAA. Drastic reductions in shoot regeneration were also observed when decreasing of concentrations of TDZ or replacing it with BAP. This effect of TDZ is in conformity with many studies where shoot organogenesis of some crops in tissue culture have been achieved using Thidiazuron (TDZ), a substituted phenylurea compound with cytokinin activity (Malik and Saxena, 1992; Kanyand *et al*., 1994; Kim *et al*., 1997; Jain and Rashid, 2001, Hosseini and Rashid, 2003; Thomas, 2003; Uranbey, 2005). Gill and Saxena (1992) suggested a crucial role of TDZ in the interaction with endogenous hormones in reprogramming the mode of morphogenesis from organogenesis to somatic embryogenesis possibly by releasing, synthesizing, protecting or even inhibiting auxins *in situ* in combination with other sub-cellular metabolic changes, particularly in key regulatory enzyme and related proteins.

Similarly, auxins such as IAA, NAA, IBA in combination with cytokinins have also great effect on regeneration (Yancheva *et al*., 2003). Frequency of shoot organogenesis may be increased with combinations of TDZ and NAA. Combinations of TDZ-NAA in the media revealed an efficient pathway for shoot proliferation in leaves of apple. In the present study, it was observed that the use of NAA with TDZ produced satisfactory responses that might be the best treatment to eliminate the secretion of phenolic substances and this effect might be also due to the oxidation of phenols by auxin oxidase.

On the other hand, regeneration of adventitious shoots is still difficult in many species and cultivars. The optimum conditions for shoot regeneration vary according to the genotype thus methods should be fitted for each genotype. Regeneration efficiencies for apples (up to 50% regeneration rate for several genotypes) reported could be further improved with new methods. Moreover, there are some cultivars without published regeneration methods (Tabori 2011). There are two possible reasons why regeneration ability in plant tissue culture (PTC) differs from study to study. In life, not all beings are born equal. In PTC, too, not all explants have the same regeneration capacity. A plethora of factors influence the organogenic outcome of an explant in PTC, but differences in production, yield and organogenic output are all measured by one factor, and one factor alone: the size of the explant. In this ground-breaking paper, we put forward a radical notion that would attempt to allow for the direct comparison of organogenic potential of PTCs of the same cultivar or species conducted in different studies or laboratories. The prototype concept, the growth correction factor or GCF, has been tested on a model species, apple (*Malus* sp.) (Teixeira da Silva and Dobránszki 2011).

Five-minute daily exposures of leaf explants to red light (651 nm) suppressed adventitious shoot formation by 80%; five-minute exposure to far-red light (729 nm) immediately following the red light counteracted the red suppression. (Liu *et al*. 1983). In our experiments, however, we incubated explants for the first three weeks in complete darkness and did not expose explants to any source of light. Dufour (1990) obtained improved yield in *in vitro* adventitious regeneration in apple cultivars ‚Granny' Smith', ‚Mark', ‚Novole', ‚Lancep' and ‚Cepiland with significant increase in the number of regenerated shoots from ‚Gala' and ‚Golden Delicious'. He regenerated Plants from callus or directly from leaves from micropropagated plants. He got 100% regenerating leaves with an average of 14.2 regenerated shoots per leaf in ‚Gala', *In vitro* adventitious regeneration in apple cultivars ‚Granny' Smith', ‚Mark', ‚Novole', ‚Lancep' and ‚Cepiland' was reported with a significant increase in the number of regenerated shoots from ‚Gala' and ‚Golden Delicious'.

Our preliminary experiments of transformation showed that treating the leaves with the non-traumatic forcep resulted in a higher and faster organogenic and transformation responses, due to severe wounding and Agrobacterium-mediated gene transfer in accord with what was observed in the previous experiments by Norelli *et al.* 1996. This also confirms earlier observations (Ferradini *et al.* 1996, Sicurani *et al.* 2001) showing that leaves are good explants for adventitious shoot formation. However, it should be pointed out that selection, excision, wounding and arrangement on the medium was time-consuming and labor- intensive. However, it worth it since wounding of tissues boosted the regeneration efficiency of transformants. Hemerly *et al.* (1993) explained that wounding of plant tissues triggers the expression of genes in cell division and differentiation. The positive effect of wounding on regeneration was demonstrated for M26

apple rootstock by Sicurani *et al.* 2001 and also in other species (Piccioni and Valecchi, 1996).

While, on the other hand, genetic engineering has been used very successfully mostly with other crops than apple, which included mainly: corn, cotton, soybean, canola, tomato and papaya to produce disease, insect, and herbicide-resistant varieties that were grown on over 170 million hectares of GM crops worldwide in 2012. Twenty-eight countries planted GM crops in 2012, but most were grown in just five countries: The United States, Brazil, Argentina, Canada and India (James 2013). However, such technology should solve many of our apple cultivation diseases. It will allow us to improve the shortcomings of our present varieties and rootstocks, without altering their desirable features, especially familiarity to nurseries and growers, and recognition in the market by brokers, supermarkets, and consumer (Aldwinckle *et al*. 2000). Polanco *et al*. (2010) summarize in their reiew the advances of genetic engineering applied to the development of resistant apple cultivars to fungus disease, with particular attention in the generation of apples resistant to apple scab Venturia inaequalis. The present work contribute to the researcher efforts to produce fungal resistant apple.

Finally, it might be relevant to mention that no environmental risk studies specific to transformed apple have been published. Probably researchers are still concerned with producing acceptable GM apple cultivars with commercial interest and having environmental benefits, such as reduction of pesticides use (James *et al*., 2003). Under these circumstances, the commercialization of transgenic apple carrying a DNA from different species or genera, in the near future is certain (Polanco *et al*. 2010).

Conclusions

- An efficient approach of regeneration from leaf discs and genetic transformation for apple cvs. 'Golden Delicious', 'Royal Gala' and 'MM111', 'M26' rootstocks were worked out for improving their fungal resistance using genetic engineering techniques.
- From the present study, it was established that cytokinins TDZ and BAP played an important role in induction of direct shoot organogenesis in in vitro leaf discs of apples studies and the response could be further improved by combination of TDZ with the auxin NAA in the regeneration medium. Though TDZ proved to be superior to BAP in inducing regention, however, BAP has economic advangave over TDZ and can replace it. Further, efficiency of direct shoot organogenesis and transformation in the presence of the selection agent PPT could be substantially improved by wounding leaves with non-traumatic forcep treatment before culturing them on cocultivation medium.
- Transgenic apples harbouring *g2ps1* gene from *Gerbera hybrida* that confer fungal resistance were obtained. Six, seven, one and six transgenic clones of the GD, RG, MM111 and M26 apples studied respectively have been obtained and confirmed by selection on the media containing the selection agent "PPT" at high concentration of 5.0 mg/l and by PCR analysis using the suitable primers in all clones obtained for the presence of the selection" bar gene and the gene-of-interest *"g2PS1*", with satisfactory transformation efficiency. These transgenic clones were multiplied further and rooted *in vitro* also in the presence of the selection agent 'PPT'. Rooted transgenic plantlets were successfully acclimatized and are being kept under-containment conditions to evaluate their performance for fungal resistance.

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