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Genetic transformation of apple for increasing its resistance to fungal diseases

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Abstract

The aim was to establish an efficient and practical reproducible approach of genetic transformation for apple cvs. 'Golden Delicious', 'Royal Gala' and 'MM111', 'M26' rootstocks for improving their fungal resistance using genetic engineering techniques. 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}. Transgenic clones of the apples studied 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 and the gene-of-interest "g2PS1". Results of DNA sequence analysis of the transgenic plants also proved the successful transformation and had 97 to 99% sequence homology with the gerbera hybrida mRNA for 2-pyrone synthase g2ps1 gene (accession no. Z38097.2). These transgenic clones were multiplied further and rooted in vitro in the presence of the selection agent 'PPT'. 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.

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, 2013). 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). Development of genetic transformation systems requires efficient and reliable methods for plant regeneration from transformed cells. Successful utilization of plant biotechnology for plant improvement is a principal need for genetic transformation studies and requires the development of an efficient and reliable in vitro shoot regeneration system from cultured cells or tissues. On the other hand, micro propagation represents a widely known method for plant propagation. However, it still has some limitations for wider applications in nurseries. Proliferation rates are not always satisfactory. Adventitious shoot proliferation prove to be a more productive pathway than auxiliary shoot proliferation which is the most commonly used method in commercial laboratories. Usually, regeneration in woody plants of Rosaceae is associated with explant infection and phenolics exudation problems. Adventitious shoot proliferation from leaf blades has demonstrated high productive potential in apple cultivars and a few clonal rootstocks as well as other woody species (Korban et al., 1992; Yepes and Aldwinckle, 1994; Ancherani et al., 1990; Caboni et al.,1996; Ferradini et al., 1996; Modgil et al., 1999; Fiola et al.,1990; Mencuccini and Rugini,1993; Famiani et al., 1994; Sicurani et al., 2001; Liu et al., 1983; Wilson and James, 2003; James et al.,1990; Famiani et al.,1994, Sriskandarajah et al., 1994; Yao et al.,1995; Puit, and Schaart ,1996, Norelli et al.,1996, 1999; Sicurani et al.,2001, Szankowski et al., 2001, Welander et al.,2004, etc.) However, prior to these successful works, efficient regeneration systems have been worked out. 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). Genetic Engineering is first instance addresses improvement of breeding material by introducing special valuable genes from different germplasm or other sources. Researchers can now take a single gene from a plant or animal cell and insert it into another species to give that species a desired characteristic, such as resistance to a destructive pest or disease. The result is commonly referred to as a genetically modified organism (GMO), or as a living modified organism (LMO), resulting from modern biotechnology. 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 oftissue 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 hybrid* (*Asteraceae*). GCHs gene utilizes acetyl-coA and 2-malonylco- 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 hybrid*.

Materials and Methods

Place of Study: This study have been carried out at GCSAR, Biotechnology Department, Genetic Engineering Division.

Plant material: Shoot cultures used in the present study were obtained from *in vitro* proliferating shoots of **MM111, M26** apple rootstocks and the most popular grown apple **cvs. Golden Delicious, Royal Gala** maintained at the Dept. of Biotechnology, GCSAR that has been sub cultured on proliferation media for three years (Al-rihani *et al.* 2008 and Al-tinawi *et al.* 2009)., The first apical 3-4 leaves were collected from the proliferated shoots for the regeneration experiments as follows: - Whole leaf (control). - Upper part of leaf -Middle part of leaf - Lower part of leaf. Explants were cultured in 90 mm-diameter Petri dishes with 20 ml of different media as shown in table 1. Adventitious shoot regeneration system for apple cvs. Golden Delicious, Royal Gala and MM111, M26 rootstocks (*Malus domestica* L.) using leaf explants obtained from 21

days-old *in vitro* grown proliferation shoots were optimized. Influence of different combinations of growth regulators at different concentrations on regeneration ability including Thidiazuron (TDZ), N6-benzylamino-purine (BAP), α -naphthalene acetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were studied. Leaves were cultured on Murashige and Skoog medium (MS), B5 vitamins, sucrose, solidified with Gerlite and supplemented with different concentrations and combinations of TDZ, or BAP in combination with NAA or 2, 4-D. Eight hormonal combinations were tried. 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 and 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). Adventitious shoot regeneration, followed by transferring regenerated shoots to rooting MS basal medium with macronutrient salts ($_{1/2}$ concentration), micronutrients, Myo-inositol, Thiamine HCl, sucrose and agar with, indole-3-butyric acid (IBA), under a light intensity of 5.0 W.m^{-2} (16 h per day).(Ali Bacha.,*et al*. 2009).

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

Contents	**Media**
MS+2.5 g/l Gelrit**e** + 30 g/l Sucrose	MS=R0
MS+2 mg/l **TDZ**+0.2 mg/l NAA+2.5 g/l Gelrite + 30 g/l Sucrose	R1
MS+ 5 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 mg/l BAP +0.2 mg/l NAA +2.5g/l Gelrite +30 g/l Sucrose	R4
MS+5 mg/l BAP + 0.2 mg/l 2,4-D +2.5 g/l Gelrite + 30 g/l Sucrose+1g/lMES	R05

Optimization of genetic transformation of apple cvs. 'Golden Delicious', Royal Gala and 'MM111', M26 root stocks: For genetic transformation, the system consisted of the following steps:

1- **Establishment of in vitro cultures** of the apple cvs. 'Golden Delicious' Royal Gala and 'MM111', M26 rootstocks,
2- **Developing an efficient regeneration system** via direct organogenesis from in vitro-grown leaf pieces,
3- **Genetic transformation of apples studied via Agrobacterium** tumefaciens harbouring *g2ps1* gene by co-cultivation and regeneration from leaf pieces in the presence of the selection agent 'Glufosinate-ammonium Pestanal' (PPT) for herbicide resistance,
4- **Multiplication of putative transformants** in the presence of the selection agent "PPT",
5- **Confirmation of transformation by PCR** for the presence of "bar" and "***g2PS1***" **genes,**
6- **In vitro rooting and acclimatization of transgenic** clones.

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 in order to evaluate its efficiency in conferring tolerance to fungal diseases. Young green leaves were used for transformation using several regeneration media. Leaves were co-cultivated for 3 days with engineered disarmed Agrobacterium tumefaciens harboring the g2ps1 gene. Putative transgenic shoots could be obtained on MS media with B5 Vitamins, 5.0 mg /l BAP, or 2.0 mg /l TDZ with 0.2 mg /l NAA in the presence of the selection agent "PPT" at 3.0-5.0 mg /l. 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 in order to get sufficient material to confirm transformation of the putative shoots obtained.

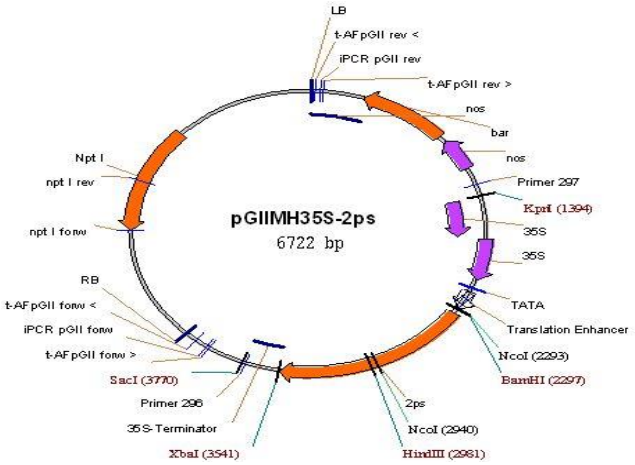


Fig. 1. Map of the pGreen II 35S-*g2PS1* harbouring the *g2ps1* used in the present transformation study.

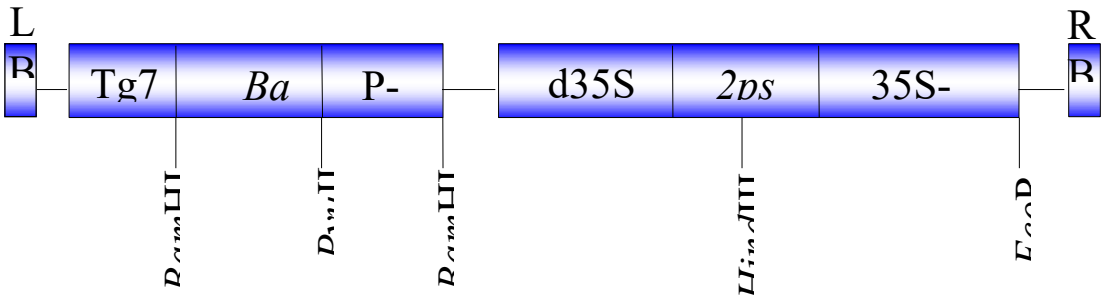


Fig.2. binary vectors witch used for Apple cv. & root stocks Transformation.

Table 2 . PCR reaction component

Volume / sample(microletter)	Material
5.3	H2O
4	5X PCR buffer
1.5	25 mM MgCl2
2	2 mM dNTPs
1	10 p.m/µl primer (f)
1	10 p.m/µl primer(r)
0.2	5 U/µl Taq
5	20 ng/µl DNA
20 µl	Total

Table 3. PCR program for Confirmation of transformation.

	Temperature /c°		Time/minet		Cycles number	
	bar	*G2ps1*	*bar*	*g2ps1*	*Bar*	*g2ps1*
Melting	94	94	3	1	1	1
Denaturation	95	95	1	1	30	30
anneling	60	60	1	1		
extension	72	72	1	1		
Final Extension	72	72	5	10	1	1

Table 4. primers sequences

Volume/ bp	Sequences	primer
1244bp	5-CCG ACG GTA CCC CCC CTG CAG GTC GAC GG-3	HK*g2ps1* (f.)
	5- GTC GGT CTA GAT CAG TTT CCA TTG GCA ACC GC-3	HK*g2ps1* (r)
264 bp	5-GCAGGAACCGCAGGAGTGGA-3	*bar* (f)
	5-AGCCCGATGACAGCGACCAC-3	*bar* (r)

Experimental design: Each treatment had four replicates consisting of Petri dishes containing 10 explants for adventitious shoot regeneration study. 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). Data given in percentages were subjected to arcsine (_X) transformation (Snedecor and Cochran, 1967) before statistical analysis. All the experiments described here were repeated at least two times and all results were pooled.

Results and Discussion

Effects of different leaf explants on regeneration and organogenesis ability: 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.

Explants were tested with special attention to the **regeneration rate**. A high regeneration frequency (92%, 95% and 90%, 94%) was obtained with leaf explants from 21days old leaves with (Golden Delicious, Royal Gala and MM111, M26) respectively on MS medium supplemented with 2 mg/l TDZ, while reducing TDZ concentration to 0.5 mg/l but with adding 0.5 mg/l BAP could result also in high regeneration rate (82%,90% and 79%,85%) respectively (Fig. 3). Adventurous shoots preferentially located along the cut basal edge of the explant were clearly visible after four to five weeks of culture. Adult leaves gave low regeneration frequency, while unfolded non-expanded young leaves, incubated on organogenic induction medium regenerated up to 95%. Under these conditions, leaf explants showed an overall expansion, about four-fold increase, within 4-5 weeks of culture (tables 5 and fig. 3). After four to five weeks, shoots appeared directly at the cut edges of explants. In all explants used, shoots could be obtained after 2 months. Young leaf organogenesis reached the highest regeneration rate producing two to four shoots per explant, while from adult leaf organogenesis, the average was at best one shoot per explants and show the organogenesis ability and regeneration % in apple cvs. "Golden Delicious" & "Royal Gala", and rootstocks, "MM111"& "M26" on different media used. A regeneration system from leaf discs of apple cvs. "Golden Delicious" &" Royal Gala" and Rootstocks, "MM111" & "M26" was established on MS based media and supplemented with 1.0 g/l MES, 5.0 mg/l *BAP or* 2.0 mg/l TDZ*, 0.2mg/L NAA,* 30 g/l Sucrose*,* 2.5 g/l Gerlite. Organogenesis did not occur on media without cytokinins. A high percentage of regenerated shoots (92, 95% and 90,94 %) and a high number of regenerated shoots per explant (4.025 & 5.615 and 4.1&4.50) could be obtained in cvs." Golden Delicious" &"Royal Gala" and "MM111"& "M26" rootstocks. respectively on MS basal medium containing 2.0 mg/l TDZ and 0.2 mg/l NAA and 3% sucrose. The morphogenetic capacity of leaf discs was dependent on the leaf maturity and the origin of the leaf disc with the young folded and expanding leaves being more regenerative than the older ones. Middle leaf segments were more prolific than the upper part of the leaf. Golden delicious, Royal Gala showed higher regenerative response (92, 95%) then MM111,M26 (90,94 %).Direct multiple shoots from leaf explants were developed within 4– 6 weeks in media containing either TDZ or BA in combination with NAA but not with 2-4-D.

Table 5. Mean shoot number regenerated *in vitro* (organogenesis ability) per explant in relation to the explant type and media tested in the apple cvs. Golden delicious & Royal Gala and MM111& M26 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	2.512c ± 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	2.00d ±0.80	1.65d ±0.80	1.500d ±0.60
R05	0.00e	0.00e	0.00e	0.00e
RO	0.00e	0.00e	0.00e	0.00e
$LSD_{0.05}$	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.

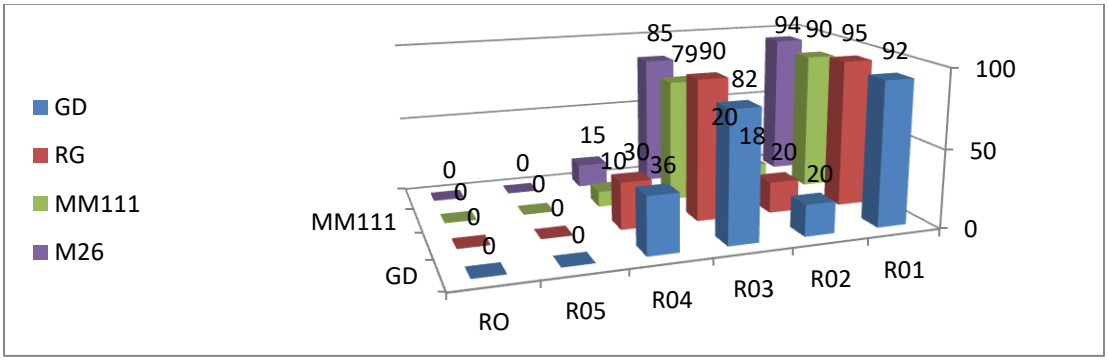
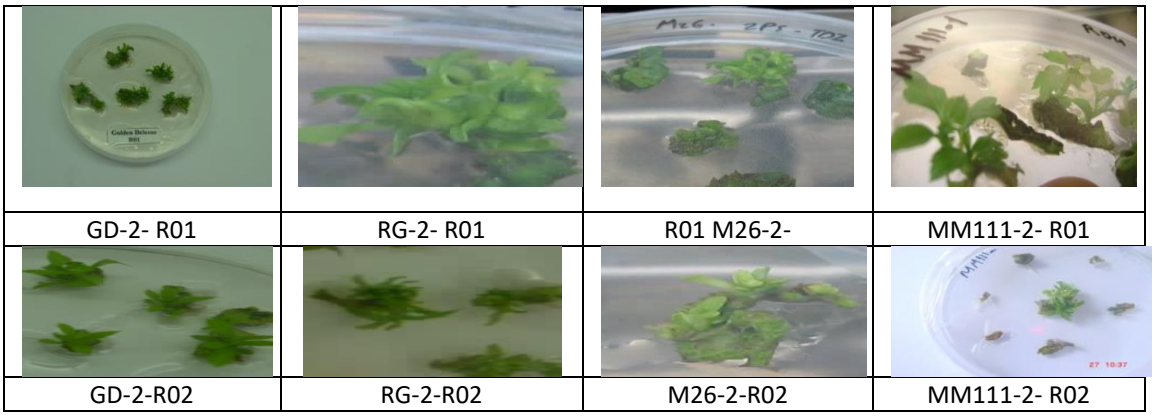


Fig. 3. Organogenesis ability per explant in relation to the explant type and media tested in the apple cv. Golden delicious and MM111 rootstock.

Most of the shoot regeneration using apple leaf explants is accomplished by cutting edges and wounds using forceps (Norelli *et al.,* 1996). Treating the leaves with the non-traumatic forceps resulted in a higher and faster organogenic response, due to severe wounding, 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. Wounding of tissues boosted the regeneration. 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). The number of adventitious shoots produced was different with different types of explants used with the medium part giving the higher regeneration rate (4.02**,** 5.615**,** 4.1 and 4.5). Meristemoids formed only on the cut margins, and on the abaxial surface of leaves, possibly to the closer contact with the regeneration medium. Furthermore, leaf parts that were closer to the petiole were more regenerative, confirm in previous observations carried out in experiment about the adventitious shoot proliferation from leaves of the same apple rootstock (Ferradini *et al.* 1996). Figure 4 shows shoot regeneration from GD, RG, MM111, M26 using mid-leaf explants on the best regeneration media



GD-2- R01	RG-2- R01	R01 M26-2-	MM111-2- R01
GD-2-R02	RG-2-R02	M26-2-R02	MM111-2- R02

Fig. 4. Shoot regeneration from Apple cvs. and rootstocks using leaf explants on the best regeneration

Appearance of shoot initials on leaf explants could be seen after 4–8 weeks of culture. 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 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. 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 micro propagated 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'. Plants were regenerated both from callus or directly from leaves from micro propagated plants. For 'direct' adventitious regeneration, leaves were collected and cultured on different media and in light or in darkness. Fourty two hormonal combinations were tried. Organogenesis did not occur without cytokinins and was enhanced in darkness. One treatment gave, on 'Gala', 100 % regenerating leaves with an average of 14.2 regenerated shoots per leaf. Two thousand plants from 'Gala' were successfully acclimatized and 1000 of them are now entering field trials to evaluate their phenotypic uniformity. 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 that BAP ((Leblay *et al.*, 1990; Korban *et al*.,1992; Escalettes and Dosba 1993; De Bond *et al*., 1996; Sarwar and Sirvin,1997 ; Hammatt and Grant,1998). Similarly, auxins such as IAA, NAA, IBA have also great effect on regeneration (Yancheva *et al*., 2003). Caboni *et al*., (2000) found that adventitious regenerates from shoot tips were significantly higher than that from leaves. For shoot regeneration from leaf discs, a range of BAP and TDZ concentrations was examined. Whilst 'Green sleeves' responded in line with published data, 'Bramley' produced significantly fewer shoots. 'Bramley' shoots were obtained from 5 mg l^{-1} BAP and 1 mg l^{-1} NAA. TDZ did not increase regeneration significantly**.** TDZ was used to induce adventitious shoot regeneration in many 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 . The differences among different parts of the same plant may be attributed to the various levels of endogenous plant growth regulators of explants from different positions (Özgen et al., 1998; Uranbey *et al*., 2005). Although induction of shoots was observed in most media tested, there was statistically difference among the the TDZ and BA (media R01 and R02: Table 1). Karhu,1997 tested three carbon sources such glucose, sorbitol and sucrose for their regeneration efficay in different apple Malus domestica explants and found that sorbitol and sucrose has similar

effects in induction of regeneration . In the present study, however, two energy sources were tested for regeneration efficiency, i.e. sucrose and sorbitol, where it was found that sucrose has better efficiency in induction of regeneration. The percentage of explants producing shoots and the number of shoots per explant were influenced by type and concentrations of TDZ and BAP tested (p.0.05). The percentage of regenerated shoots fluctuated between 6 - 95%. The highest percentage of regenerated shoots (92%,95%,94% and 90%) and the highest number of shoots per explant (**4, 5.6 , 4.5** and 4.1) occurred with 2.0 mg/l TDZ and 0.20 mg/l NAA in case of Golden delicious, Royal Gala, M26 and MM111 respectively.

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. The shoot organogenesis of some crops in tissue culture have been recently 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, Singh et al., 2002; 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, synthesising, protecting or even inhibiting auxins *in situ* in combination with other sub-cellular metabolic changes, particularly in key regulatory enzyme and related proteins. 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. The use of NAA with TDZ might be best treatment to eliminate the secretion of phenolic substances and this effect might be also due to the oxidation of phenols by auxin oxidase. No abnormality, necrosis was observed during the culture. Differential response of the explants tested was observed on the different media for shoot multiplication. The effects of combinations of BAP x NAA and explants on shoot multiplication were statistically significant. Also, explant types and plant growth regulators interaction was 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 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 (90%) was obtained on a medium containing 0.5 mg/l BAP, 0.5 mg/l TDZ + 0. 2 mg/l NAA. Selection of a suitable explant at correct developmental stage plays a key role in the successful establishment of culture under *in vitro* conditions.

Morphological integrity of an explant along with the proper choice of plant growth regulators strongly influence induction of optimal callus and shoot regeneration (Khawar et al., 2005). The multiple shoot induction rate and morphogenetic response significantly varied to a greater extent according to the explant type and plant growth regulators concentrations (Özgen *et al*., 1998; Uranbey, 2005). Type of explant and culture medium with specific growth regulator concentrations influenced the organogenesis in the present study and leaf explants could be used for rapid clonal propagation with optimized culture medium. All regenerated shoot tips (20 - 35 mm length) were excised and rooted readily in half strength MS medium supplemented with IBA. Rooting was observed from the cut ends of the shoots within 30 days in most media tested. All of the developing roots were physically vigorous and healthy. 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.

Rooted plantlets were acclimatized to ambient conditions and later established under greenhouse conditions and finally in the field under natural field conditions.

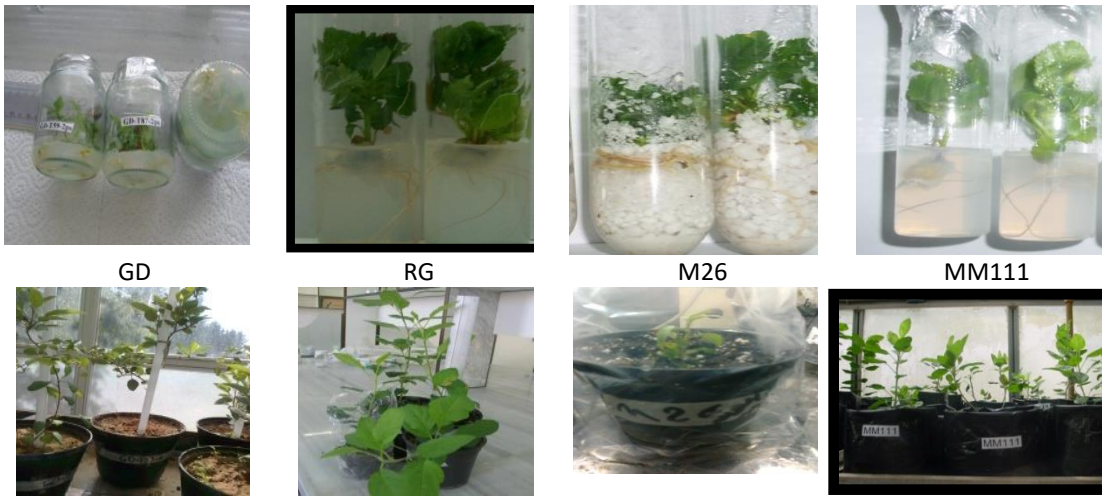


Fig.5. Rooted plantlets from transformed Apple cvs. rootstock s

Explants started regeneration after at least 4 weeks of beginning of the regeneration experiment and continued until the eighth week where no regeneration could be seen afterwards. Numerous shoots were produced in each Petri dish between 4-8 weeks. Explants were tested with special attention to the regeneration rate. A high regeneration frequency (92% and 90%) was obtained with leaf explants from 21- days old leaves with Golden Delicious and MM111 respectively on MS medium supplemented with 2 mg/l TDZ, while reducing TDZ concentration to 0.5 mg/l, but with adding 2.5 mg/l BAP could result also in high regeneration rate (82% and 79% respectively). Adventitious shoots preferentially located directly along the cut basal edges of the explant which were clearly visible after four to eight weeks of culture. Adult leaves gave very low regeneration frequency, or no regeneration at all, while unfolded young leaves, incubated on organogenic induction medium regenerated up to 92%. Under these conditions, leaf explants showed an overall expansion with about four-fold increase within 4-8 weeks of culture. In all explants used, shoots could be obtained after 2 months. On control media free of growth regulators, no shoot regeneration could be observed. Observations of different leaf parts allowed the identification of more organogenetic leaf areas. Meristemoids formed only on the cut margins, and on the abaxial surface of leaves, possibly 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 about the adventitious shoot proliferation from leaves of the same apple rootstock (Ferradini *et al*. 1996). Appearance of shoot initials on leaf explants could be seen after 4 – 8 weeks of culture. The results presented here 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. 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 mg l^{-1} BAP and 1 mg l^{-1} NAA, whereas TDZ did not increase regeneration significantly**.** Our results, however, are in contrast to such findings, where TDZ proved to be more efficient in inducing regeneration in apples studied than BAP. Dufo (1990). obtained improved yield in *in vitro* adventitious regeneration in apple cultivars 'Granny' Smith', 'Mark', 'Novole', 'Lancep' and 'Cepiland with significant increase in number of regenerated shoots from 'Gala' and 'Golden Delicious'. He regenerated Plants from callus or directly from leaves of micro propagated plants. He got 100 % regenerating leaves with an average of 14.2 regenerated shoots per leaf in 'Gala'. Organogenesis did not occur without cytokinins and was enhanced in darkness. 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 that BAP (Korban *et al*.,1992; DeBond *et al*., 1996; Hammatt and Grant,1997). Similarly, auxins such as IAA, NAA, IBA have also great effect on regeneration (Magyarne *et al.* 2001). Caboni *et al*., (2000) found that adventitious regenerants from shoot tips were significantly higher than that from leaves. For shoot regeneration from leaf discs, a range of BAP and TDZ

concentrations was examined. Whilst 'Greensleeves' responded in line with published data, 'Bramley' produced significantly fewer shoots. 'Bramley' shoots were obtained from 5 mg l^{-1} BAP and 1 mg l^{-1} NAA. TDZ did not increase regeneration significantly**.** TDZ was used to induce adventitious shoot regeneration in many 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 and apple (Theiler-Hedtrich, and Theiler-Hedtrich 1990; McAdam-O Connell *et al.* 2004; Van Nieuwkerk *et al*. (1986); Fasolo *et al.* 1990). The differences among different parts of the same plant may be attributed to the various levels of endogenous plant growth regulators of explants from different positions (Özgen *et al.,* 1998; Uranbey *et al.,* 2005). In the present study, however, although induction of shoots was observed in most media tested, TDZ proved to be more efficient than BA in induction of shoots. Selection of a suitable explant at correct developmental stage plays a key role in the successful establishment of culture under *in vitro* conditions. The percentage of explants producing shoots and the number of shoots per explant were influenced by type and concentrations of TDZ and BAP tested (p.0.05). The percentage of regenerated shoots fluctuated between 7-92%. The highest percentage of regenerated shoots (92% and 90) and the highest number of shoots per explant of about 4-fold increase occurred with 2.0 mg/l TDZ and 0.20 mg/l NAA for both Golden delicious and MM111. 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. The shoot organogenesis of some crops in tissue culture have been recently 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, synthesising, protecting or even inhibiting auxins *in situ* in combination with other sub-cellular metabolic changes, particularly in key regulatory enzyme and related proteins. In the present study, frequency of shoot organogenesis could 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 apples studied. No abnormality, necrosis or chlorosis was observed during the culture. The use of NAA with TDZ might be best treatment to eliminate the secretion of phenolic substances and this effect might be also due to the oxidation of phenols by auxin oxidase. 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 (92 %) 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. Using 2,4-D induced callus formation and inhibited adventitious shoot regeneration. Morphological integrity of an explant along with the proper choice of plant growth regulators strongly influence induction of optimal callus and shoot regeneration (Khawar *et al*., 2005). The multiple shoot induction rate and morphogenetic response significantly varied to a greater extent according to the explant type and plant growth regulators concentrations (Özgen *et al*., 1998; Uranbey, 2005). In the present study, it was shown that type of explant and culture medium with specific growth regulator concentrations influenced the organogenesis It could be proved that leaf explants can be used for rapid clonal propagation with optimized culture medium.

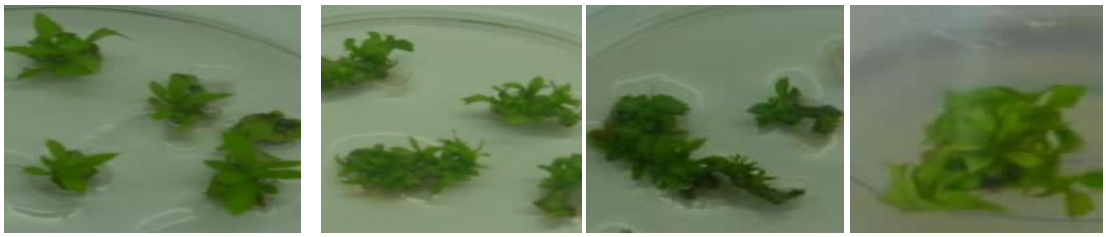
Effects of leaf wounding on regeneration and organogenesis ability: Most of the shoot regeneration using apple leaf explants is accomplished by cutting edges and wounds using forceps (Norelli *et al.,* 1996). In the preliminary experiments of the present study, treating the leaves with the non-traumatic forceps as suggested by Norelli (personal communication) resulted in a higher and faster organogenic response, due to severe wounding, which is in line with what was observed in the previous experiments by Norelli *et al.* 1996. Wounding of tissues boosted the regeneration. 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, Royal gala (Norelli et al. 1996) and also in

other species (Piccioni and Valecchi, 1996). Overall organogenesis was satisfactory in all treatments both in terms of regeneration rate and of adventitious shoot production. However, excessive wounding could be determental initially by slowing down regeneration, due to oxidation and necrosis of the tissues.

Effects of carbon source and gelling agent on regeneration and organogenesis ability: Karhu,1997 tested three carbon sources: glucose, sorbitol and sucrose for their regeneration efficay in different apple *Malus domestica* explants and found that sorbitol and sucrose has similar effects in induction of regeneration. Jamborne and Dobranszki (2005) also reported different effects of carbon sources (sucrose, sorbitol and glucose) on regeneration in apple. In the present study, however, two energy sources were tested for regeneration efficiency, i.e. sucrose and sorbitol, where it was found that sucrose has better efficiency in induction of regeneration, while replacing Sucrose with Sorbitol negatively affected on the regeneration ability of new shoots. In the present study, gelrite was better than agar or a combination of both. It was shown that adding Agar in combination with Gelrite had an inhibitory effect of regeneration. Verification was not observed because of gelrite.

Transformants Evaluation on media contacting the selection agent: Putative transformatants subcultures on media with PPT at concentrations of 3-5 mg/l could survive in the presence of the selection agent "PPT", while the non-transformed explants were died (Fig. 6). These were subcultures and proliferated on media containing PPT (Fig. 7).

Acclimatization of rooted transgenics: Rooted transgenic plantlets were successfully acclimatized with 70% efficiency and kept in the greenhouse to evaluate their performance for fungal resistance under containment conditions (Fig. 9). **In vitro rooting of transgenics:** Roots were formed easily within 2-4 weeks with 85 % efficiency (fig. 9)



GD-R2-2ps1-ppt RG-R1-2ps1-ppt M26-R1-2ps1-ppt MM11-R1-2ps1-ppt

Fig. 6. Regenerated culture of transformed Apple cvs. and rootstocks in the presence of PPT.

GD-T27-ppt *g2ps1* RG- T01-ppt *g2ps1* M26-T17-ppt *g2ps-1* MM111-T2,T5-ppt *g2ps-ppt1*

Fig. 7. Growth of putative cultures of Transformed Apple cvs. and rootstocks in the presence of PPT.

GD RG M26 MM111

Fig 8. Rooted transformed Apple cvs. and Rootstocks. In present ppt.

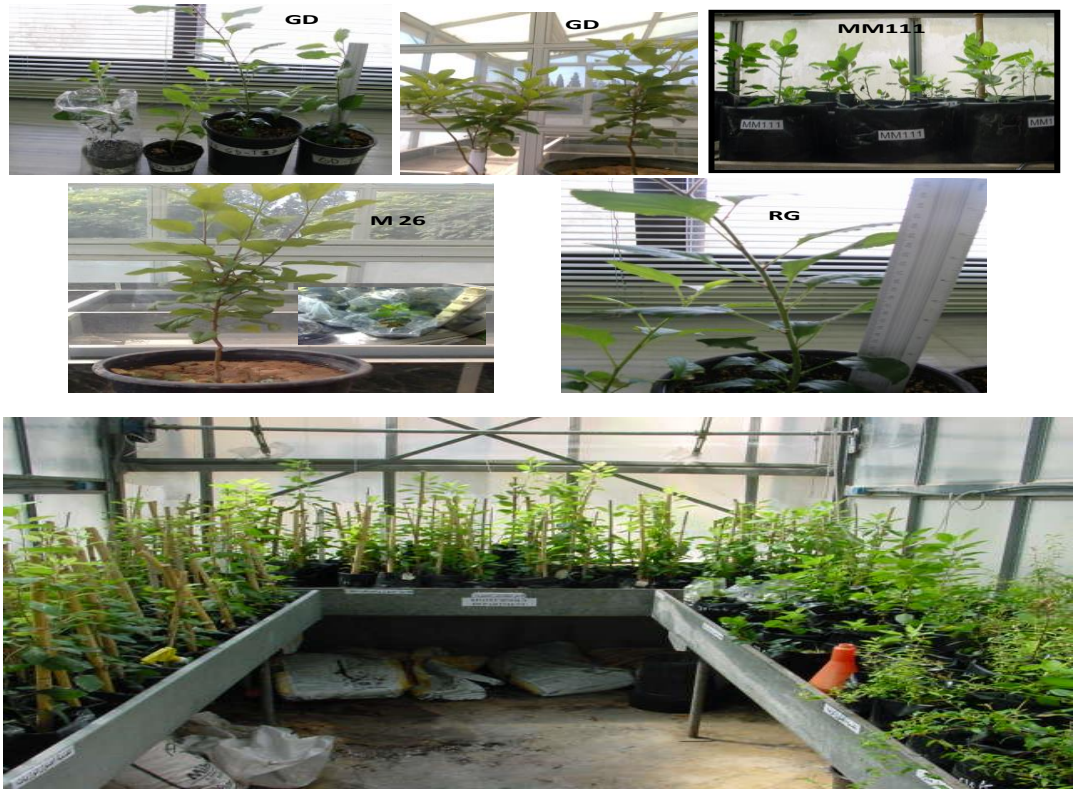


Fig 9. Acclimatized Transformed Apple cvs. and rootstocks.

Molecular Confirmation of Transformation PCR: Confirmation of putative transgenic regenerates was done by PCR. Specific primers for detection of the selection "*bar*" gene and also for the gene of interest *"g2PS1"* showed the transfer of the gene according to the expected band size of 447 bp or 265 bp for *bar* gene and 1244 bp for the *g2PS1* gene (Figs. 8 and 9), while no band shown in the negative control not transformed apple. Also, no band shown in the negative control (well containing no DNA).

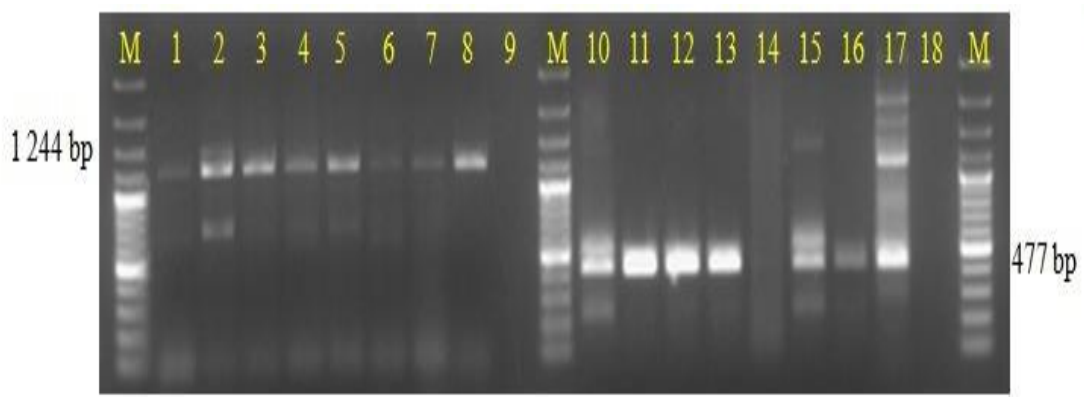


Fig10. Wells (1,2) :Golden Delicious.(3,4): M26.(5,6): Royal Gala,(7): MM111.(8): positive control (plasmid). (9):water.(10): Golden Delicious. (11,12):M26. (13):Royal Gala.(14):water.(15): MM111.(18): negative control (DNA isolated from non transformed apple).M: Marker100 pb.

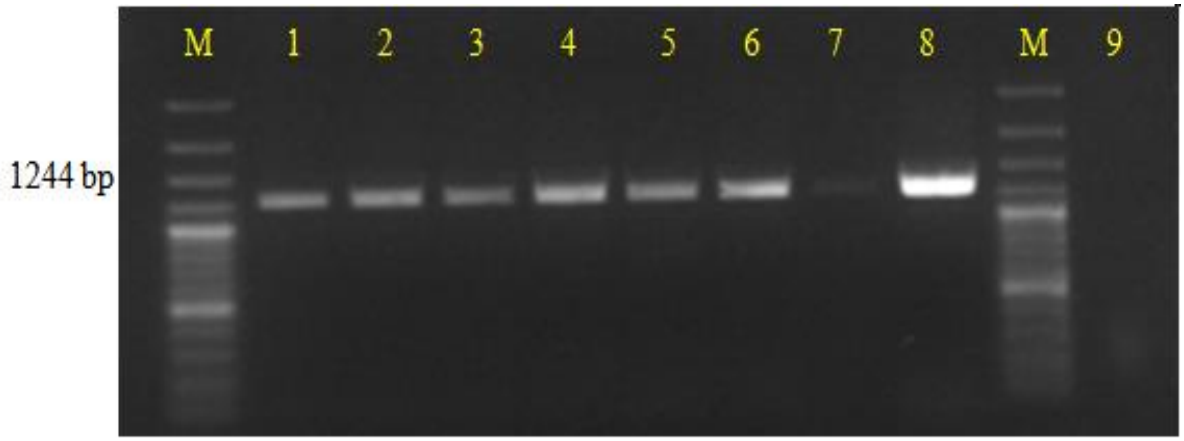


Fig 11. Acclimatized Transformed Apple cvs. and rootstocks. PCR for g2ps1. Welles : M: Marker. 1,2: Golden Delicious. 3:Royal Gala. 4-5: M26. 6: MM111. 7: water. 8: plasmid posetiv. 9: negative Golden

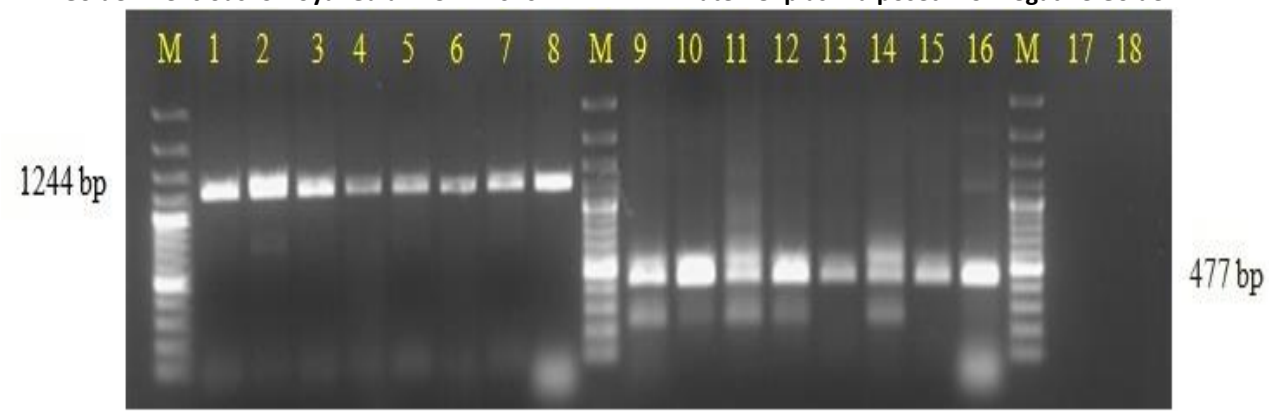


Fig 12. Acclimatized Transformed Apple cvs. and rootstocks. PCR.
For "g2ps1" gene and "bar" gene.
Wells (1,2) :Golden Delicious.(3,4): M26.(5,6): Royal Gala,(7): MM111.(8): : positive control (plasmid). (9, 10): Golden Delicious. (11,12):M26. (13,14):Royal Gala.(15): MM111. (16): positive control. (17): water. (18): negative control (DNA isolated from non transformed apple). M: Marker 100 pb

Conclusions

- This work focused on the development and improvement of knowledge and expertise in application and utilization of biotechnology techniques to improve some horticultural crops, as well as the development of the technology and genetically modified plants resistant to diseases.
- The long-term work aimed to Promote the production and use of biotechnology for development and integration through capacity building, creation and exchange of new knowledge and problem-oriented research that are of major concern to the country.
- As a prerequisite of the development of transformation system for apple, a leaf disc regeneration system have been developed. The results indicated that organogenesis in apple using leaves is a good pathway for regeneration as a prerequisite for genetic transformation using g2ps1 gene to confer fungal resistance to the studied apples.
- Transgenic shoots were obtained from leaf explants of apples studied using *Agrobacterium tumefaciens* strains that produced transgenic apples resistant to fungal diseases such as apple scab and powdery mildew (but this still to be confirmed under containment conditions).
- As a prerequisite of the development of transformation system for apple, a leaf disc regeneration system has to be developed. The results presented in the current investigation indicate that organogenesis in apple using leaves is a good pathway for regeneration as a prerequisite for genetic transformation which is in progress using g2ps1 gene and to confer fungal resistance in the studied apples. Cut leaves maintain their regenerative ability and can be multiplied and rooted.

- In conclusion, the results recorded during the present investigation clearly suggest that leaves obtained from 21 day-old proliferating cultures of apple are very important explant type for efficient shoot regeneration.
- Furthermore, the present study underlines the importance of combinations of TDZ and NAA or TDZ , BAP and NAA for high shoot regeneration from leaves by organogenesis and may be used easily transformation studies. We have been trying to obtain transgenic shoots from leaf explants of various genotypes using *Agrobacterium tumefaciens* strains that will potentially lead to large scale production of transgenic apple.

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