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In vitro studies on regeneration and transformation of some pomegranate genotypes

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Abstract

The present study aimed to develop an *in vitro* regeneration of plantlets of four commercial pomegranate genotypes (Manfalouty, Tahrir, Badr and Araby) grown in Egypt. Shoot tips were selected and separated from 7-year-old mother plants as explants. The MS media supplemented with different dosage of growth substances were examined under controlled conditions. Gene transformation mediated by *Agrobacterium tumefaciens* was examined for only two genotypes (Manfalouty and Araby) which recorded highest and lowest regeneration capacity, respectively. The results showed that regeneration of pomegranate was mainly controlled by genotype, whereas the response of the explants was largely affected by the environmental factors. The data also show that the embryonic calli were formed within six weeks in the presence of MS media supplemented with 1 mg L^{-1} of 2,4-D. Shoots emerged successfully from the embryonic callus in the presence of 6 mg L^{-1} BA. Addition of 1 mg L^{-1} IBA to the MS basal medium increased the shoot forming, roots ratio (100%) and plantlet growth in all pomegranate genotypes. The highest recorded regeneration frequency was 79.75% in genotype Manfalouty followed by 70%, 63% and 55% in Tahrir, badr and Araby, respectively. The regenerated putative transgenic plantlet inoculated and co-cultivated with *Agrobacterium tumefaciens* were able to grow vigorously in the media containing 50 mg l^{-1} Kanamycin with high degree of gene stability. Fresh weight (FW) of the regenerated plantlets was increased, whereas the concentration of H_2O_2 was decreased due to the infection with *A. tumefaciens* in both genotypes**.** Similarly, the infected plants showed higher specific activities of CAT and SOD compared to control. The PCR analysis showed the integration of the transgene into the infected plant genome corresponding to the relevant sequence of both of the NPT-11 gene and the 35 S promoters. This protocol can be used to produce tolerant to stress pomegranate plantlets and needs further investigation.

Keywords: Pomegranate, gene stability, *Agrobacterium tumefaciens*, Neomycin, phosphotransferase-11, CAT, SOD, H2O2.
Abbreviations: CAT_Catalase, CIF_Callus induction frequency, FW_Fresh weight, IBA_Indol butyric acid, MS_Murashige and Skoog nutrient media, PRF_Plantlet regeneration frequency, SOD_Superoxide dismutase, ST_Shoot tip, 2,4D_2,4-Dichlorophenoxyacetic acid.

Introduction

Pomegranate (*Punica granatum* L.) belongs to Punicaceae family and is one of the most important and oldest known edible fruit tree growing in arid zones because of its resistance to drought and its ability to grow under different conditions (Özgüven et al., 2009). This fruits is cultivated extensively in Iran, Afghanistan, India Mediterranean countries and into some extent in the USA, China, Japan and Russia (Sarkhosh et al., 2006). In Egypt, the total cultivated area reached 8080 hectare and produced about 42934 metric tons (FAO, 2012). Economically, pomegranate is an important species of tropical and subtropical regions of the world due to its delicious edible fruits and pharmaceutical and ornamental uses (Jayesh and Kuman 2004). It is considered as a valuable fruit that is associated with fertility as well as medicinal and ornamental properties. Some parts such as leaves, roots, and flowers have featured in industry and medicine for thousands of years (Feng et al., 1998). There is an increased demand for pomegranate fruits to meet the needs of international markets (Deepike and Anwar, 2010). The agriculture policy in Egypt aimed to increase its production by expanding into the new reclaimed region. The evaluation of pomegranate trees has been done all over the world using fruit products for processing and to determine the degree of polymorphism and similarities among genotypes (Pasad and Banker, 1999; Sarkhosh et al., 2009) and/or to develop an index for cultivar selections for production and breeding programs in the future (Varasteh et al., 2009). However, genetic variation studies in pomegranate is required to breed cultivars that are high yielding and resistant to several biotic and abiotic stresses. It is known that perennial fruits like pomegranate are unable to bloom and bear fruits for several years after seedlings are planted. This condition, with no reproductive capability, is termed as juvenility. For breeding and commercialization of promising pomegranate cultivars, a precise determination and discrimination of genotypes is required. The study of diversity of pomegranate germplasm in Tunisia based on fruit characteristics has been performed by Mars and Marrakchi

Table 1. Analysis of variance and heritability.

SOV	d.f	ST responded %	CIF	SIF	PRF
Replicate	3	0.0033	0.366	0.533*	0.322*
Levels(L)	3	-	-	44.322*	10.677*
Genotype(G)	3	0.0066	3.333*	1.111*	9.003*
G × L	9	-	-	0.566*	1.333*
Error	46	0.002	0.109	0.0111	0.266

SOV = source of variance, d.f = degree of freedom, ST = shoot tip, CIF = embryos induction frequency, SIF = shooting induction frequency, PRF = plantlet regeneration frequency.

(1999). Although, fruit characteristics are important considerations for breeding of fruit trees, functions of genes expressed in fruits cannot be analyzed for several years because of juvenility. A long juvenile phase is a serious constraint in the traditional breeding and cultivation because large fields and many laborers are required to maintain numerous in-nursery plants over a long time period (Terakami et al. 2007). Therefore, plant improvement through conventional breeding method is slow, time consuming and labor-intensive. Tissue culture and molecular genetics are essential as a complement to standard breeding methods (Lichtenstein and Draper, 1985). Several studies have been conducted on micropropagation of pomegranate trees over the past several years. Protocols have been developed for regeneration of *Punica granatum* L. plantlets *in vitro* through either organogenesis or callus derived from leaf segments (Murkuta et al., 2002), cotyledons (Kanwaret al., 2010), anthers (Moriguchi et al., 1987), embryogenesis of various seedling explant (Jaidka and Mehra 1986), petals (Nataraja and Neelambika 1996) and immature zygotic embryos (Kanwaret al., 2010). *In vitro* propagation of pomegranate through axillary shoot proliferation of nodal segments (Kanwaret al., 2004), shoot tips (Murkute et al., 2004) and cotyledonary nodes (Sharon and Sinha, 2000) have also been reported. However, to date there have been rare reports concerning the physiological behavior of pomegranate regenerated by the reproducible protocol for its regeneration and transformation. Therefore, pomegranate has become an object of extensive tissue culture studies and its relation to the two essential components of molecular breeding, variability and selection efficiency. It is well documented that the balance of auxin and cytokinin in the culture media regulates organogenesis (Moghieb et al., 2006) through its effects on the physiological and various cellular processes. The regeneration is highly variable and genotype specific (Phogat et al., 2000). Moreover, the efficient *Agrobacterium*-medieted transformation methods require a reliable and efficient callus induction and plantlet regeneration procedures (Riemenschiender et al., 1988). On the other hand, *Agrobacterium*-mediated transformation has advantages over particle bombardment because one of a few copies of the transgene are integrated and the transgene is less fragmented (Hadi et al., 1996). Thus, the present investigation also aimed to develop an efficient protocol for pomegranate regeneration and transformation using four commercial genotypes grown in Egypt. Certain physiological aspects were evaluated in the presence or absence of *Agrobacterium tumefaciens*.

Results and Discussion

The regeneration

Analysis of variance and heritability

Four pomegranate genotypes were studied for response and nodular calli percentage of shoot tip (ST) explants. Moreover, the combined data over four levels of growth substances were evaluated and some parameters such as callus induction frequency (CIF), shoot induction frequency (SIF) and plantlet

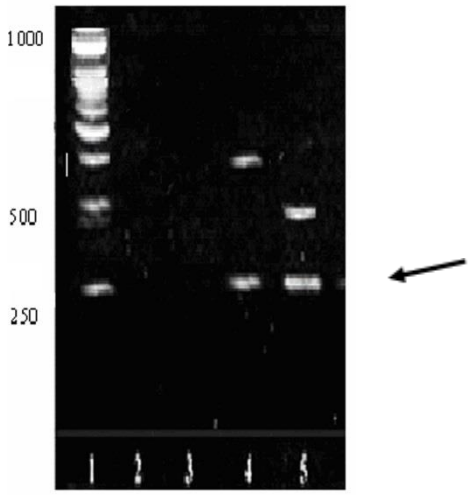

Fig 1. PCR analysis confirming the transformation of a foreign gene with T-DNA in the Tiplasmid of agrobacterium *A. tumefaciens* into regenerated pomegranate genome, showing the stable integration of the NPT-11gene into pomegranate genome.

regeneration frequency (PRF) were subjected to ordinary analysis of variance. The results of mean squares presented in Table 1. show the presence of significant differences between the studied genotypes for the studied traits except for ST response percentages. This finding emphasized that CIF, SIF and PRE are controlled by the genotypes of donor plants. Similar results were reported by Sarkhosh et al. (2006) on Iranian pomegranate and Helaly and Hanan El-Hosieny (2011) on citrus genotypes.

The total variances of the studied traits were partitioned into its components; genetic variance (σ^2_g) and environmental variance (σ^2_e) in addition to growth substances levels (σ^2_L). The interaction levels of genotypes variance (σ^2_{gxL}) such as CIF, SIF, and PRF were estimated. Moreover, these parameters were used for estimating heritability in broad sense for these traits. Table 2. shows that the genetic variance is positive and the estimated heritability was less than 50% for the studied traits except for CIF. These findings indicated that calli regeneration is mainly controlled by genetic, while the other traits are mainly affected by the environmental factors. The mean performances of pomegranate genotypes were determined for all the studied *in vitro* traits and the obtained results are presented and discussed below.

Nodular callus induction frequency (CIF)

ST explants exhibited an initial swelling within three weeks followed by callus formation within six weeks of incubation on MS media supplemented with 2,4-D (data not presented). No significant differences were observed in swelling between the various studied genotypes. Moreover, there was no difference

Table 2. Nodular callus induction frequency (CIF).

Parameters	ST responded	CIF	SIF	PRF
σ^2_g	0.002	0.666	0.02	0.60
σ^2_L	-	-	1.66	0.27
σ^2_{gL}	-	-	0.22	0.24
σ^2_e	0.004	0.233	0.02	0.20
σ^2_{ph}	0.005	0.899	1.92	1.31
Hb%	33.33%	74.08	1.04	45.80

σ^2_g = genetic variance, σ^2_L = growth substances level, σ^2_{gl} = levels by genotypes interaction, σ^2_e= environmental variance, σ^2_{ph} = phenotypic variance, ST = shoot tip explant responded percentage, CIF = callus induction frequency, SIF = shooting induction frequency, PRE = plantlets regeneration frequency

between the swelling degree under the various 2,4-D levels. However, data in the Table shows that CIF and the embryogenic callus (Nodular callus %) were significantly different within the genotype depending on the 2,4-D level applied. The highest percentage of explants that formed nodular callus was recorded at 1 mg L^{-1} (Table 3) and decreased at 5 as well as 15 mg L^{-1}. These results were obsereved in all studied genotypes with the superiority for Manfalouty.

The effectiveness of auxins (low amounts) on increasing calli regeneration was previously reported by many investigators. Altaf et al. (2009) examined different types of auxins and found that 2,4-D is more effective for the promotion of somatic embryos compared with NAA or NOA in plant tissue culture. They found 2,4-D can reach to the highest intracellular concentration as free auxin, and is also the most efficient in promoting hypermethylation and morphogenetic activity. Instead, if auxin is retained in the medium, the embryogenic progression stops before globular stage. There is a positive correlation between the level of added auxins and the level of methylation in DNA (Chao and Krueger, 2007).

Regarding the variable effects of auxins on the different genotypes, Hornung et al. (2000) recorded that the swelled explants and induction of their establishment is dependent on the genotype and the explants. The establishment period can be extended to about six months by continuously exposing the explant to high auxin levels such as 2,4-D. El-Bellaj et al. (2000) found that IAA's oxidase activity in the embryogenic calli was three fold than that of in non-embryogenic calli. Moreover, IAA oxidase activity was higher than ionic oxidases in embryogenic calli, whereas no differences were detected in non- embryogenic calli. In addition, the point of arrest is more or less typical of each cell line. Thus, the establishment of the explants in pomegranate depends on the genotype and medium component, especially plant growth substances used and its level. This conclusion was supported by (Moghaieb, 2006) who found a correlation between DNA fingerprinting using AFLP technique for five canola cultivars and the embrygenic callus formation.

Shooting regeneration frequency (SIF)

The exogenous BA supplementation to the MS basal media induced the development of nodular structures in embryogenic calli and promoted direct development into somatic embryos and shoot bud formation within nine weeks from the induction of calli of all examined genotypes. Data in Table 3 also shows that, shoot induction frequency was increased due to an increase in BA level up to 6 mg L^{-1}, thereafter it tended to decrease but was still higher than the control in all studied genotypes. Several investigators reported

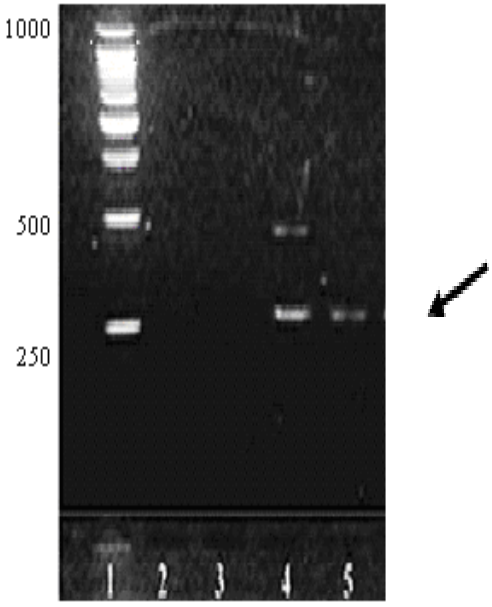

Fig 2. PCR analysis confirming the transformation of a foreign gene with T-DNA in the Ti-plasmid of *A. tumefaciens* into the transformed pomegranate genome, showing the detection of the 35-5 promoter. 1 = MW (XVI-250 bp ladder, Roche), 2 and 3 = non-transgenic plantlets of Manfalouty; M and Araby; A genotypes respectively and 4 and 5 = transgenic plantlets of M and A respectively.

that shoot production efficiency increased when the medium was supplemented with high concentrations of BA ranging from 2-4 mg L^{-1} (Wang et al., 2003). The genotype Manfalouty and Tahrir showed higher capability for production of somatic embryos and shoots at 3 mg l^{-1} BA, compared with the other levels and genotype. Three weeks after sub-culturing, Manfalouty showed higher regeneration percentage followed by Badr while the genotype Araby showed least regeneration frequency. The data in the present investigation support the assumption that regeneration in pomegranate is genotype specific (Abd el maksoud, 2003; Sarkhosh et al., 2009).

The positive effects of auxins and cytokinin on growth may be related to their effects on the availability of water and certain nutrients as well as cell division and elongation as previously observed by Hatwing (2004). The auxins could increase the absorption of mineral nutrients and GA into the plant tissues, whereas it decreases levels of both plant inhibitors and endogenous ethylene. These changes in plant hormones may exert an important influence on the physiological processes such as assimilation, transport and

Table 3. Nodular callus induction frequency (CIF).

Genotype	Treatments 2,4-D mg L^{-1}				
	MS alone (control)	MS+1	MS+5	MS+10	Mean
	Nodular callus induction frequency (CIF)				
Manfalouty	38^{e}	87^{a}	74^{b}	55^{d}	63.5^{A}
Tahrir	32^{f}	83^{a}	65^{c}	52^{d}	58^{B}
Badr	28^{g}	77^{b}	56^{d}	43^{e}	51^{C}
Araby	25^{g}	61^{c}	50^{d}	30^{f}	41.5^{D}
Mean	24^{D}	77^{A}	61.25^{B}	37.5^{C}	
LSD at 5% for					
Genotype (G) = 2.11 Treatment level (L) = 3.96 G × L = 5.33					
Shoot induction frequency (SIF)					
Genotype	Treatments 2,4-D mg L^{-1}				
	MS alone (control)	MS+3	MS+6	MS+9	Mean
Manfalouty	31^{f}	74^{a}	65^{b}	44^{d}	53.5^{A}
Tahrir	27^{f}	66^{b}	54^{c}	46^{d}	48.3^{B}
Badr	24^{gf}	58^{c}	45^{d}	38^{e}	41.2^{C}
Araby	20^{g}	44^{d}	37^{e}	30^{f}	32.75^{D}
Mean	25.5^{D}	53.75^{A}	50.25^{B}	39.33^{C}	
LSD at 5% for					
Genotype (G) = 1.99 Treatment level (L) = 3.33 G × L = 5.36					

Means with the same letter are not significantly different at 5% level**.** The capital letters in the columns are comparing the genotypes overall treatment level. The capital letters in the rows are comparing the effects of treatments overall genotypes. The small letters refer to the interaction (genotype x treatment level).

Table 4. Plantlet regeneration characters (PRF).

Genotype	IBA (mg/l)	PRF %	Shoot forming roots%	Shoot(S) height(cm)	Root(R) length (cm)	FWg/plantlet S	FWg/plantlet R
	0	65^{c}	65^{i}	2.9^{d}	3.6^{f}	2.7^{d}	3.0^{h}
Manfalouty	1	100^{a}	95^{a}	4.8^{a}	4.8^{a}	3.4^{a}	3.8^{a}
	2	84^{b}	92^{b}	4.2^{b}	4.6^{b}	3.2^{b}	3.7^{b}
	3	70^{b}	85^{d}	3.6^{c}	4.0^{d}	$3.0b^{c}$	3.3^{f}
	Mean	79.75^{A}	84.25^{A}	3.88^{A}	$4,25^{A}$	3.08^{A}	3.45^{A}
	0	49^{e}	62^{j}	2.8^{d}	3.4^{f}	2.6^{d}	3.0^{h}
Tahrir	1	100^{a}	92^{b}	4.8^{a}	4.5^{b}	3.3^{a}	3.6^{c}
	2	79^{b}	88^{c}	4.0^{b}	4.3^{c}	$3.0b^{c}$	3.4^{e}
	3	52^{d}	82^{f}	3.7^{c}	4.2^{c}	$3.0b^{c}$	3.2^{g}
	Mean	70^{B}	81^{A}	3.83^{A}	4.1^{A}	2.98^{C}	3.3^{B}
	0	44^{e}	58^{l}	2.5^{d}	3.4^{f}	2.6^{d}	3.0^{h}
Badr	1	100^{a}	84^{e}	4.2^{b}	4.6^{b}	3.4^{a}	3.6^{c}
	2	62^{c}	80^{g}	3.6^{c}	4.0^{d}	$3.1b^{c}$	3.5^{d}
	3	46^{e}	73^{h}	3.4^{c}	3.8^{e}	$3.0b^{c}$	3.3^{f}
	Mean	63^{C}	73.75^{B}	3.43^{B}	3.95^{B}	3.03^{B}	3.35^{B}
	0	29^{g}	50^{m}	2.2^{de}	$3.2f^{g}$	2.7^{d}	2.8^{i}
Araby	1	100^{a}	73^{h}	$3.8b^{c}$	$4.1c^{d}$	3.2^{b}	3.4^{e}
	2	52^{d}	65^{i}	3.3^{c}	3.8^{e}	$3.1b^{c}$	3.2^{g}
	3	39^{f}	60^{k}	3.0^{c}	3.5^{f}	2.8^{c}	3.0^{h}
	Mean	55^{D}	62^{C}	3.08^{C}	3.65^{D}	2.95^{C}	3.1^{C}
	0	45.25^{D}	65.5^{D}	2.6^{D}	3.4^{D}	2.65^{D}	2.95^{D}
Mean	1	100^{A}	86^{A}	4.4^{A}	4.5^{A}	3.33^{A}	3.53^{A}
	2	69.25^{B}	81.25^{B}	3.78^{B}	4.18^{B}	3.1^{B}	3.43^{B}
	3	51.75^{C}	75^{C}	3.43^{C}	3.88^{C}	2.95^{C}	3.2^{C}
LSD at 5% for Genotype(G)		3.33	0.36	0.21	0.07	0.03	0.01
Treatment level (L)		4.11	0.41	0.27	0.09	0.09	0.03
G × L		5,96	0.76	0.39	0.10	0.10	0.04

Means with the same letter are not significantly different at 5%level**.** For each trait, The capital letters in the columns are comparing the genotypes overall treatment level. The capital letters in the rows are comparing the effects of treatment overall genotypes. The small letters refer to the interaction (genotype x treatment level).

others including anatomical and physiological changes, promotion or retardation of growth and development as well as restriction and resistance to the unfavorable condition. El-Meskaoui and Tremblay (2001) suggested that ethylene could cause tissue browning and activate the synthesis of oxidative enzymes or could inhibit the synthesis of protective enzymes. These effects of nutrient and hormonal modifications reflected on vigorous vegetation growth such as FW growth value and number of shoots and secondary embryos.

Arteca (1996) noted that, auxins and cytokinin increased cell division and cell enlargement caused by an increases of water uptake as a result of an increase in the osmotic potential of the cells. Jiaqiang et al. (2003) reported that cytokinins play a critical and important role in plant growth and development by stimulating cell division, cell differentiation and cell division. Tokuji and Kuriyama (2003) added that cytokinin regulates the early stage of auxin induced somatic embryogenesis in carrot. Moreover, cytokinin nullified the inhibitory effects of purine riboside, an anticytokinin, on direct somatic embryogenesis. Helaly et al. (2008) found that addition of cytokinin during somatic embryo development of date palm resulted in some embryos remaining in globular stage with mild signs of enlargement but with no later development. However, El- Meskaoui and Tremblay (2001) mentioned that to produce mature somatic embryos of black spruce, ethylene production in the high capacity cell line was less than in the low capacity cell line.

Plantlet regeneration characters (PRF)

Table 4. shows that PRF, shoot forming roots ratio, plantlet height, root length as well as FW of the shoot and root system were increased in all pomegranate genotypes due to the supplementation with IBA to the MS basal medium. However, IBA at 1 mg L^{-1} was preferably better since it recorded 100% PRF values in all genotypes. However, the increasing rate tended to decreased with the increase of IBA level. Similarly, the plantlet growth represented with height and root length as well as fresh weights of the root and shoot system which increased due to IBA application up to 1 mg L^{-1}; thereafter, tended to decrease but still higher than the control. The lowest values were recorded under MS alone (control; auxin-free medium) in all genotypes. It was found that IBA at the levels more than 1 mg L^{-1} increased shoot forming roots (%) as well as plantlet height and produced strong shorter roots and bigger diameter with Tahrir, Manfalouy, Badr and Araby genotypes in a descending order. The interaction treatments showed that IBA at 1 mg L^{-1} was more effective on increasing shoot forming roots (%) and plantlet height in all pomegranate genotypes. All genotypes grown under 1 mg L^{-1} IBA produced higher number and stronger (shorter and thicker) roots. Plant roots grown in the IBA at levels more than 1 mg L^{-1} and the control plantlets recorded least values in this respect.

El-Rewainy et al. (2004) attributed the increase in shoot number and fresh weight caused by auxins to their important role in breaking plant apical dominance. They suggested that increasing the shoot number will increase the leaf number. This is of great interest due to leading plants to the higher photosynthetic area and the net assimilates which finally can be directed to the developing pomegranate plantlets with higher surviving ability and acclimatization.

The positive relation between root formation and the presence of auxins in the media, in all four genotypes of pomegranate studied here, are quite similar to those obtained by Wang et al (2003), who stated that auxins, especially NAA, promoted rooting of date palm plantlet. They applied NAA, by which the root growth increased due to its effect on increasing ethylene production, inducing cell radial expansion of ground tissue (Fki et al., 2003). Auxins are known to encourage cell division and formation of the new cells as well as the total growth and development of the plantlet (Seckin et al., 2009). The addition of exogenous auxins at low level promotes cell expansion and enlargement that caused by water uptake increase. Anjarne and Zaid (1996) demonstrated that the best tissue growth and organogenesis i.e. root, shoot, or leaf formation occurred on media with low auxin levels, whereas root initiation was promoted by the high levels especially with NAA. Al-Mazroui (2008) attributed the increase in growth of date palm tissues to application of auxins and cytokinin, not only to the effects on stimulation nitrogen fixation activity but also to the production of GA and others promoters substances. Furthermore, the hormonal balance helps in greater absorption of nutrients and water uptake from the media and alter plant metabolism to growth.

Gene transformation effects

Frequencies of callus, shooting and plantlets regeneration

Table 5. shows that infected ST explants from Manfalouty and Araby genotypes with or without *A. tumefaciens* cultivated on specific MS media (MS + 1 mg L^{-1} 2,4-D) for three weeks initiated callus formation. Sub-culturing the initiated calli on fresh MS specific media supplemented with 50 mg L^{-1} Karanycin improved its growth vigorously to form friable and nodular structures and emberyogenic calli during the selection process. Shoots were usually regenerated well in MS basal media supplemented with 6 mg L^{-1} BA at the same condition for the same period previously mentioned. At the end of incubation period of nine weeks, the average percentages of shooting and regenerated plantlets were increased for the two tested pomegranate genotypes. Regeneration percentage frequency was also stimulated due to *A. tumefaciens* infection in both genotypes, Manfalouty and Araby produced plantlets without infection. These results are in agreement with those reported by Xiang et al. 2000 and Moghaieb et al. (2006) on canola.

On the other hand, the untransformed explants produced least callus formation and eventually bleached within two weeks from incubation. Shooting and formed plantlets grown slowly, were small in size, showed stunted growth and unhealthy in general appearance especially with Araby genotype. In addition, the plantlets showed obvious change in color if compared to the infected ones. Moreover, regeneration was also found to be markedly genotype dependent. Manfaluti genotype recorded higher values than Araby.

The efficiency of *A. tumefaciens* infection on increasing frequencies of callus and shoots formation as well as plantlets regeneration may be due to the co-cultivation effects on stimulation of growth and proliferation of the tissue (Neri et al., 2006) through one or more of increasing cell division, cell viability, vitamin content, membranes permeability, nutrient uptake especially N, P, K, Ca, and Mg, respiration, endogenous enzymes and hormonal levels. The synthesis of specific protein to act as an organic catalyst, cytokinins and auxins production, activities of antioxidant enzymes (SOD, GR, AsP), synthesis of tocopherol, ascorbic acid and carotenoids which protect photosynthetic apparatus of PS II

Table 5. Frequencies of callus, shooting and plantlets regeneration.

Treatments	Developmental regeneration stages						Mean	
	CIF%		SIF%		PRF%			
	M	A	M	A	M	A	M	A
Control (without infection)	87^{b}	61^{b}	74^{b}	44^{b}	56^{b}	29^{b}	72.33^{B}	44.66^{B}
Infection with *A. tumefaciens*	95^{a}	81^{a}	90^{a}	73^{a}	78^{a}	62^{a}	87.66^{A}	72^{A}
Mean	91^{A}	71^{A}	82^{B}	58.5^{B}	67^{C}	45.5^{C}		
LSD at 5% for Infection (I) = 2.33 Genotype (G) = 3.16 I xG = 4.34								

Means with the same letter are not significantly different at 5% level. The capital letters in the columns are comparing infection overall developmental regeneration stages. The capital letters in the rows are comparing the effect of developmental regeneration stages over all infection treatments. The small letters refer to the interaction (infection x developmental regeneration stages). Five shoot tips were used for each treatment and the explant were repeated thrice.

Table 6. Hydrogen peroxide; H_2O_2 concentration ($\mu Mg^{-1}FW$), specific activities ($\mu g^{-1}FW$) of Catalase; CAT and suberoxide dismutase; SOD enzymes as well as fresh weight; F.Wt of the regenerated plantlet (g/plantlet) of Manfalouty; M and Araby; A pomegranate genotypes as affected by infection with or without *A. tumefaciens.*

Genotype	Treatments	H_2O_2	CAT	SOD	FW
Manfaluty	Without infection with *A. tumefaciens*	1.07^{a}	107^{b}	13^{b}	5.2^{b}
	With infection with *A. tumefaciens*	0.5^{b}	140^{a}	15^{a}	6.6^{a}
	Mean	0.79^{B}	123.5^{B}	14^{A}	5.9^{A}
Araby	Without infection with *A. tumefaciens*	1.6^{a}	110^{b}	8^{b}	4.7^{b}
	With infection with *A. tumefaciens*	0.7^{b}	139^{a}	11^{a}	5.6^{a}
	Mean	1.15^{A}	124.5^{A}	9.5^{B}	5.15^{B}
Mean	Without infection with *A. tumefaciens*	1.34^{A}	108.5^{B}	10.5^{B}	4.95^{B}
	With infection with *A. t tumefaciens*	0.6^{B}	139.5^{A}	13^{A}	6.1^{A}
LSD at 5% for					
Genotype (G)		0.11	0.02	0.16	0.01
Treatments (T)		0.23	0.96	0.21	0.26
G × T		1.66	2.03	0.76	0.86

Means with the same letter are not significantly different at 5% level. The capital letters in the columns are comparing infection overall genotypes. The capital letters in the rows are comparing the effect of genotypes over all infection treatments. The small letters refer to the interaction (infection x genotype).

(Zhang and Schmidt, 2000), sugar accumulation and nucleic acid metabolism may be the other factors to stimulate the growth.

H_2O_2 and activities of CAT and SOD enzymes

Data in Table 6. shows that fresh weight and FW of the regenerated plantlets were increased, whereas the concentration of hydrogen peroxide (H_2O_2) was decreased due to the infection with *A. tumefaciens* in both genotypes of pomegranate. Similarly, the infected plantlets showed higher specific activities of catalase (CAT) and superoxide dismutase (SOD) than the control (non-infected). El-Meskaoui and Tremblay (2001) suggested that ethylene activate the oxidative enzyme synthesis or inhibit the synthesis of protective enzymes. Comparing the genotypes, the data in the same table shows that, in general, FW as well as the specific activities of CAT and SOD were higher in Manfalouty genotype compared with Araby. On the contrary, the latter contained more H_2O_2 level than Manfalouty over all infection treatments.

The increasing effects of *A. tumefaciens* infection on FW may be due to its effects on protein and antioxidants, on increased mitotic activity, cell division and synthesis of hydroxy praline-rich protein. Moghaieb et al. (2006) found that *A tumefaciens* triggered some gene expressions and was the precursor of phytochelaties. The protective role of *A. tumefaciens* against H_2O_2 accumulation may be due to its effects on gene action. It acts as an antioxidant in the detoxification of reactive oxygen species (ROS) in aerobic cells. The interaction effects between genotypes and the infection with or without *A. tumefaciens* on antioxidant enzyme activities indicated that *A tumefaciens* induced oxidative stress. Inducing antioxidative enzyme activities is important to overcome oxidative stress due to the imposition of environmental stress (Foyer and Harbinson, 1994). This induction may be due to an enhanced gene (s) activity, since over-expression of genes encoding these enzymes in several transgenic plant species confers protection against free radical-induced oxidative stress. Smeets et al. (2005) reported that plant cells respond to elevated levels of oxidative stress by activating their antioxidative defence system and the first group of enzymes involved in this defense are the ROS-quenching enzymes such as CAT, Peroxidase (PODs) and SOD. Cheng (2003) recorded that CAT, PODs and SOD are important enzymes for plant adaptation to environmental stresses as the harmonious interaction of the three enzymes maintain the balance between ROS production and elimination; thus, keeping the level of ROS in plant tissues low to prevent the injury of cells. In peroxisomes, H_2O_2 can be destroyed by CAT. CAT produces molecular oxygen and water from two molecules of H_2O_2. Since these two molecules must impinge simultaneously at the active site. CAT has a very high maximum velocity. PODs play an important role in scavenging H_2O_2 and organic peroxide (Smeets et al., 2005). Moreover, Helaly and El Housiny (2011) reported that SOD causes the catalytic dismutation of potentially toxic superoxide anion radical (O^{2-}) to H_2O_2, whereas CAT decomposes H_2O_2 to water and oxygen

molecule. Both enzymes provide an efficient mechanism for the removal of free radicals from the cells in lemon.

PCR analysis

To confirm the stable integration of the T-DNA into the plant genome and its presence in the pomegranate regenerated plantlets, the plantlets co-cultivated with *A. tumefaciens* were subjected to PCR analysis. The putative transgenic pomegranate plantlets were analyzed by PCR using NPT-11 gene and 35-S promotor specific primers as shown in Figs 1 and 2.

The PCR analysis indicated that all the non-transformed generated plantlets showed a clear band corresponding to the relevant sequence of both of the NPT-11 gene and the 35 S promoter (Figs 1 and 2); however, the gene was detected at 325kDa fragment in pomegranate plantlets infected with *A tumefaciens*. The other nonspecific bands noticed in the Figs, maybe due to the heritability (Table 1) and or lines possibility of somatic embryogenesis mutation throughout micropropagation process. In this context, the first report on the transformation of pomegranate was carried out by Terakami et al. (2007).

Several workers recorded different and contradictory data against the co-organisms or co-cultivation depending on the primer used and plant species (Thorpe 1993; Hillis et al., 1996, El-Tarras et al., 2001 and Terakami et al., 2007). It is important to note that some clonal variation may be occurring through tissue culture technique and the genetic background varied according to the primers used. Markers are unlimited in number and they are not affected by the environmental stresses like drought, salinity, microorganism, etc. but may detect genetic and somaclonal variation (Sarkhosh et al., 2006). However, El-Tarras et al. (2001) concluded that RAPD can be used as a complement to pomological studies to distinguish new berry cultivars. The difference detected on confirmed polymorphism under the two primers used may be possibly due to their different effects on gene expression and/or their refection on the horticultural important characteristics and needs to be further investigated. The efficiency of *A. tumefaciens*–mediated transformation technique is influenced by cultivar specificity, donor plant age and explant type (Poulsen, 1996). Regeneration is also markedly genotype-dependent (Helaly et al., 2008).

It could be concluded that the reported regeneration system of pomegranate and the transformation protocol are repeatable and can be easily used to regenerate transgenic plants. Pomegranate plants express the genes present in the *Agrobacterium* binary vector T-DNA. The protocol may produce pomegranate plantlets having ability to tolerate stress condition need further investigation.

Materials and Methods

The present investigation was carried out at the plant tissue culture laboratory at the Horticulture, Research Institute of the Agricultural Research Center (ARC) and Agricultural Botany Department of the Faculty of Agriculture at the Mansoura University in Egypt during the period from 2010 to 2011.

Mother plant sources and explant material

Four seven-year-old pomegranate genotypes of Manfalouty, Tahrir, Badr and Araby grown at the Horticulture Research Station orched of Seds in Beni-swef Governorate in Egypt were used as a mother plants in this study. These trees were planted 5×5 m apart in clay soil. Pomegranate single stem trees were selected with approximately the same vigour and received the same horticulture practices. Shoot tips 2-4 mm in width and 5-8 mm in length were separated with a hatchet iron stick and sharp knife and always sprayed with ethyl alcohol 70%. The shoot tips (ST) of pomegranate genotypes were used as an explants and sterilized with 70% ethanol for 1 min followed by soaking in 50% commercial disinfectant Clorox (5.25% NaOCl) for 20 min and two drops of Tween 20 as wetting agent. The explants were washed three times with sterilized distilled water to remove all traces of the disinfectant.

Callus induction

Several shoot tips from each cultivar were used as an initial explant for the regeneration. They were cultured on MS (Murashige and Skoog, 1962) basal nutrient media containing sucrose (30 g L^{-1}) , solidified with (7 g L^{-1}) agar (Defco, India), different concentrations of dichlorophenoxyacetic acid (2,4-D), at graded levels (0.0, 1,0, 5,0 and 10,0) mg L^{-1} and the final pH was adjusted to 5.8. Media were dispersed in jars (325 ml) containing 50 ml from the specific media. The culture jars were immediately capped with polypropylene closures and autoclaved at 121^{o}C and 15/1bs/inch^{2} for 20 minutes.

Each treatment was replicated four times. The sterilized explants were cultured on the specific medium at the rate of five explant/jar and incubated at 25 ±1^{o}C under a 16/8 h day/night photoperiod (1000-Lux) for 4 weeks (Supplementary Figs) .

The survived explants were transferred and sub-cultured on the same fresh specific media for four more weeks. At the end of incubation period, callus induction frequency (CIF) were calculated (Moghaieb et al., 2006) as follows:

CIF = Number calli-producing explants/ Total number explants in the culture × 100

Shooting regeneration

The produced calli of each genotype were transferred to the shooting media in jars containing MS salts supplemented with benzyladenine (BA) at graded levels (0,0, 3,0, 6,0, and 9,0 mg L^{-1}) and adjusted to pH 5.8. Each jar contained 3 callus pieces and all the treatments were replicated four times. The jars were incubated at the same condition as calli induction. The explants were sub-cultured after three weeks on corresponding medium freshly prepared (Supplementary Figs). At the end of incubation period, shoot induction frequency-(SIF) were calculated (Moghaieb et al., 2006) as follows:

SIF = Number shoots-producing explants/Total number explants in the culture x 100

Rooting and plantlet formations

The developed shoots were cultured individually in cultured jars containing 35 ml of half strength MS medium supplemented with 3 g L^{-1} activated charcoal and differently IBA levels 0, 1, 2 and 3 mg L^{-1}. Each treatment was replicated four times and they were incubated in the growth room at the same condition as calli induction previously maintained for 3 weeks (Supplementary Figs). At the end of incubation, the mean values of the shoot formed roots were recorded. As for acclimatization, the obtained plantlets were selected based on their size. The plantlets which showed a well-developed root system (4-5 cm in length) were planted

individually in plasic pots (5.5 × 6.5 cm tyrpido) containing sterilized peatmoss + sand + perlite (1+1+1v/v) and irrigated with one tenth MS solution in a humid chamber at 25°C under a 16/8 h day/night cycle. After acclimatization for 6 weeks, the plants were grown under greenhouse conditions. The plantlet regeneration frequency (PRF) was calculated according to the following formula: PRF = Number plants-producing shoots/Total number of shoots initiated in the culture × 100 Shoot height, root length as well as fresh weight of the plantlets shoots and roots were also estimated.

Bacterial strain mediated gene transfer in pomegranate

The *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pBI- 121 was grown overnight in 30 ml of LB medium containing 50 mgl L^{-1} kanamycin sulfate (Sigma-Aldrich, Japan) at 28°C and then collected by centrifugation at 1000 × g for 5 min. The pellet was re-suspended in MS medium containing 100 µM acetosyringon. The shoot tip explants of the genotypes Manfalouty (showed higher regeneration) and Araby (showed lower regeneration) were selected for immersing in the bacterial suspension for 5 min. They were blotted on sterilized filter paper, placed onto a co-cultivation medium, which consisted of growth regulators free MS medium containing 100 µM acetosyringon and incubated under dark conditions.

After co-cultivation for three days, the explants were transferred to the callus induction medium-(CIM) containing 1 mg L^{-1} 2,4-D, (which showed best results) 500 mg L^{-1} cefotaxime and 50 mg L^{-1} kanamycin sulfate. Two weeks later, the explants were transferred onto shooting medium-(SM) supplemented with 6 mg L^{-1} BA (which showed best results) and 500 mg L^{-1} cefotaxime in addition to 50 mg L^{-1} kanamycin sulfate. The plates were sealed with parafilm and incubated at 25°C under a 16/8-h light/dark photoperiodic regime (1000-Lux). The explants were sub-cultured weekly on corresponding freshly prepared medium up to shooting regeneration. The experiment was repeated thrice and five shoot tips were used for each treatment. Frequencies of callus (CIF), shooting (CIF) and plantlets regeneration (BRF), were estimated as previously mentioned. In addition, fresh weight of the regenerated shoots was recorded and analyzed for H_2O_2 as well as the activities of catalase and superoxide dismutase. DNA and PCR analysis were also estimated.

Enzymes activities determination

According to Velikova et al. (2000), 0.5g of the regenerated shoots was homogenized in an ice bath with 5 ml of trichloroacetic acid (TCA) 0.1% (w/v). The homogenate was centrifuged at 12.000 g for 15 min(s) and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM K-P buffer (pH 7.0) and 1 ml of 1M KI. The absorbance of the mixture was measured at 390 nm and the concentration of H_2O_2 was determined using a standard curve. Catalase (CAT) activity (EC 1.11.1.6) was determined by the degradation of H_2O_2 as follows: 1 ml of the reaction mixture contained 10 mM k-P buffer (pH 7.0), 33 mM H_2O_2 and 20 µl of the enzyme extract prepared by grinding 1 g of the explant in 10 ml of 0.1 M K-P buffer-(pH 6.8) containing 1mM Na-EDTA using a chilled mortar and pestle. The homogenate was then filtered through cheese cloth, centrifuged at 2000 g for 15 min(s) and the supernatant was used for the enzyme assays. All operations were performed at 4°C. The decrease in H_2O_2 was followed as a decline in optical density at 240 nm. Superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined according to the method described by Van Rossun et al. (1997) as follows: 50 µl of the enzyme extract (EE) was added to solution containing 13 mM L-methionine, 75 µM nitroblue tetrazolium chloride (NBT), 100 µM EDTA and 2 µM riboflavin in a 50 mM K-P buffer (pH 7.8). The reaction took place in a chamber under illumination of a 30 W-flurescent lamp was started formazane produced through NBT's photoreduction and was measured as the increase in absorbance at 560 nm. Control RM had no EE. The blank solution had the same components included in the complete RM but was kept in the dark. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction in comparison with tubes lacking the EE.

PCR analysis

To confirm the stable integration of the T-DNA into the plant genome and its presence in the regenerated plantlets, the plantlets regenerated from culture with or without *Agrobacterium* infection and selected with Kanamycin were subjected to polymerase chain reaction (PCR) analysis using *NPT-II* gene and 35 S-promoter specific primers. DNA samples were isolated from both of the transformed and non-transformed (control) plantlets according to the method described by Rogers and Bendich, (1985) and determined according to Hillis et al. (1996) as follows: genomic DNA extraction was heated at 60^{o}C for 1 hr. Half a volume of M LiCl was added after one hour and centrifuged at high speed in a microcentrifuge to pellet the precipitated DNA. Supernatant was removed and the pellet re-suspended in water or TE to allow precipitation of DNA away from mucopolysaccharides that inhibit PCR.

Extracted DNA was determined using UV-sectrometer at 260 nm to measure the concentration of DNA and determine the degree of purity.

The forward and reverse primers as well as their nucleotides sequence of the *NPT-II* gene were 5`-CGCAGGTTCT-CCGGCCGCTTGGGTGG-3` (position: 24-49) and 3`CTG-AAGCGGAAGGGACTGGCTGCT-5` (position: 254-277). The nucleotides sequences of the primers for the 35S promoter detection were 5`AAAGGAAGGTGGCTCCTAC-AAAT-3`and 3` CTCCAAATGAAATGATCC-5`, respectively. Thirty PCR cycles were performed. Each cycle consisted of the following steps: [i- 94 ^{o}C for 30 s (Denaturation), ii- 55 ^{o}C for 30 s (Annealing) and iii 72 ^{o}C for 1.5 min (Extension). PCR products were separated on horizontal agarose gel electrophoresis using an EC 370 Mini system (Hybaid instrument, Holbrook, New York, USA) containing 1.0% (w/v) agarose in migration buffer.

Statistical analysis

All data were subjected to analysis of variance according to Steel and Torrie (1980) using SAS system (2003). Subsequently, the partitions of phenotypic variances of its components were done. Heritability in broad sense was estimated as follows:

$$Hb\% = \sigma^2_g / \sigma^2_{ph} \times 100$$

Where: Hb = Heritability

σ^2_g = genetic variance

σ^2_{ph} = phenotypic variance

Conclusions

A. tumefaciens infection can developed the regeneration of *Punica granatum* L. plantlets *in vitro* by increasing

frequencies of callus and shoots formation as well as plantlets regeneration. Moreover, the new protocol can produce pomegranate plantlets tolerant to stress conditions by increasing their antioxidative defence system.

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