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Effect of Agrobacterium- Mediated Transformation on Regeneration Efficiency of Tomato Explants

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Abstract

The objective of this study was to study the effect of Agrobacterium- mediated transformation on regeneration efficiency of tomato explants. In vitro regeneration frequencies of cotyledon and hypocotyl of one local (Allah Kareem) and two imported (Peto86, Strain-B) tomato cultivars were investigated in a regeneration medium supplemented with different concentrations and/or combinations of phytohormones. The highest regeneration capacity was recorded for cultivar Peto86 (86% for cotyledon and 74% for hypocotyl) followed by Allah Kareem (82% for cotyledon and 74% for hypocotyl) and Strain-B (74% for cotyledon and 62% for hypocotyl). After transformation, results showed that The regeneration frequencies for cv. Peto86were significantly decreased from 24% to 15.3% and from 27% to 19.4%, for hypocotyls and cotyledonary explants, respectivel. Hypocotyls of cv. Strain B showed no regeneration after transformation. However, regeneration % of cotyledonary explants of the same cultivar was slightly decreased from 29.9% to 27.7%. Regeneration of Allah Kareem hypocotyls had sharply declined from 27% to 6.6%, after transformation. However, for coteledonary explants of Allah Kareem, the regeneration % was decreased from 34.6 to 26.9%.

Keywords: Tomato, Cotyledon, Hypocotyl, Regeneration, Transformation

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Introduction

Tomato (Lycopersicon esculentum Mill.), a member of the family Solanaceae, is a major vegetable crop that being consumed all over the world (Mueller et al., 2005). The United States is one of the world's leading producers of tomatoes, second only to China and followed by Greece, Italy, Spain and India (FAOSTAT, 2011). The main areas of production in Sudan include Khartoum, Northern Gezira, White Nile and Managil extension (Emam, 2011) with an estimated annual yield of 14.2857 tonnes per hectare (AOAD, 2010). Tomato production is seriously affected by both bioticand abioticstresses (Zhu, 2002; Bhatnagar- Mathur et al., 2008). In Sudan, cultivated tomatoes suffer from many diseases that are caused by many species of fungi, bacteria and viruses (Abbo et al., 2009). The advent of plant transformation and advanced molecular techniques for plant breeding provides powerful tools to enhance resistance to fungal diseases (Melchers and Stuiver, 2000).

The ability to introduce genes into plant species, via biotechnological techniques, has revolutionized fundamental research and allowed for the fastest development of new varieties in the history of commercial agriculture (Flavell, 2003). In this respect, plant transformation has probably been the most important technique for development of improved crops varieties (Flavell, 2000). Regeneration is a pre-requisite procedure for genetic improvement of crops through plant transformation and subsequent selection of interesting variants (Suslow et al., 2002). In vitro plant regeneration has been found to depend on many factors including genotype, explants, composition of basic medium, growth regulators, gelling agent, light intensity and quality, photoperiod, temperature, cultivation vessels and vessel covers (Reed, 1999). However, cultivar, explants type and medium composition are the most important in many plant species. The current protocols used for tomato transformation are based on shoot regeneration from leaf disc tissue co-cultivated with disarmed A. tumefaciens harboring binary vectors (Fillatti et al., 1987). However, the efficiency of transformtion is generally low because most of the transformed leaf/cotyledons cells do not develop into shoots (Hamza et al., 1993; Frary et al., 1996). Thus, the main objective was to study the effect of transformation process on the regeneration frequencies of tomato cultivars.

Materials and Methods

Preparation and regeneration of Explants

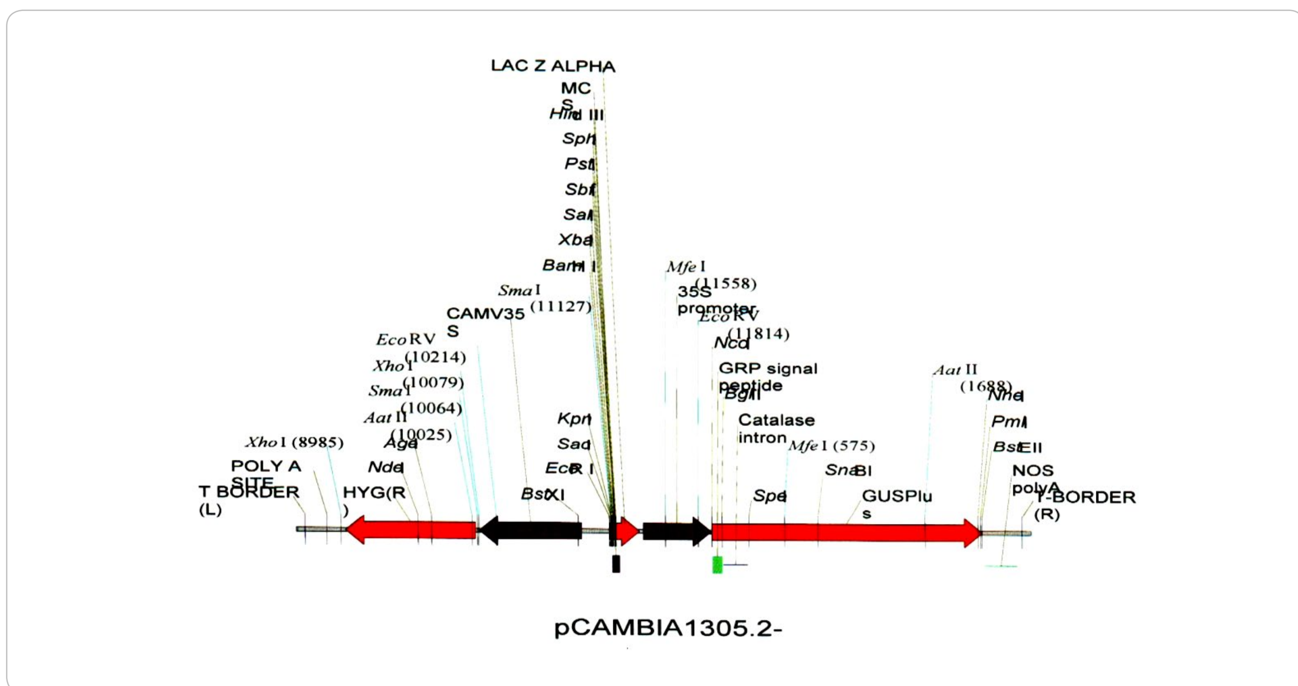
Two imported tomato cultivars, namely Peto86, Strain B and one

local cultivar (Allah Kareem) were used throughout this study. For sterilization, seeds were first dipped in 70% (v/v) ethanol for one min followed by immersion in 70% (v/v) clorox for 15 min. Seeds were then rinsed several times in sterile distilled water and cultured in sterilized full Murashige and Skoog (MS) and/or half strengthen (½MS) media (Murashige and Skoog, 1962) for germination. The seeds were initially kept for two days in the dark at 27 ± 1°C and then maintained under a 16-hrs. photoperiod at 50µmol/m2/S, with day/night temperature of 25°C/20°C.

Hypocotyl and cotyledonary explants were cut from 6-days-old seedlings. Hypocotyls were cut into two segments and placed horizontally on the surface of the regeneration medium (MS) supplemented with 30 g/L sucrose and various concentrations of various hormone treatments (RM1: MS + 2.5mg/l 6-Benzylaminopurine (BAP); RM2: MS + 2.5mg/l BAP + 1mg/l Zeatin (ZEA); RM3: MS + 2.5mg/l BAP +1.5mg/l ZEA; RM4: MS + 2.5mg/ l BAP + 2.5mg/l ZEA; RM5: MS + 1mg/l ZEA; RM6: MS + 1.5mg/l Kinetin (KIN) + 0.5mg/l Indole-3-acetic acid (IAA). Each medium pH was adjusted to 5.8 prior to autoclaving. Each cotyledon was transversally cut at the tip and base (proximal and distal) and placed with the adaxial surface in contact with the regeneration medium. Explants sub-cutting was made in 1/3MS liquid medium and pieces were blot-dried and placed into the medium. Induced shoots were elongated in MS medium supplemented with 1 mg/L ZEA. For root induction, elongated shoots were excised and cultured on MS medium supplemented with 1mg/L IAA + 0.5mg/L Gibberellin acetic acid (GA3) ; pH 5.8.

Transformation procedure

For transformation Agrobacterium tumefaciens EHA105 strain harbouring a pCAMBIA 1305.2 vector was used. The construct genes were under the transcriptional control of cauliflower mosaic virus 35S promoter (CaMV-35S), nopaline synthase (Nos) terminator, hygromycin phosphotransferase (hpt) conferring hygromycin resistance and neomycin phosphotransferase II (nptII) (conferring Kanamycin resistance) genes as selection markers for transformant plants and bacteria, respectively and GUS-intron (uidA-β-glucuronidase) as a reporter gene, a diagrammatic representation of the construct is as follows:



Primary culture of transformed Agrobacterium was prepared by inoculating 30 ml of LB medium with a single colony. The secondary culture was initiated by inoculating 1 ml of the primary culture in 50 ml of LB medium without antibiotic. The culture was then incubated at 28°C and 120 rpm for 48 h until the optical density (O.D600) of the culture broth was in the range of 0.5 to 0.8. The culture was then centrifuged at 6000 rpm for 10 min. The bacterial pellets so formed was diluted to an O.D600 = 0.3-0.6 by suspending in MS basal medium.

Transformation and regeneration of transformants

Before Transformation, explants were precultured in MS medium supplemented with 2.5mg/l BAP + 1mg/l ZEA or 1mg/l ZEA for four days. Pre-cultured explants were carefully submerged in Agrobacterium inoculum with gentle swinging. The explants were dried on sterile filter papers and transferred to co-cultivation MS medium supplemented with + 2.5mg/L BAP + 1mg/L ZEA or 1mg/l ZEA and incubated, in the dark, at 28°C for one day. Explants were then washed by washing medium (liquid regeneration media), dried on sterile filter papers and transferred to regeneration medium supplemented with 200mg/L Augmentin for pre-selection. The explants were left to regenerate at 25°C, with a 16h/8h (light/dark) photoperiod in the culture room. After 10 days explants were transferred to selection medium containing 200mg/L Augmentin + 15mg/L Hygromycin. Survivor shoots were transferred weekly to a fresh selection medium for four weeks; the fresh shoots were sub-cultured in a shoot elongation medium. Elongated shoots were then transferred to a rooting medium for root development. A set of explants which were not co-cultivated with Agrobacterium were also regenerated, as described above. Regeneration frequency was expressed as a percentage of the number

of putative transformants recovered relative to the number of the hygromycinresista- nt explants. Any rooting shoot were considered as putative transformants.

Results and Discussion

Both hypocotyls and cotyledonary explants, from 6-day-old seedlings, were tested using different growth regulators. The two types of explants showed comparable results in their capacity to initiate shoots in a given concentration/combination of growth regulators (Table 1). However, although sometimes non-significant, in all cultivars, cotyledonary explants showed higher shoot induction percentages compared to hypocotyls explants. Inconsistent with this, Moghaieb et al. (1999) recorded higher regenera¬tion frequency for hypocotyls (70.2%) compared to cotyledon (35.3%) for three studied tomato cultivars. The average number of initiated shoots was 7 to 10 shoots per explant for both types. Peto86 showed higher shoot induction percentage for both hypocotyls and cotyledonary (86% and 82%, respectively) explants, compared to Allah Kareem (74% for cotyledon and 74% for hypocotyls, respectively) and Strain B which showed (74% for cotyledon and 62% for hypocotyl). Palana et al. (2005) reported that, tomato adventitious shoot capacity depends on explants source. Gubis et al. (2003) reported that the frequency of adventitious shoot regeneration differed depending on the type of explants and concentration of growth regulators added to the regeneration medium. Most of the reports about adventitious regeneration in tomato deal with regeneration induction using hypocotyl or cotyledonary explants (Asakura et al., 1995; Ichimura & Oda, 1995; Moghaieb et al., 1999). Results reported here are comparable to those obtained by Nogueira et al. (2001) and El Siddig et al. (2009). Results showed that the media containing only BAP or ZEA as cytokinins demonstrated the lowest shoot initiation % in the range of 0% to 30 %, recorded for cultivars Peto86 and Strain-B. When a combination of 2.5mg/l BAP plus 1.0mg/l ZEA or 2.5mg/l BAP plus 1.5mg/l ZEA were used in the regeneration medium, significantly higher shooting percentage in the range of 40% to 86 % was obtained. The exception was Cultivar Allah Kareem which showed highest shooting percentage (82 and 74% for cotyledons and hypocotyls, respectively) when explants were cultivated on 1.0mg/l ZEA. However, the medium containing 2.5mg/l BAP plus 1.5mg/l ZEA initiated shoots within two weeks (Table 1) while the medium containing 2.5mg/l BAP plus 1mg/l ZEA initiated shoots within three weeks for cultivars Peto86 and Strain-B. It worth mentioning that the medium containing (mg/l) 2.5 BAP plus 1 ZEA induced high numbers (7-10) of shoots/explant when compared to the medium containing (mg/l) 2.5 BAP plus 1.5 ZEA which induced less (2-4) shoots/explant. Considering these previous results, two media were selected for regenerating putative transformed explants in subsequent experiments. These are: 2.5mg/l BAP plus 1mg/l ZEA (for Peto86 and Strain-B) and 1mg/l ZEA for Allah Kareem. Our result supported the results of other authors (Ichimura and Oda, 1995; Nogueira et al. 2001) who found that the most efficient medium for in vitro regeneration of tomato being induction medium supplemented with a cytokinin ZEA. Park et al. (2003) reported that media containing ZEA as a cytokinin were significantly better than BAP for regeneration in all of their tested cultivars and for all explants.

Cotyledons and hypocotyls explants of tomato cultivars Allah Kareem, Peto86 and Strain-B were used for transformation. The results showed that the cultivar Peto86 was more prone to transformation and regeneration than other cultivars. The percentage of explants which were Hygromycin resistant after four successive selections were 28% for Peto86, 15.6% for Allah Kareem and 11% for Strain-B.

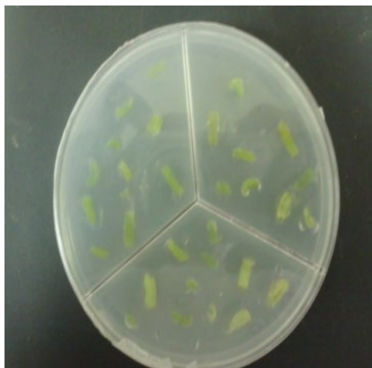
Transformed tissues were maintained on shoot induction medium for regeneration. Shoot induction was achieved in 5-6 weeks during which explants were transferred to fresh medium every 10 days. Long shoots were cut off and vertically inoculated into root induction medium for rooting. A representative result is given in Plate 1. Not all of the transformed plantlets that have shown Hygromycin- resistance initiated shoots. Transformation process decreased the regeneration efficiency for all of the tested cultivars. The regeneration frequencies for cv. Peto86were significantly decreased from 24% to 15.3% and from 27% to 19.4%, after transformation, for hypocotyls and cotyledonary explants, respectively (Tables 2). Higly significant difference in regeneration frequencies was recorded for hypocotyls of cv. Strain B which showed no regeneration after transformation. However, regeneration % of cotyledonary explants of the same cultivar was slightly decreased from 29.9% to 27.7% (Table 3). Results obtained in Table 4 indicate that regeneration % of Allah Kareem hypocotyls had sharply declined from 27% to 6.6%, after transformation. However, for coteledonary explants of Allah Kareem, the regeneration % was decreased from 34.6 to 26.9%. High frequency of regeneration in the untransformed tissue may be attributed to the competence of the cells for regeneration (Velcheva et al., 2005). On the other hand, decreasing of regeneration frequencies after transformation process can be explained by the effect of transformation parameters such as antibiotics used to eliminate Agrobacterium growth. The wide range between the concentrations of antibiotics used by several authors demonstrates the different effects of these compounds in plant tissue cultures, where they can have a phytotoxic and harmful effect, even favouring the regeneration of transformed plants, therefore affecting the growth of plant cells and the organogenesis and somatic embryogenesis processes (Mayolo et al., 2003; Nauerby et al., 1997). However, Augmantin was found not to affect regeneration of tomato and other plants (Quisen et al., 2009). It is also reported by Peña et al. (2004) that not every transformed cell can be regenerated into plants and that the best conditions for regeneration are not necessarily the best for transformation. It has been demonstrated previously that co-cultivation of safflower explants with Agrobacterium decreases regeneration frequency compared with non-transformed controls and the addition of AS further reduced safflower regeneration because of the increased bacterial infectivity and resulting hypersensitive response (Orlikowska et al., 1995). Necrosis at the proximal end of the cotyledons following co-cultivation with Agrobacterium was resulted in poor regeneration from transformed cells (Belide et al., 2011).

Furthermore, hyperhydration of transgenic shoots was considered as a major problem that result in reduction of regeneration of transgenic shoots in many plants. Of these Eucalyptus (Whitehouse et al., 2002) and Safflower (Carthamus tinctorius L.) (Belide et al., 2011).

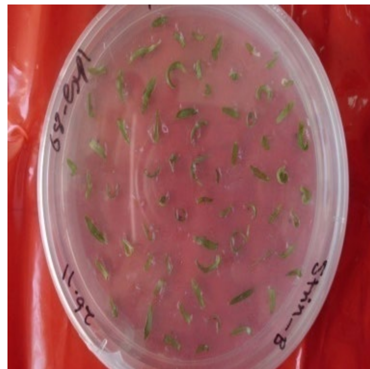
cultivar / Media	Peto86		Strain-B		Allah Kareem	
	Coty.	Hypo.	Coty.	Hypo.	Coty.	Hypo.
MR1	*30 **(4)	22 (5)	26 (4)	20 (5)	0 (6)	0 (6)
MR2	86 (3)	74 (3)	74 (3)	62 (3)	14 (4)	0 (4)
MR3	62 (2)	60 (2)	68 (2)	58 (2)	18 (4)	4 (4)
MR4	20 (4)	16 (4)	60 (4)	54 (4)	20 (4)	12 (4)
MR5	0 (8)	0 (8)	0 (8)	0 (8)	82 (3)	74 (3)
MR6	34 (6)	30 (6)	20 (6)	12 (6)	22 (6)	16 (6)

Table 1: Effect of explant type and growth regulators on regeneration of different tomato cultivars

Plate 1: Various stages during transformation and regeneration of tomato

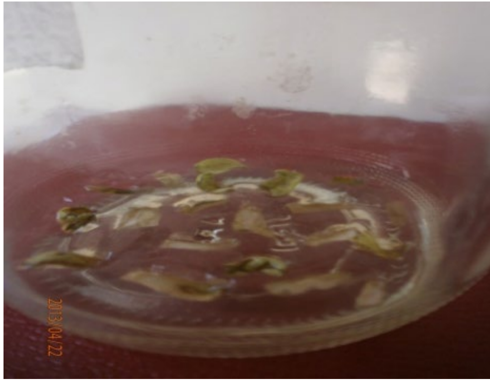


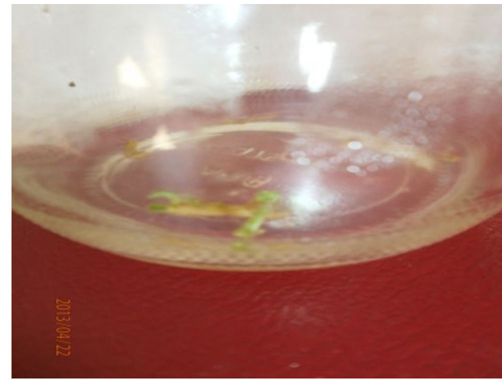
(a): Pre-cultured explants



(b): Co-cultivated explants

(c): Pre-selected explants


(d): Hygromycin-resistant explants


(e): Initiated shoots

(f): Shoot elongation

(g): rooted plantlets

Table 2: Regeneration percentage* of transformed (co-cultivated) and untransformed (control) tomato cultivar Peto86

Stage	Co-cultivated		Control	
	Coty.	Hypo.	Coty.	Hypo.
No. of explants	237	192	378	404
Hygromycin resistant explants	67	39	-	-
Shoot initiation	19	13	287	203
Rooting	13	6	102	97
Regeneration%	19.4%	15.3%	27%	24%

*Results based on pooled data of three replicates

Table 3: Regeneration percentage* of transformed (co-cultivated) and untransformed (control) tomato cultivar Strain B

Stage	Co-cultivated		Control	
	Coty.	Hypo.	Coty.	Hypo.
No. of explants	164	195	194	200
Hygromycin resistant explants	18	5	-	-
Shoot initiation	9	0	168	105
Rooting	5	0	58	33
Regeneration%	27.7%	0%	29.9%	16.5%

*Results based on pooled data of three replicates

Table 4: Regeneration percentage* of transformed (co-cultivated) and untransformed (control) tomato cultivar Allah Kareem

Stage	Co-cultivated		Control	
	Coty.	Hypo.	Coty.	Hypo.
No. of explants	166	213	280	293
Hygromycin resistant explants	26	30	-	-
Shoot initiation	10	4	202	187
Rooting	7	2	97	79
Regeneration%	26.9%	6.6%	34.6%	27%

*Results based on pooled data of three replicates

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