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Agrobacterium-mediated Transformation of Tomato Plants Expressing Defensin Gene

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ABSTRACT

The aim of this study was to develop a protocol for transformation/regeneration of tomato plant (cv. Summer set) using a plant antifungal gene (defensin). Transformation was carried out using disarmed *A. tumefaciens* strain LBA4404 harbouring a binary vector pITB-AFP. Sequencing of plasmid DNA extracted from this strain indicated that it contains defesin gene (*AFP*), an antifungal protein-coding gene, under the control of a CaMV 35S promoter and Nopaline Synthase (NOS) terminator, hygromycin phosphotransferase (*hpt*) and β-glucuronidase (GUS) genes, as selectable and marker genes, respectively. The factors that affect transformation/regeneration protocols were optimized in a series of experiments. Results indicated that exposure of cotyledonary explants to *Agrobacterium* inoculums of 0.8 $O.D_{600}$ for 30 min, selection on Hygromycin-containing medium (Hygromycin concentration of 25 mg L^{-1}) and subsequent regeneration on MS medium supplemented with 2.5 mg L^{-1} BA as a cytokinin and 1.0 mg L^{-1} IAA as an auxin resulted in transformation efficiency of 7%. GUS expression was observed in transformed tomato shoots but never in the control plants. PCR amplification of DNA extracted from the transformed tissues demonstrated the generation of the expected amplicon, corresponding to AFP gene. This result strongly verifies the successful transformation of the tomato cultivar Summer set, an endeavour which is reported for the first time in Sudan. Moreover, this protocol paves the way for problem solving-applications encompassing other Sudanese crops of economic importance.

Key words: *Agrobacterium*, defensin, summer set, regeneration, *AFP* gene

INTRODUCTION

Tomato (*Lycopersicon esulentum* Mill.) is the major vegetable crop grown worldwide, with a production estimates of 95 million Mt (FAOSTAT, 2002) and its production is concentrated in semi-arid regions (Santa-Curz *et al*., 2002). Presently, tomato is becoming increasingly important in Sudan for local consumption and for export. It is cultivated throughout the year under irrigation in an area that exceeds 36540 hectares with an average yield of 17.57 tons per hectare (AOAD, 2007). The most important grown cultivars are the canning types such as Strain B, Strain C, Summer set, Peto86, Peto111 and CastleRock in addition to few local varieties (Mukhtar *et al*., 2009).

In Sudan, as in other parts of the world, cultivated tomatoes suffer from many diseases that are caused by viruses, bacteria and fungi. Among virus diseases, tomato mosaic root knot and tomato

yellow leaf curl (Geneif, 1986) are considered as the most important. Bacterial diseases include bacterial wilt (*Pseudomonas solanacearum*) (Jinnah *et al*., 2002), *Verticillium* wilts (*Verticillium dahliae*), powdery mildews (*Leveilula taurica*) and early and late blights which are caused by *Alternaria solani/alternata* and *Phytophthora infestans*, respectively (Abbo *et al*., 2009).

While the use of disease resistant cultivars may present an effective way of controlling the above diseases, genetic engineering techniques continue to play a major role in the development of disease resistant cultivars (Bhatia *et al*., 2004). Various factors that affect the development of techniques for the isolation and identification of many genes involved in plant disease resistance, morphology and development have been studied (Ling *et al*., 1998). In addition, different factors such as *Agrobacterium* cell density (Murray *et al*., 1998), regeneration and co-cultivation conditions (Hu and Phillips, 2001), addition of acetosyringone and cell competence after wounding (Murray *et al*., 1998) and gene constructs (Krasnyanski *et al*., 2001) were found to play a major role in tomato transformation. The first report of tomato transformation was forwarded by McCormick *et al*. (1986) and since then, there have been numerous publications on transformation of various tomato cultivars (Vidya *et al*., 2000; Hu and Phillips, 2001; Raj *et al*., 2005; Sun *et al*., 2006; Shahriari *et al*., 2006; Saker *et al*., 2007). Standardization of tomato transformation procedures is, still, incomplete as different tomato cultivars vary in their response to specific treatment. The present study was undertaken to develop an efficient procedure for the production of fungal-resistant transgenic tomato (cv. CastleRock) plants expressing defensin gene.

MATERIALS AND METHODS

This study was conducted during June 2009 to Jan. 2010. *Agrobacterium*-mediated tomato transformation was performed in accordance to the protocol of Park *et al*. (2003) with some modifications.

Plant tissue: Seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar Summer set were surface sterilized in 10% (v/v) sodium hypochlorite solution, with 2 drops of Tween-20, for 15 min followed by three rinses in sterile water. Seeds were then germinated on MS (Duchefa Biochemie) inorganic salt (MSO) medium (Murashige and Skoog, 1962) with 30 g L^{-1} sucrose, pH 5.7 and solidified using 7 g L^{-1} TC agar (GFS Chemicals and reagents, Texsas, USA). Hypocotyl explants and cotyledonary sections either with distal or proximal ends from 8-day-old seedlings were used. Explants sub-cuttings made in MSO liquid medium were blot-dried and placed on MS medium supplemented with 1 mg L^{-1} of both 6-benzyladenine (BA) and Naphthalene Acetic Acid (NAA) for one day before co-cultivation with *Agrobacterium*.

Bacterial strain and plasmid: A defensin (antifungal, AFP) gene cloned in *Agrobacterium tumefaciens* LBA4404 strain was used. This strain harbours the pITB-AFP plasmid vector which contains defensin (*AFP*) gene under the transcriptional control of cauliflower mosaic virus 35S promoter (CaMV-35S) and nopaline synthase, Nos, terminator, hygromycin phosphotransferase (*hpt*) gene and GUS-intron (*uid*A-β-glucuronidase) as a reporter gene. Bacteria were grown on LB medium supplemented with streptomycin (30 mg L^{-1}) and kanamycin (100 mg L^{-1}) with shaking at 200 rpm to an OD_{600} = 1.0. The bacterial suspension was then diluted with MSO medium in a ratio of 1: 5 and used for transformation experiments.

Transformation and regeneration protocol: The precultured explants were carefully submerged in an *Agrobacterium* inoculum in a Petri dish (Ø9 cm) for 30 min with gentle swinging.

The explants were then blot dried on sterile filter paper, transferred to Medium B and incubated in the dark for three days at 25°C. Explants were then transferred to plates containing a selection medium (Medium C) supplemented with 25 mg L^{-1} hygromycin. The plates were sealed with parafilm and explants were left to regenerate at 25°C, with a 16/8 h (light/dark) photoperiod in the culture room. Hygromycin-resistant calli obtained after a second round of selection were transferred to a fresh selection medium. Once in every two weeks, the fresh and healthy looking hygromycin-resistant calli were sub-cultured in a fresh selection medium for shoot regeneration. After approximately 6-8 weeks, shoots were excised and transferred to Medium D for shoot development. The shoots obtained were transferred to rooting medium (Medium E) for root development. A set of explants which was not co-cultivated with *Agrobacterium* was also prepared, as described above, as a negative control. The experiment was made in three replications in a completely randomized design. The number of surviving calli in each case was determined and the data was subjected to analysis of variance. Transformation frequency was expressed as a percentage of the number of shoots recovered from hygromycin-resistant calli relative to the total number of incubated hygromycin-resistent calli. Any rooting shoot on the selection medium containing 25 mg L^{-1} hygromycin was considered as a transformant.

Media composition: The culture media used in the transformation and regeneration experiments were solidified with 7g L^{-1} TC agar and its pH was adjusted to 5.7.

Medium B (Co-cultivation medium): MS salts and vitamins, 30 g L^{-1} sucrose, 1 mg L^{-1} BAP and 1 mg L^{-1} NAA.

Medium C (Selection and shoot regeneration medium): MS salts and vitamins, 30 g L^{-1} sucrose, 2.5 mg L^{-1} BAP, 0.1 mg L^{-1} Lndole-3-Acetic Acid (IAA), 250 mg L^{-1} cefotaxime and 25 mg L^{-1} hygromycin.

Medium D (Shoot eleongation medium): MS salts and vitamins, 30 g L^{-1} sucrose, 1 mg L^{-1} BAP, 0.1 mg L^{-1} IAA and 250 mg L^{-1} cefotaxime.

Medium E (Rooting medium): MS salts and vitamins, 30 g L^{-1} sucrose, 1 mg L^{-1} IAA and 250 mg L^{-1} cefotaxime.

Effect of Cefotaxime on callus growth and shoot regeneration: To examine the influence of cefotaxime on callus growth and shoot regeneration (before and after transformation), 100 cotyledonary explants (25 pieces per Petri dish) were separately cultured on MS medium containing 0, 50, 100, 200, 250 and 300 mg L^{-1} cefotaxime. Regeneration frequency was calculated as a percentage of the number of regenerated explants/total number of explants (Ling *et al*., 1998).

Effect of the inoculation period: To assess the effect of different periods of transfection (the period for which explants were immersed in the bacterial suspension) on the transformation frequency, explants were submerged in the bacterial broth for 10, 20, 30 and 35 min.

Effect of *Agrobacterium* density: Bacterial densities (OD_{600}) ranging from 0.3 to 2.0 were tested to determine the optimum density for transformation.

Sensitivity of tomato explants to hygromycin: To determine the effect of hygromycin (water solution of hygromycin B) on the regeneration of tomato explants and to screen for an appropriate hygromycin concentration for transformants selection, cotyledonary and hypocotyl explants were transferred to the induction medium supplemented with different concentrations of hygromycin (0, 5, 10, 15, 20, 25, 30, 35 and 40 mg L^{-1}) in Petri dishes (10 cm in diameter) each containing 100 explants. The explants were incubated for 2 weeks in the dark at 25°C and then transferred to a 16/8 h (light/dark) photoperiod of 80 mol m^{-2} sec^{-1} at 25°C. The number of regenerated shoots was recorded after 5 weeks.

Effect of plant growth hormones combination on regeneration: To evaluate the effect of growth regulators on transformation efficiency, different plant growth regulator combinations were examined. These combinations were in mg L^{-1}: BA 1.0/NAA 0.1 for 4 days (a one-day preculture period and a 3-day co-cultivation period) followed by transfer to BA 2.5/IAA 0.1; Zeatin 1.0/IAA 0.1; Zeatin 2.0; or to Zeatin 2.0/IAA 0.1.

Histology and histochemical analysis: GUS activity was analyzed in putative transformed primary explants, shoots and leaves from regenerated plants according to Jefferson (1987). Plant cells were incubated at 37°C for 24 h in GUS-staining solution (0.5 mM of X-gluc, 10 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, in 0.1 M phosphate buffer, pH 7.0). GUS activity was visualized under stereoscope after washing the stained explants in 70% ethanol.

Molecular verification of the transformants: To confirm the presence of the AFP gene in the regenerated transformed plants, total DNAs were isolated from both transformed and untransformed (negative control) plant samples and were used as templates for PCR. Plasmid DNA was also isolated and used as a positive control.

Rapid plant DNA extraction: DNA extraction was carried out following CTAB (cetyl trimethyl ammonium bromide) protocol, which is a modification of the method of Doyle and Doyle (1987).

Polymerase Chain Reaction (PCR): The presence of *AFP* gene was investigated by PCR amplification. Specific oligonucleotide primers for *AFP* gene were 'forward': 5'- CGC GGA TCC ATG GCG AGG TGT GAG AAT TTG GCT-3' and 'reverse': 5'-TGC TCT AGA ATG GCG AGG TGT GAG AAT TTG GCT-3'.

Each PCR reaction was performed in 25 μL (total volume) of the reaction mixture that consisted of 1X reaction buffer, 10 ng plant DNA from a putative transgenic plant as a template, 200 mM dNTPs, 1.5 mM $MgCl_2$, 2 mM of each primer and 0.5 unit of Taq DNA polymerase. Amplification was carried out in a Thermal Cycler (Biometra) under the following conditions: 94°C for 3 min for initial denaturation, 94°C for 1 min for denaturation, 54°C for 1.5 min for annealing, 72°C for 1 min for elongation, 7 min at 72°C final extension and 35 cycles of amplification.

Amplified DNA fragments were electrophoresed on 1.0% agarose gel and detected by ethidium bromide staining and photographed under ultraviolet light. The presence of the target band in the transformants and its absence in the untransformed plants is considered as a proof of successful transformation.

RESULTS

A total of 300 cotyledonary leaves and 300 hypocotyls of tomato plants (cv. Summer Set) were used for transformation. Trials revealed great variations in the transformation frequency depending on the explant type (cotyledon or hypocotyl). The results showed that cotyledonary explants were more efficient for regeneration and subsequent transformation than hypocotyl explants (Table 1). In both explant types, callus formation was observed in more than 85% of the cultured plant tissues within 15-25 days (Fig. 1).

Detection of transformed cells was made, after 5 days of co-cultivating the explants with *Agrobacterium*, by placing individual explant pieces on Hygromycin-containing medium. Green calli were obtained, on average, after 4 weeks of incubation (Fig. 2). 47% and 35% of cotyledonary and hypocotyl explants, respectively, showed Hygromycin resistance after three selections. Transformed tissues were maintained on selection media until the appropriate size for transfer to shoot induction medium. Shoot induction was achieved in 6-8 weeks during which explants were transferred to fresh medium every two weeks. Well-grown shoots were cut off, vertically inserted into root induction medium and incubated for 10 days. A representative result is given in Fig. 3. The highest transformation frequencies were 7 and 3% for cotyledonary explants in media containing BA and Zeatin, respectively (Table 2). The results of survival of transformed and untransformed plantlets at various stages are summarized in Table 3. The frequency of shoot initiation for transformed plantlets was found to be 2.0% (6/300) in the medium containing Zeatin

Table 1: Effect of explants type on the rate of transformation

Explant type	Cotyledons	Hypocotyls
Total No. of explants	300	300
Callus formation (%)	100	100
Regenerant (%)	89	86
Hygromycin resistant calli (%)	47	35

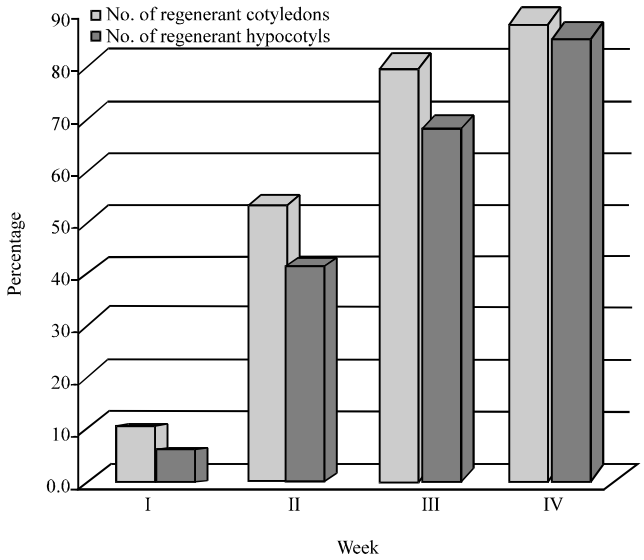

Fig. 1: Percentages of regenerant tomato explants during 4 weeks

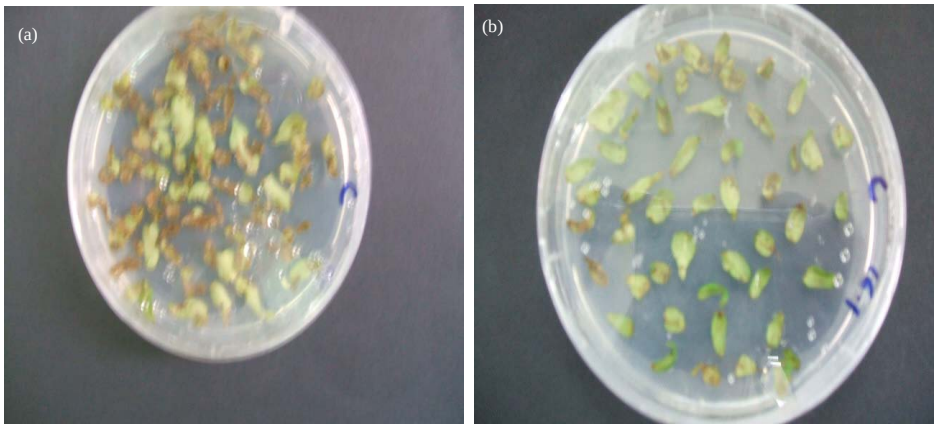

Fig. 2: Recovery of Hygromycin resistant transgenic tomato explants on Hygromycin-containing medium. (a) Transformed cotyledonary explants culture on a hygromycin-containing medium and (b) transformed cotyledonary and hypocotyl explants cultured on a medium without hygromycin (note the browning of the untransformed tissues in between the resistant transformed tissues)

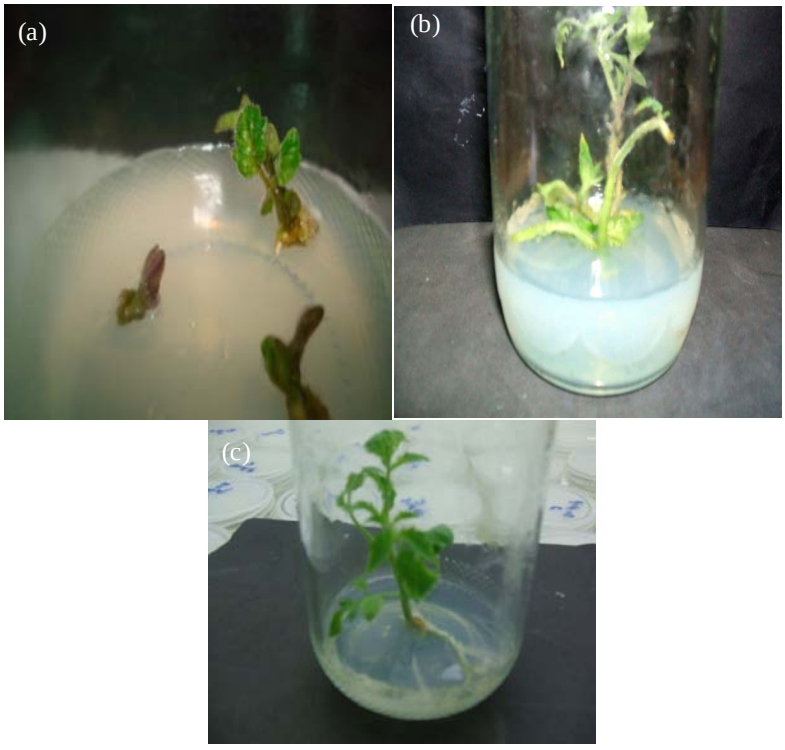

Fig. 3: Various stages during regeneration of transgenic tomato plantlets: (a) callus induction and shoot initiation, (b) shoot elongation and (c) rooted plantlets

Table 2: Effect of explants type and growth regulators on the transformation frequency of tomato

	Plant growth regulators			
	2.5 mg L^{-1} BA + 1.0 mg L^{-1} IAA		1.0 mg L^{-1} Zeatin + 0.1 mg L^{-1} IAA	
Type of explants	Cotyledonary explant	Hypocotyl explants	Cotyledonary explants	Hypocotylexplants
Total No. of explants	120	100	120	100
No. of explants initiated shoot primordial	87	62	80	56
No. of shooted explants	8	5	4	1
No. of rooted explants	8	5	4	1
Transformation frequency with regeneration (%)	7	5	3	1

Table 3: Survival percentage* of transformed (co-cultivated) and untransformed (control) tomato plants at various stages of development

	Co-cultivated				Control			
	Survival/total		Percentage		Survival/total		Percentage	
Stage	BA medium	Zeatin medium	BA medium	Zeatin medium	BA medium	Zeatin medium	BA medium	Zeatinmedium
Co-cultivation	220[a]	202[a]	73.3	67.3	295[b]	291[b]	98.3	97.0
Shoot initiation	25[a]	9[b]	8.3	3.0	270[c]	243[c]	90.0	81.0
Shoot elongation	22[a]	7[b]	7.3	2.3	255[c]	228[c]	85.0	76.0
Rooting	22[a]	6[b]	7.3	2.0	246[c]	225[c]	82.0	75.0

*Results based on pooled data of three experiments. Any two readings of the same stage having the same alphabetical letter are not significant at 0.05 level

and 7.3% (22/300) in the BA medium. For untransformed plantlets, shoot initiation frequency was 75.0% and 82.0% in Zeatin and BA media, respectively. The shoots were subsequently transferred on fresh selection medium containing BA for shoot elongation. Survival of the transformed shoots during shoot elongation period indicated successful transformation.

Verification of the transgenic plants

GUS histochemical assay: The histochemical GUS activity of selected regenerants showed a uniform dark blue color in the transformed tissues. GUS expression should also mimic the normal expression pattern of the gene of interest. On the other hand, non-transformed tissue did not exhibit such a color under identical assay conditions. GUS expression was found in some parts of the cotyledonary leaves and the hypocotyl pieces either at high (dark blue) or low levels (light blue). The possibility of false GUS positive was ruled out because the GUS gene was interrupted by an intron.

DNA isolation and PCR analysis: Genomic DNA from six randomly selected transformed plants as well as from untransformed ones were isolated and subjected to PCR analysis. The putative transgenic plants, when analysed by PCR using *AFP*-specific primers, gave the expected PCR amplicon (300 bp), whereas such an amplicon was not observed in untransformed (negative control) plants (Fig. 4). This result clearly demonstrates the presence of the *AFP* gene in tomato planlets co-cultivated with *Agrobacterium* containing the *AFP*-disarmed plasmid construct.

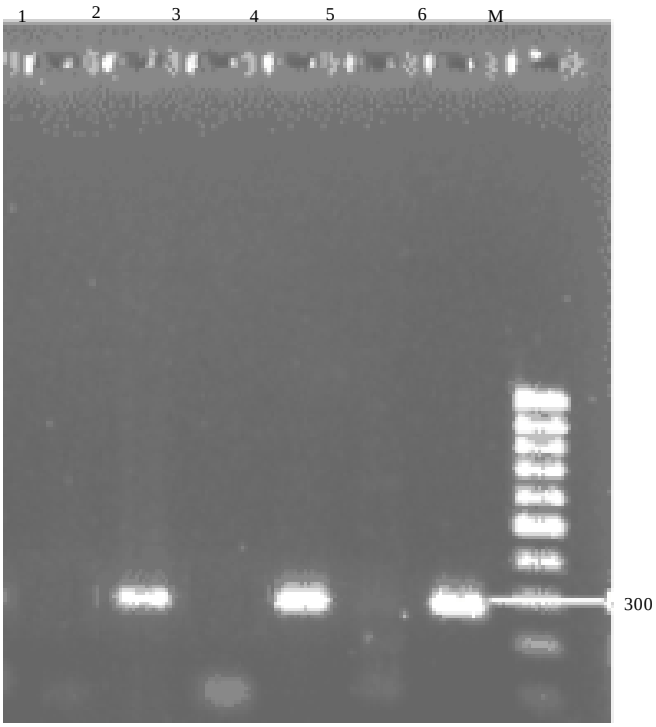

Fig. 4: PCR confirmations of regenerated transformed plants using AFP primers. Lanes: 1: Negative control; 2 and 6: Transformed plants; 4: Positive control; 3 and 5: Untransformed plants; M: 1 kb marker

DISCUSSION

Transformation of variuos tomato cultivars was previously reported by various authors. Transformation frequencies have ranged from 6% in cv. Pusa Ruby (Vidya *et al*., 2000) to 40% in cv. Micro-Tom (Sun *et al*., 2006). In spite of reported successes in tomato transformations, most of the transformation procedures were cumbersome and relied on feeder layers (petunia, tomato, or tobacco), time consuming media formulations or successive subcultures (Qiu *et al*., 2007).

No simple general procedure for tomato transformation exists due to many unknown factors that affect transformation and/or regeneration efficiencies. It has been reported that *Agrobacterium tumefaciens*-mediated gene transfer is influenced by strain (Stomp *et al*., 1990), genotype response to specific treatments (Park *et al*., 2003), explant type (Humara *et al*., 1999), temperature (Dillen *et al*., 1997), size of T-DNA (Park *et al*., 2003) and constitutive expression of the virulence genes (Hansen *et al*., 1994; Rossi *et al*., 1996). Van Roekel *et al*. (1993) reported significant effects of culture media and *Agrobacterium* strains on the transformation frequency of tomato. High-frequency transformation using *A. tumefaciens* depends not only on the efficiency of the plant *in vitro* regeneration system but also on the subsequent elimination of bacterial cells from transformed tissues (Tang *et al*., 2004).

A reliable plant regeneration protocol determines the successfulness of any transformation trial. Therefore, plant regeneration conditions have to be optimized for a given plant species/cultivar and type of explant (Farid Uddin *et al*., 2004). Results on regeneration trials indicated that callus

induction and regeneration from tomato explants are quite permissive over a range of plant growth regulators. Previuos studies (McCormick *et al.*, 1986; Van Roekel *et al.*, 1993; Frary and Earle, 1996; Costa *et al.*, 2000) have suggested specific types and combinations of plant growth regulators for effective regeneration of transformed tomato plants.

In this study, a reproducible transformation system for Summer set tomato cultivar using *Agrobacterium tumefaciens* and cotyledonary explants was developed. The results presented are the average of three independent experiments with 100 explants in each trial. Both explant types have shown high capacities for regeneration. However, cotyledons were found to be superior compared to the hypocotyls. In addition, the type and the concentration of phytohormones in the induction media were found to be crucial factors controlling tomato regeneration.

Compared to the transformed tissues, high frequency of shoot induction was observed in the untransformed tissues. This may be attributed to the competence of the cells for regeneration (Velcheva *et al.*, 2005). Direct shoot initiation, rather than the callus, was observed at the edge of the cotyledonary proximal end. Similar observations were reported by Raj *et al.* (2005) and Peres *et al.* (2001) who attributed this to be due to hormonal metabolism and/or genetic background of organogenetic competence. The possibility of non-transformed shoot survival on the elongation medium is generally reported in similar transformation trials giving a false positive indication (Velcheva *et al.*, 2005). The regeneration of non-transformed plants was explained by non-efficient selection due to the protection of non-transformed cells presumbly caused by the occurrence of chimeric plants (Ghorbel *et al.*, 1999; De Almeida *et al.*, 2003; Velcheva *et al.*, 2005) or alternatively that the antibiotic used for selection (hygromycin) was partially or completely phosphorylated by cells expressing *hpt* gene (Bashir *et al.*, 2004; Velcheva *et al.*, 2005).

Previous studies aiming at improving shoot regeneration of transformed tissue from many tomato cultivars indicated the necessity of using a tobacco, petunia or tomato feeder layer for pre-culture and explant-*Agrobacterium* co-cultivation (McCormick *et al.*, 1986; Fillatti *et al.*, 1987; Delannay *et al.*, 1989; Van Roekel *et al.*, 1993; Agharbaoui *et al.*, 1995; Frary and Earle, 1996; Ling *et al.*, 1998; Tabaeizadeh *et al.*, 1999). Hamza and Chupeau, (1993) showed that pretreatment with feeder cells stimulated tomato cell transformation but significantly reduced regeneration of transformed cells. Although, in this study, no feeder layer was used for pre-culture or *Agrobacterium* co-cultivation, an adequate transformation frequency (7%) was obtained. (Park *et al.*, 2003) reported similar results on other tomato cultivars with a transformation frequency of 20%.

CONCLUSION

When grown off-season under tropical conditions of the Sudan, tomato is likely to suffer from fungal infections. We have established a transformation/regeneration system for successful insertion of an antifungal protein (plant defensin) into tomato cv. Summer set using *A. tumefaciencs* as a vector. The result is considered to be a significant step in the development of commercial fungal resistant transgenic tomato cultivars.

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