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Callus tissue induction and analysis of *GUS* reporter gene expression in tomato (*Solanum lycopersicum* L.) transformed with *Agrobacterium tumefaciens*

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Abstract

Tomato is one of the most important crops of Solanaceae familyand one of the largest products consumed in Ecuador. The productivity of this important crop has been affected due to the involvement of some abiotic and biotic factors. For this crop the application of genetic improvement techniquesinvolves as a first step the production of dedifferentiated in vitrotissue culture. The objective of this research is to generate callus formation from tomato leaves, and a protocol for the analysis of GUSgene expression by histochemical tests for further investigation regarding the genetic transformation with various applications for this important crop. The sterilization procedure consisted in using 15% w/v detergent and sodium hypochlorite (0.5% v/v and 0.8% v/v) and for the callus induction the explants were inoculated on MS medium with 2,4-D (0.25 to 3 mgL^{-1}) and KIN (0.25 and 0.5 mgL^{-1}). The transfer of the reporter gene was performed through tomato callus co-culture with Agrobacterium tumefaciens($O.D_{600nm}$ 0.2 to 1) for 10 to 20 min, the plasmid used for transformation being pCAMBIA1305.2. Temporal expression in transgeniccells after 3 and 7 days of co-culture showed significant differences between the treatments applied. Treatment with $O.D_{600nm}$ 0.8, inoculation for 20 min and 5 days co-cultured with A. tumefaciens provided necessary conditions for T-DNA insertion into genome cells, being the most promising protocol for further important genetic transformation issues of this species.

Keywords: callus, *Solanum lycopersicum, Agrobacterium tumefaciens, GUS* reporter gene.

1. Introduction

Tomato is one of the most important crops appertaining to *Solanaceae* family, and the second crop most cultivated in the world. It is a nutritious food which can be cultivated in tropical, subtropical and temperate areas (ABU-EL-HEBA *&* al. 2008 [1]), also being one of the most consumed product in Ecuador. According to the Ministry of Agriculture, this crop has been planted on 1.688 hectares with a productivity of 36.221 tons per year during 2011 (MAGAP, 2012 [2]).

Culture techniques applied for crop improvement in Ecuador, consist only on crossing with resistant species for integrating to the progeny the resistance gene. However, these type of projects specifically for these species in Ecuador remain still under research (INIAP, 2012 [3]).

In vitro culture technique is a biotechnology tool for various applications (MANOLE A., 2014 [4]) including the production of virus-free plants and genetic transformation. The successful application of this methodology leads to the stabilization of an efficient cultivation system, with good genetic properties and competence to different culture conditions (OSMAN

&al. 2010 [5]). The process of callus formation in tomato starting on apices, leaves, roots and nodal segments is suitable for the plant regeneration (JATOI & al. 2001 [6]).

Application of breeding techniques involves the transfer of genetic material from one organism to another one. It has been limited because growing conditions will differ according to the explant (type, size and age), medium (plant growth regulators, carbon source, macro and micronutrients) and environmental conditions such as temperature (HERRERA & al.2004 [7]; BHATIA & al. 2004 [8]).

Although tomato production through micro propagation techniques and *A. tumefaciens* mediated transformation have been done in other countries then Ecuador, the protocols not being applied in the same way in our country because of many different factors such as environment, human sources and laboratory conditions. For this reason, generating our own protocols for callus formation *in vitro* (an essential pre-requisite breeding), and a protocol for *GUS* gene expression analysis after *A. tumefaciens* mediated transformation is important for future breeding projects, to achieve sustainable tomato production that contains a stable feature of heritability.

2. Materials and methods

Plant material and plasmid vector

Tomato plants about two months old were selected, and they were moved into Molecular Biology greenhouses (Sangolquí, Ecuador). A phytosanitary control was performed to prevent fungal growth every three days. For this study was used a pCambia1305.2 conventional vector carrying the *GUS* reporter gene, an *npt*II gene as the selectable marker gene for kanamycin resistance, and a CaMV 35S promoter.

Callus tissue induction

Solanum lycopersicum calli were developed from young leaves. Standard size leaves were collected and washed with run water for 20 minutes to remove impurities (ROBACKER, 1993 [9]). Further the leaves were immerse din 0.2% (v/v) Phytonsolution for 5 minutes and rinsing with sterile distilled water. Consequently, the protocol proposed by ZUBEDA & all (2010) [10] was modified and the explants were sterilized with 1.5% (w/v) detergent solution for 15 min at low agitation, and grapefruit juice for 5 min. Three washes with sterile water were performed after the sterilization of the plant material with 0.5% (v/v) sodium hypochlorite (NaCl) containing few drops of Tween 20 during 5 minutes (Fig 1). The leaves were washed three times with sterile distilled water.

The explants were inoculated on Murashige& Skoog (MS) medium supplemented with 30 g L^{-1} sucrose and 2 g L^{-1}phytagel, adjusted to 5.8 pH. The disinfection process of tomato leaves consists in different combination variants of sodium hypochlorite concentrations (0.25%, 0.5%, and 0.75%) and different immersion times varying by 5 to 8 minutes. These have been evaluated through a factorial arrangement 3x2 with six treatments.

Tomato calli were obtained on MS medium modified with 2,4-dichlorophenoxyacetic acid (0.5 to 3 g L^{-1}), kinetin (0.25 to 0.5 g L^{-1}), 30 g L^{-1} sucrose and phytagel as gelling agent, adjusted to 5.8 pH (Table 1). The conditions for the explants incubation were represented by 20 ± 2 °C temperature, 30-50% relative humidity, under complete darkness in 4 weeks after explant inoculation and then used in the transformation process. Calli were subculturedon new culture medium to maintain nutrients conditions for the development.

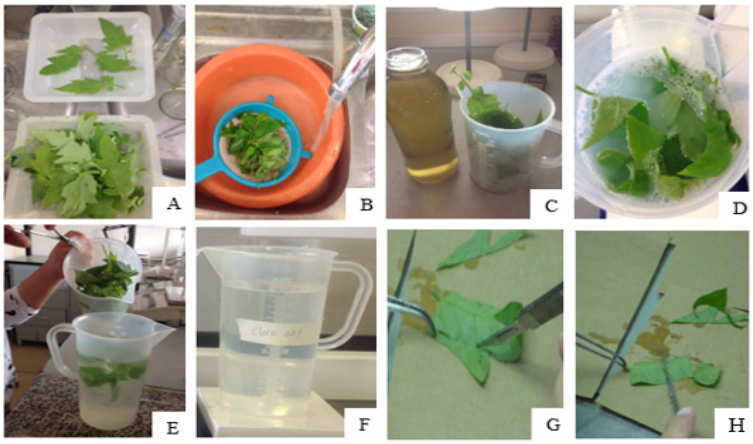

Figure1. Different stages of tomato leaves in the sterilization process.
A: Young leaves selection; B: Run water rinse; C: Immersion fungicide; D: Immersion detergent; E: Immersion Grapefruit extract; F: Immersion in sodium hypochlorite; G and H: explants.

Table 1. Culture medium for tomato callus induction.

Treatments	2,4-D (mg L^{-1})	Kinetin (mg L^{-1})
TC1	0.5	0.25
TC2	1	0.25
TC3	1.5	0.25
TC4	2	0.25
TC5	2.5	0.25
TC6	3	0.25
TC7	0.5	0.5
TC8	1	0.5
TC9	1.5	0.5
TC10	2	0.5
TC11	2.5	0.5
TC12	3	0.5

Agrobacterium tumefaciens mediated transformation

Agrobacterium strain (stored at -80^0 C) activation was achieved on selective LB (Luria Broth) medium supplemented with 50 g L^{-1} kanamycin and 100 g L^{-1}rifampicin (CHÁVEZ & al. 2002 [11]). One of the colonies was taken for subculture on LB medium with antibiotics to prevent non-transformed bacteria growth under 28^0 C and darkness conditions for 48 h. After incubation time, bacteria were inoculated on LB liquid medium with antibiotics and acetosyringone (1.962 mL L^{-1}) to obtain optical density of 0.2 to 1 (600 nm absorbance) (PARAMESH & al. 2010 [12]).

Transformation was performed immersing calli in *A. tumefacienciens* suspension for 10 to 20 minutes. The excess bacteria were dried with filter paper two times, and the calli were transferred to a new culture medium with acetosyringone to ensure T-DNA insertion for 3 to 5 days. After co-cultured, the calli were inoculated in a selectable medium to avoid bacteria proliferation and non-transgenic calli (RATNAYAKE & al. 2010 [13]).

GUS histochemical assay

Transgenic tomato callus were histochemically tested for β-glucuronidase activity (JEFFERSON & al, 1987 [14]) after co-cultivation time of 3 and 5 days. The materials for staining were incubated for 72 h at 30±1°C in X-Glucbuffer (phosphate buffer, metanol,

0.1%(v/v) Tritón X-100, EDTA 0.5 M, X-Gluc stock) (PARAMESH & al. 2010 [12]; RATNAYAKE & al. 2010 [12]). Finally, the tissues were examined under a stereo microscope. Three repetitions per treatment were performed with 24 experimental units. For the evaluation of the results were investigated the variables as Bacteria Optical Density (600 nm) (0.2 to 1), inoculated time (10 and 20 minutes) and co-cultured time (3 and 5 days). Factorial design was made with 20 transformation treatments. After4 weeks of incubation, callus morphological changes were observed.

Data Analysis

An ANOVA test was achieved to evaluate if data type followed a normality behavior. This hypothesis was proved by Shapiro-Wilks (modified) test. Comparison between treatments was performed by Kruskal Wallistest (no parametric), and Duncan test was applied for parametric variables with 5% of significance. All analysis was made through InfoStat program.

3. Results

Plant material induction

Results of the sterilization protocol shown that 0.5% (v/v) sodium hypochlorite concentration for 5 min, generated a lower rate of contamination (90% available explants), while the increase immersion time of leaves in sodium hypochlorite solutions showed a high percentage of contamination. This event was closely related to tissue necrosis. The eight minutes of immersion indicated „a high contamination" level in the three concentrations and these treatments cause tissue damage. The conclusion is that less time and lower concentration of hypochlorite was proving a significant percentage of contamination.

There is no a relation between immersion time and concentration because contamination results depend of time. The results indicate that less time in any chlorine concentration is giving a contamination index lower than higher time.

The treatment of 0.5% (v/v) hypochlorite concentration for 5 minutes has the lower statistic media (0.1) to contaminated explants percentage with a standard deviation of 0.31. This proved to be the best treatment for leaves sterilization and for maintaining the proper leaves properties.

Callogenesis

Callus induction results indicate that all treatments were inducing the presence of calli on the explants. TC5 and TC6 treatments had no significant difference during4 weeks. All tomato tissue was undifferentiated in 100% of the explants but was some variation in the calli amount on the tissue.

High percentage of callus induction in tomato young leaves could be obtained using high 2,4-D concentration (3 mg L^{-1}) and low kinetin concentration (0.25 mg $L^{-1)}$). In contrast, tissue-undifferentiating process took more time used high concentration of kinetin, so only 47% of explants had callus (Fig 2). TC5 treatment (2.5 mg L^{-1} 2,4-D and 0.25 mg L^{-1} kinetin) reached an average of 1.3 g fresh weight, being the highest weight compared to the other treatments.

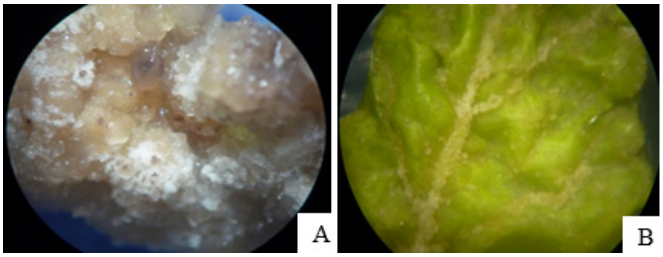

Figure 2.A: Dedifferentiated tomato tissue. **B:** Explant without callus formation.

Gus assay

One week after *Agrobacterium tumefaciens* mediated transformation, transformed callus tissues were histochemically tested for *GUS* activity. The putative transgenic tomato callus tissues indicated *GUS* activity as determinated from the blue spots observed in the histo chemical test that are proving the occurrence of transformation.

After 5 weeks, the putative transgenic calli showed *GUS* activity, indicating that *GUS* gene expression was relatively stable after transformation (Fig 3).

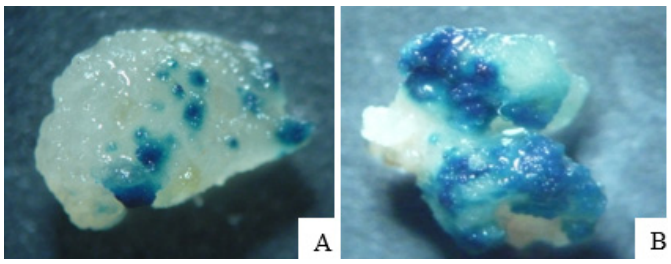

Figure 3.A: Temporal *GUS* activity (1 week);
B: Stable *GUS* activity (5 weeks)

Treatments with high bacteria concentration (O.D. 1) obtained the highest rate of transformed tissues (100% transformed callus). However, both inoculation time in the bacterial suspension and post-culture time influenced in the process of transferring the T-DNA. The explants co-cultured for 5 days reached a higher percentage of transformed callus than explants co-cultured for 3 days. Using 0.6, 0.8 and 1 O.D. were obtained 50%, 90% and 100% of calli with T-DNA respectively (Table 2).

		Inoculate time (min)			
		10		20	
	Co-culture time (d)	3	5	3	5
Bacteria O.D	0.2	0	10	0	10
	0.4	0	0	30	30
	0.6	10	10	50	40
	0.8	20	30	40	90
	1	20	90	30	100

Table 2. Transformed callus percentage

Evaluation of temporal *GUS* gene expression, blue spots counting was carried out as evidence of metabolism of X-Glucsubstrate for each one of the treatments. Treatment no.16 showed a greater number of small blue spots on the surface of explants with 88 pellets. Treatment no.19 and 20 showed 49 and 51 blue spots respectively.

Unlike other treatments at lower co-cultivation time and low bacteria concentrations, the number of blue spots as a signal GUS activity was lower. Treatments no. 1, 2, 5, and 7 as control (non-transformed tomato cells) didn't show any blue points. Blue dots were shown after 48 hours of X-Glucincubation (Table 3).

		Inoculate time (min)			
		10		20	
	Co-culture time (d)	3	5	3	5
Bacteria O.D	0.2	0	2	0	11
	0.4	0	0	5	6
	0.6	1	1	25	13
	0.8	6	22	7	88
	1	4	49	8	51

Table 3. *GUS activity* (blue spots number) in tomato calli.

4. Discussion

A rapid morphogenic response of the explants dependent on the growth regulators, which are added to the culture medium (LUTFUN & al. 2013 [15]). Moreover, Osman & al. (2010 [4]) reported that *in vitro* callus induction depends on endogenous and exogenous plant growth regulators.

Combination of 2,4-D and Kin in the culture medium will promote callus induction in 10 days, the same results being obtained by Ali & al. (2012 [16]). In their study, cytokinin-cytokinin interaction for induction of callus and plant regeneration of tomato varieties showed that there weren't statistically significant differences among treatments in all tested genotypes during 6 and 10 days. Cultured explants showed callus formation signals for different concentrations of cytokines.

Transient transformation assay after 72 hours co-culture on 1.28 $O.D_{600nm}$ bacteria indicated that were reached 20% (10 min inoculate time) and 30% (20 min inoculate time) transgenic explants. These results were more effectively than the protocol developed by Barrero (2008 [17]), were only just 6% of explants showed blue dots indicating the X-Gluc metabolism. Devi& al. (2012 [18]) obtained a significant percentage of *GUS* activity using a low bacterial concentration and 30 minutes inoculation time. Around 12.03% gene activity was achieved while was used a double bacteria concentration.

After treatments application, about 100% of tomato callus co-cultured (1.28 $O.D_{600nm}$) were viable for tissue regeneration, unlike work done by Barrero (2008 [17]) in another *Solanaceae* family plant at same bacterial concentration, only 32% of the explants were viable because of the existing oxidation and chlorosis sings on the tissues.

Treatments with 120 days co-culture and 10 to 20 minutes on highest bacterial concentration showed 90% and 100% transformants, and 88 blue spots using vector pCAMBIA1302.5. This score was low, compared with those obtained by Sánchez (2010 [19]). His protocol reached an average of 488 ± 18 blue points using pCAMBIA1301 vector in banana 'Williams' (AAA) embryogenic cell. This result may be because we worked on tomato callus that is an amount of accumulated cells, in addition tissue was not enough injured by scalpel to T-DNA insertion. Banana study used cell suspensions that allowed better access to T-DNA.

5. Conclusion

The efficiency of the transformation process depends on several factors such as the type of crop, the age of explant, *Agrobacterium* strain and bacterial density; inoculation time, co-cultivation time and selective medium regeneration. Histochemical analyzes confirmed that tomato cells in animmersion time of 20 minutes in the bacterial suspension and co-cultivation time for 5 days with *A. tumefaciens,* were engineered. The use of *Agrobacterium tumefaciens* for transformation process in *Solanum lycopersicum* permits the insertion of important genes that could improve production and disease resistance.

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References

(1) ABU-EL-HEBA G, HUSSEIN G, ABDALLA A, A rapid and efficient tomato regeneration and transformation system. *Agric. For. Res*. 58, 103-110 (2008).

(2) MAGAP. Ministerio de Agricultura, Ganadería, Acuacultura y Pesca, Producción: Tomate riñón: Superficie, producción y rendimiento a Nivel provincial, (2012). <http://servicios.agricultura.gob.ec>
(3) INIAP, Instituto Nacional de Investigaciones Agropecuarias, (2013). <http://www.iniap.gob.ec/web/#>
(4) A. MANOLE PAUNESCU, Biotechnology for endangered plant conservation. In M.R. Ahuja & K.G. Ramawat (Eds) Biodiversity and Biotechnology. Springer International Publishing, Switzerland, ISBN 978-3-319-09380-2:181-202 (2014).
(5) OSMAN M, ELHADI E, KHALAFALLA M, Callus formation and organogenesis of tomato (*Lycopersicumesculentum* Mill, C.V. Omdurman) induced by thidiazuron. *Afr. J. Biotechnol*., 9, 4407-4413 (2010).
(6) JATOI S, SAJID G, SAPPAL H, BALOCH M, QURESHI A, ANWAR R, Differential In vitro response of tomato hybrids against a multitude of hormonal regimes. *Online J. Biol. Sci*., 1, 1141-1143 (2001).
(7) HERRERA L, SIMPSON J, MARTÍNEZ M, Transgenic plants: Methods and protocols. *Ed Humana*, 286, 3–31 (2004).
(8) BHATIA P, ASHWATH N, SENARATA T, MIDMORE D, Tissue culture studies of tomato (*Lycopersiconesculentum*). *Plant C., Tissue and Organ Cult*. 78, 1-21 (2004).
(9) ROBACKER C, Somatic embryogenesis and plant regeneration from Muscadine grape leaf explant. *Hort Sci.,* 28, 53-55 (1993).
(10) ZUBEDA C, SIDRA A, AZRA Y, HAMID R, HABIB A, AKBAR M, Tissue culture studies in tomato (*Lycopersicum Esculentum*) var. Moneymaker Pak. *J. Bot*., 42, 155-163 (2010).
(11) CHÁVEZ O, RUPERTO A, Evaluación del efecto de la aplicación de micorrizas en la producción de Tomate riñón (*Solanum lycopersicum*) bajo invernadero. *Rep. ESPOCH*. (2010).
(12) PARAMESH H, FAKRUDIN B, KURUVINASHETTI M, Genetic transformation of a local variety of tomato using gus gene: an efficient genetic transformation protocol for tomato. *J AgricTechnol*, 6, 87-97 (2010).
(13) RATNAYAKE R, HETTIARACHCHI G, Development of an efficient Agrobacterium mediated transformation protocol for Sri Lankan Rice Variety-Bg 250. *Trop. Agr. Research*, 22, 45-53 (2010).
(14) JEFFERSON R, KAVANAGH T, BEVAN M, GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO journal*, 6, 3901 (1987).
(15) LUTFUN N, NASAR A, ZINNAH K, NAYEM M, ASHRAFUZZAMAN M, *In Vitro* Growth Media Effect for Regeneration of Tomato (*Lycopersicon esculentum*) and Evaluation of the Salt Tolerance Activity of Callus. J. *of Agri. and Sustainability*, 3, 132-143 (2013).
(16) ALI A, YOSSEF T, BANNA A, Cytokinin-Cytokin in interaction ameliorates the callus induction and plant regeneration of tomato (*Solanum lycopersicum* MILL.). *Act. Agronomic H*., 60, 47-55 (2012).
(17) BARRERO I, Expresión GUS en explantes de *Solanum phureja* (Juz. et. Buk) Var. Criolla Colombia, transformados con *Agrobacterium tumefaciens. Acta biol.Colomb*., 13, 1, 119-130 (2008).
(18) DEVI R, DHALIWAL M, GOSAL S, A simple and efficient *Agrobacterium*-mediated transformation of tomato. *Vegetable Science*, 39, 113-117 (2013).
(19) SANCHEZ L, Estandarización del protocolo de transformación genética de células embriogénicas de banano de la variedad" WILLIAMS"(AAA) mediada por *Agrobacterium tumefaciens* (*Doctoral dissertation*) (2010).