Organogenesis *in-vitro* using three tissues types of tree tomato [*Solanum betaceum* (Cav.)]

Organogénesis *in-vitro* usando tres tipos de tejidos de tomate de árbol [*Solanum betaceum* (Cav.)]

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ABSTRACT

Tree tomato is a fruit with great economic potential due to its high nutritional value. The induction of direct organogenesis in this species is a great alternative for clonal propagation of disease-free plants, and also useful for genetic transformation. In this study was assessed the induction of organogenesis *in vitro* using three different tissues: leaves, petioles and sexual seeds of the variety Common. All tissues were cultured on MS supplemented with agar, sucrose and TDZ or BAP phytohormones at 0.5 to 3 mg L^{-1}, combined or not with the auxins IAA and NAA. Although petioles and seeds had a great potential for regeneration of plantlets, leaves produced more shoots (average of 18.4 shoots/explant) on medium containing TDZ 0.5 mg L^{-1}. Additionally, its easy handling and resistance to physical damage may be useful traits to perform genetic transformation. The highest average of shoots produced from seeds and petioles were 4.3 and 3.1 shoots/explant, respectively. The production of multiple shoots in petioles was influenced by low concentrations of auxins. The emergence of the radicle in the seeds was important for the formation of shoots.

Key words: clonal propagation, shoot induction, tamarillo, tissue culture, phytohormones.

RESUMEN

El tomate de árbol es un frutal con gran potencial económico debido a su alto valor nutricional. La inducción de organogénesis directa en esta especie presenta una gran alternativa para la propagación clonal de plantas libres de enfermedades, y también es útil para transformación genética. En este estudio fue evaluada inducción de organogénesis *in vitro* utilizando tres diferentes tejidos: hojas, pecíolos y semillas sexuales, de la variedad Común. Todos los tejidos se cultivaron en MS suplementado con agar, sacarosa y las fitohormonas TDZ o BAP de 0.5 a 3 mg L^{-1}, combinadas o no con las auxinas AIA y ANA. Aunque los pecíolos y las semillas tuvieron un gran potencial para la regeneración de plántulas, las hojas produjeron más número de brotes (promedio de 18.4 brotes/explante) en medio que contenía TDZ 0.5 mg L^{-1}. Además, su fácil manejo y resistencia al daño físico pueden ser rasgos útiles para realizar transformación genética. El promedio más alto de brotes producidos a partir de semillas y pecíolos fue de 4.3 y 3.1 brotes/explante, respectivamente. La producción de brotes múltiples en pecíolos fue influenciada por bajas concentraciones de auxinas. La emergencia de la radícula en las semillas fue importante en la formación de brotes.

Palabras clave: propagación clonal, inducción de brotes, tamarillo, cultivo de tejidos, fitohormonas.

Introduction

Tree tomato [*Cyphomandra betacea* (Cav.) Sendt.], also known as tamarillo or *tomate de árbol* in South America, belongs to the Solanaceae family and it is native of the South American Andean zone (Bonnet and Cárdenas, 2012). Tree tomato has hanging egg-shaped fruits with exocarp from yellow to orange, but it can also be colored red to purple. Its pulp color varies from yellow to orange. This exotic species has been introduced in many subtropical areas, but production and exportation have recently increased mainly in Colombia, Ecuador and New Zealand (Schotsmans *et al.*, 2011). In Colombia, the variety named 'Común' (Common), which has yellow-orange pulp and reddish-orange exocarp is the most widely cultivated. According to Agronet for Colombia, in 2015 194.355 t of tree tomato fruits were produced, with a cultivated area of 13.804 ha and a harvested area of 10.109 ha, being Antioquia the department with the greater production. Its price ranges from $1.880 to $3.400 COP/kg (Agronet, 2016).

Its use lies mainly on the fresh fruit as it is used in juices and salads or prepared food as jams and desserts. It is characterized by a high content of antioxidants and fibers,

vitamins B6, C and E, pro-vitamin A, low carbohydrate and high of amino acids contents (Vasco *et al.*, 2009). Due to its high antioxidant content, tree tomato may have desirable properties to struggle against vascular diseases and cancer, among other illnesses (Cerón *et al.*, 2011; Kadir *et al.*, 2015).

Tree tomato growers usually use seeds and cuttings for its propagation, which possess a problem for cultivar identity. Pollination is mainly autogamic, which means a low rate of hybridization (Pringle and Murray, 1991) and explains the reduced genetic diversity found within populations. Additionally, the main constrains for tree tomato production are the availability of high-yielding varieties, with good fruit quality, resistant to pests and diseases. The costs for disease control in tree tomatoes correspond to 28 to 45% of total production costs. Despite the effort made by the producers, losses can reach up to 50%, and postharvest can reach 80-100%; which may lead to the abandonment or substitution of the crop (Tamayo, 2001; Alvarez, 2016).

Biotechnological methods have facilitated the propagation of fruit species of commercial interest, allowing selection of elite material and reducing phytosanitary problems, with minimal space requirement, independent of agricultural inputs. By direct organogenesis it is possible to regenerate whole seedlings from plant tissues, without passing through a stage of dedifferentiation, i.e., callus and/or somatic embryo formation (Hussain *et al.*, 2012), thus maintaining genomic stability in regenerated plants. However, it's worth remembering that simple *in vitro* micropropagation may induce epigenetic changes, i.e., altered methylation patterns in the genome of clonally propagated species, i.e., in *Manihot esculenta* Crantz (Kitimu *et al.*, 2015). *In vitro* cultivation of plant tissues has also been useful for genetic transformation, being the starting point for establishing working material, and to obtain and propagate seedlings that have been transformed. Genetic improvement serves as a supporting tool for breeding clonal crops like tree tomato.

A variety of *in vitro* culture technologies have been applied in *C. betacea* using different explant types (Correia and Canhoto, 2012). However, direct organogenesis has been little reported and greater emphasis has been given to somatic embryogenesis. Obando *et al.* (1992) regenerated axillary shoots starting from nodal segments and they reported some of the first responses of organogenesis from leaf segments. They used the basal MS culture medium (Murashige and Skoog, 1962) with naphthaleneacetic acid (NAA), 6-bencil amino purine (BAP) and gibberellic acid (GA$_3$), all at 0.2 mg L^{-1}, and zeatin at 2.5 mg L^{-1}. In the case of leaves, thidiazuron (TDZ) at concentrations of 0-10 mg

L^{-1} were added to the culture medium. A great number of reports highlight indirect regeneration of tree tomato *in vitro* through the induction of callus or somatic embryogenesis, with subsequent formation of seedlings (Obando and Jordan, 2001; Correia et al., 2011; Correia and Canhoto, 2012; Chacón-Cerdas *et al.*, 2013). In these indirect methods it have being used among other tissues, leaves and petioles as explants, however, to our knowledge, the use of sexual seeds for direct induction of multiple shoots has not been reported so far.

The purpose of this study was to seek a tissue from the plant in which a direct formation of shoots via organogenesis could be induced. So, three tissues of tree tomato var. Common were selected: young leaves, seed tissue, and petioles to determine which had the greatest regenerative potential for clonal propagation of fully regenerated seedlings, and later on be used for genetic transformation, which requires an efficient system for plant regeneration. We thus discuss the effect of combining explants and phytohormones on the shoot regeneration ability of tissues in vitro.

Methods

The plant material was supplied by the laboratory of plant Biotechnology of the Universidad Nacional de Colombia in Medellin, Colombia. Tree tomato (var. Common) *in vitro* plants were used as source of leaf and petiole explants. For seeds, the source of explants were fruits in a good phytosanitary conditions, purchased in local supermarkets in Medellin, Colombia.

Explants from *in vitro* plants were not subjected to any disinfection process since they came from aseptic plants. In the case of seeds, fruits were disinfected externally with sodium hypochlorite (3% NaOCl), seeds extracted, mucilaginous aril removed and immersed in 0.5% NaOCl for 5 min; then seeds were dried on sterile absorbent paper. Finally, four washes with distilled water were performed. The entire process was conducted in a laminar flow bench.

The culture media was selected based on previous experience of this research group on tree tomato *in vitro* cultivation (unpublished). Explants were placed on a culture medium composed of MS salts and vitamins, supplemented with agar (Sigma®, A1296) 6.5 g L^{-1}, sucrose 20 g L^{-1}, with the addition of growth regulators as it is shown in table 1.

For leaves, they were used segments of 0.5 cm^2 of young leaves (position 1-3 counting from the apex) and placed with adaxial side in contact with the culture medium. Four replicas by treatment, each with four explants, for a total 16

explants per treatment were made. For petioles, transverse segments of approximately 2 mm were used, each treatment had 20 explants. Disinfected seeds were placed on culture medium either with or without mechanical scarification. Eight replicas each with five seeds were explanted, for a total of 40 seeds per treatment. The scarification process consisted of removing a small portion of the seed coat, to stimulate germination.

Shoots developed in the different treatments were transferred to growth and development medium (GDM) composed of MS medium supplemented with agar 6.5 g L^{-1}, sucrose 20 g L^{-1} and growth regulators GA$_3$ (0.025 mg L^{-1}), NAA (0.025 mg L^{-1}), IAA (0.02 mg L^{-1}) and indole-3-butyric acid (IBA, 0.05 mg L^{-1}). Finally, after one month the total number of shoots from leaves and petioles was recorded and after transferred to GDM. Similarly for seeds, the number of shoots was recorded after two months then transferred to GDM.

TABLE 1. Combinations of Plant growth regulators (mg L^{-1}) tested for *in vitro* shoot induction in three tissues types from tree tomato. Phytohormone combinations were taken as treatments for statistical analysis.

Treatment	Tissue evaluated	TDZ	BAP	IAA	NAA
1	Leaf	0.5	-	-	-
2	Leaf, petiole	0.5	-	0.02	0.025
3	Leaf, petiole	1.0	-	-	-
4	Leaf, petiole	1.0	-	0.02	0.025
5	Leaf, petiole, seed	2.0	-	-	-
6[1]	Seed	2.0	-	0.02	0.025
7	Leaf, petiole	2.0	-	0.02	0.025
8	Leaf	3.0	-	-	-
9	Leaf, petiole	-	1.0	-	-
10	Leaf, petiole	-	1.0	0.02	0.025
11	Leaf	-	2.0	-	-
12	Leaf, petiole	-	2.0	0.02	0.025
13	Leaf, seed	-	3.0	-	-
14[1]	Seed	-	3.0	-	-
15 control	Leaf, petiole, seed	-	-	-	-

[1] Seeds were scarified before explanting them on media.

The pH of media was adjusted to 5.8 with NaOH and HCl 1N solutions and it was autoclaved at 121°C and 15 psi. The cultures were kept at 23±2°C, with a photoperiod of 12 h in a growth room. Treatments in which leaves and petioles were used as explants, were established in Petri dishes with 15 mL of culture medium. Treatments whose explants were seeds, were established in 100 mL glass flasks, with 10 mL of culture medium each. For the transferring of plantlets developed from shoots 225 mL glass flasks with 15 mL of GDM were used.

Data on shoot establishment were analyzed using the statistical program R version 3.2.2. Analysis of variance ANOVA and Tukey means multiple comparisons analysis were conducted ($P \leq 0.05$). In the case of the petiole tissue, the non-parametric tests Kruskal-Wallis and Dunn's multiple comparisons of sum of rank analysis were performed.

Results and discussion

The analysis of variance (ANOVA) showed that the number of means differ between groups in at least one of the treatments for leaf explants (P=1.484 x 10^{-7}), with a 95% confidence level. The beginning for the shoot formation was 20 d after sowing. The highest number of average shoots was observed in the treatment 1 with 0.5 mg L^{-1} (18.4 shoots/explant), followed by treatment 4 with 1 mg l^{-1} in combination with 0.02 mg L^{-1} IAA and 0.025 mg L^{-1} ANA (13.6 shoots/explant). In control explants, shoot formation was not detected. Concentrations above 2 mg L^{-1} TDZ or BAP were not adequate, since organogenesis was inhibited or arose in very low numbers (Tab. 2, Fig. 1A and 1B). Greater concentrations than 1 mg L^{-1} of TDZ also caused signs of hiperhidricity. The percentage of explants that produced shoots per treatment ranged between 100% (treatment 4), 20% (treatment 8) and 0% (control). The treatment 1, in which the largest number of shoots was arisen, obtained response in 94% of the explants.

TABLE 2. Averages of shoot explants in the different evaluated treatments, for each type of tissue.

Treatment	Shoots/leaf explant ± 1.842 (SE)	Shoots/seed ±0.564 (SE)	Shoots/petiole explant ±0.422 (SE)
1	18.4 a	NE	NE
2	9.7 a,j,k,o	NE	0.5 a,b
3	10.8 a,k	NE	0 a
4	13.5 a,m	NE	1.3 a,b
5	10.1 a,k,n	0.5 a	0 a
6	NE	3.2 b,d	NE
7	3.9 b,k,l	NE	1.7 a,b
8	1 c,l,n,o	NE	NE
9	0.7 d,j,k,o,p	NE	0 a
10	4.5 e,j,k,m,o,q	NE	0.9 a,b
11	7.7 f,j,k,m,o,r	NE	0 a
12	2.5 g,j,k,l,n,o	NE	3.1 b
13	3.7 h,j,k,o,s	1.5 a,b	NE
14	NE	4.3 c,d	NE
15 (control)	0 i,l,p,q,r,s	0 a	0 a

NE indicates that the treatment was not evaluated for a specific tissue.

Means with different letters indicate a significant difference according to Tukey's test ($P \leq 0.05$).

The procedure of Tukey's multiple comparison of means, performed for leaves explants, showed significant differences between treatment 1 and the most of the evaluated treatments, including control, except with treatments 2, 3, 4 and 5; and they showed significant differences with respect to control. Finally, there were also differences between treatment 3 and 8.

Similarly, the Anova for seed explants showed that the number of means differ between groups in at least one of the treatments ($P=1.572 \times 10^{-5}$). The beginning of shoot formation was evident with the seed germination, which was rather slow and low in the treatments with phytohormones in comparison with the control, which had no phytohormones, that presented the greater percentage of germination with a 67.5%, and it could be evidenced around 15 d after sowing. In the moist chamber under aseptic conditions was found that the germination was facilitated, with a percentage of 95%. The process of seed scarification improved seed germination, and at the same time the shoot formation. The greater average was observed in the treatment 14 with 3 mg L^{-1} BAP, in which the seeds were scarified (4.3 shoots/explant), with a 57.5% of seeds geminated, of which the 78% produced shoots. The second best average was for the treatment 6 (3.2 shoots/explant), with a 47.5% of germinated seeds of which 63% produced shoots. Treatments 13 and 5 presented a percentage of germination of 50 and 40% and shoot formation of 45 and 25%, respectively (Tab. 1 and 2, Fig. 1C and 1D).

The procedure of Tukey's multiple comparisons of means for seed, reveled significant differences between the treatment 14 and the others evaluated treatments and the control, except with the treatment 6. It was found significant differences among treatment 6 and 5, and control too.

For petiole explants, using Kruskal-Wallis analysis, was found that the number of medians differ between groups in at least one of the treatments ($P=1.6882 \times 10^{-5}$). The response begins around 22 d, although in the most treatments
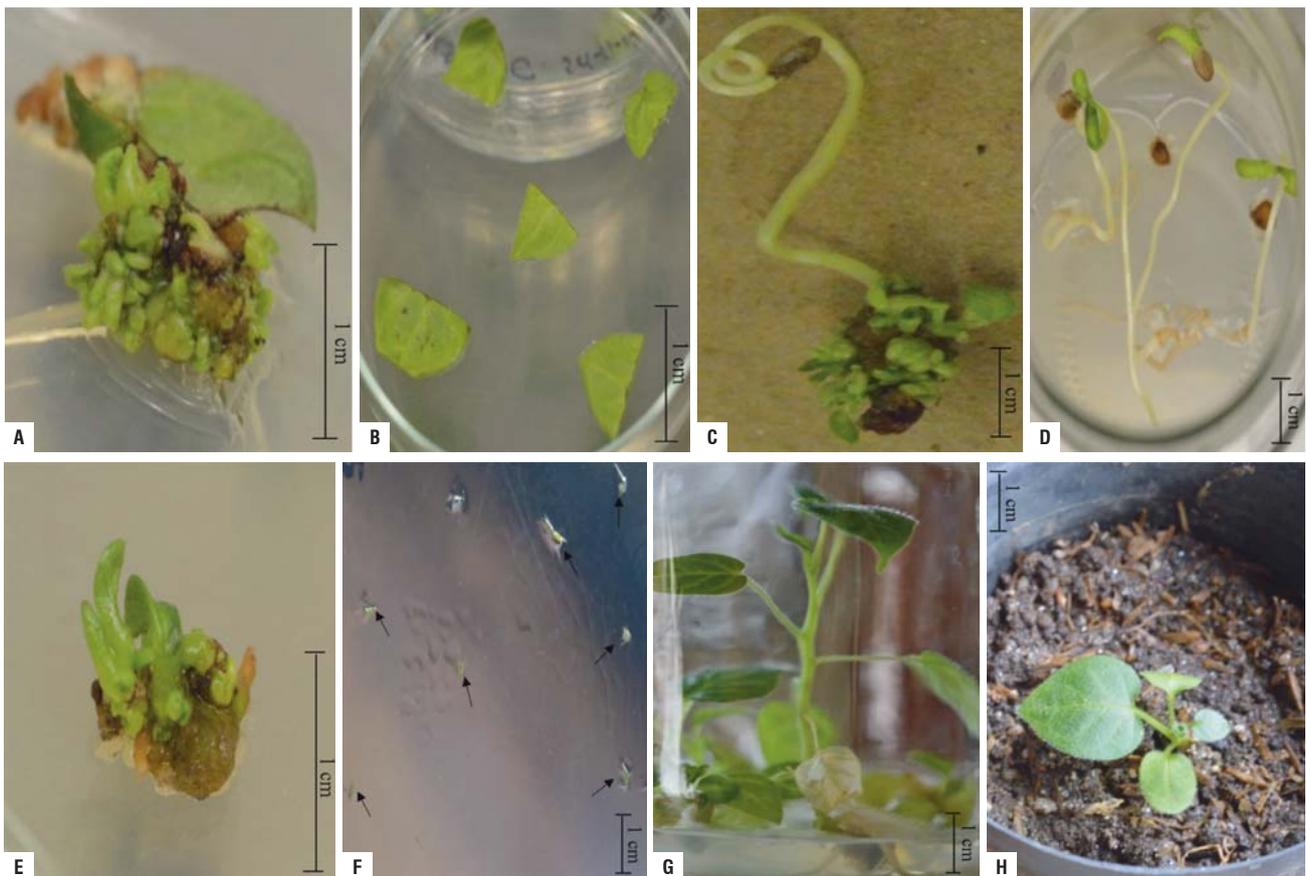

FIGURE 1. Direct in vitro organogenesis of explants from common tomato tree. Treatments in which the highest average shoot formation were shown. A. Leaf treatment; B. Control; C. Seed treatment 14; D. control; E. Petiole treatment 12; F. Control; G. *In vitro* plant on medium with TDZ 0.5, developed from shoots of tree tomato; H. Seedling adapted to soil conditions in greenhouse.

the response was null or very low. Some response only was found in treatments containing NAA 0.025 mg L^{-1} and IAA 0.02 mg L^{-1}. The treatment with the greater average of shoots was for the one contained 2 mg L^{-1} BAP and NAA and IAA to the concentrations previously mentioned, with 3.1 shoots/explant. That treatment had a 40% of explants with organogenic response. Followed by the treatment 7 with 1.7 shoots/explants and 25% of the explants showed shoots. In the rest of treatments, the percentage of explants that formed shoots ranged between 30 and 0%, including the control with a 0% (Tab. 1 and 2, Fig. 1E and 1F). The analysis of Dunn's multiple comparisons based on rank sum, revealed few significant differences. They were between treatments 12 and control, and treatments 9, 11, 3 and 5.

For the shoots that formed plantlets in all tissues tested and treatments, the occurrence of roots was facilitated once they were transferred to MS medium with low concentrations of GA$_3$ (0.025 mg L^{-1}), NAA (0.025 mg L^{-1}), IAA (0.02 mg L^{-1}) and IBA (0.05 mg L^{-1}) phytohormones and in 225 mL flasks, rooting took place around 2 weeks.

The direct induction of shoots by organogenesis counts with few reports in *C. betacea*. However, the use of TDZ in tamarillo has been studied by Obando *et al.* (1992), using high concentrations of TDZ (5 mg L^{-1} and 10 mg L^{-1}) in leaf explants, they obtained the highest percentage of explants with shoots with a 48%. The addition of IAA in amounts greater than 1 mg L^{-1} reduced the formation of shoots and the appearance of callus. NAA concentrations lower than 0.02 mg L^{-1} in nodal segments allowed the best answer for shoot induction. In the mentioned work, plantlets did not achieve to produce roots. While Atkinson and Gardner (1993) found better results using 1 mg L^{-1} BAP and 0.01 mg L^{-1} NAA, with shoot response formation up to 63% of the explants. None of those work revealed the number of produced shoots/explant, data that would be more representative at the time of searching organogenesis induction at a good rate. Obando and Jordan (2001) also obtained the best results using high concentrations of TDZ (5 and 10 mg L^{-1}), starting from mature tissues. In leaf, callus was first formed and then shoots, values of 2.1 to 16.3 shoots/explants were obtained. The response was obtained in a month or a little more time. The investigators also evaluated the tissue of hypocotyl and petiole, obtaining callus and somatic embryogenesis in presence of callus, respectively, due to the used phytohormone (5 mg L^{-1} of 2,4-D).

In contrast to the above, in this study, the effect of the hormonal concentration varied depending on the tissue. Leaf explants showed the highest rate of shoot formation by organogenesis, in MS media with low concentrations of TDZ (0.5 mg L^{-1}) with a comparable average regarding the other reports, 18.4 shoots/explant. Greater concentrations than 2 mg L^{-1} reduced shoot formation and in some cases, hiperhidricity in the tissues was evidenced. While in seed explants, high values of cytokinins (3 mg L^{-1} BAP and 2 mg L^{-1} TDZ) induced organogenesis. In the case of petiole explants, the shoots induction was influenced by the presence of auxins IAA and NAA at low concentrations (0.02 and 0.025 mg L^{-1}, respectively) for this work, what has been reported in tamarillo nodal segments and leaf explants (Obando *et al.*, 1992; Atkinson and Gardner, 1993). These auxins acted in conjunction with BAP or TDZ to stimulate the organogenic response in petiole explants, despite being very low (not significant statistical differences between the responses for treatments). The conjunction effect of auxins-cytokinins could also be seen in leaves explants, as for example in the treatment 4 (Tab. 2).

Organogenic response of common tree tomato seed explants depends on its germination, for instance the emergence of the radicle tissue or hypocotyl tissue, which are the first tissues arising during germination, appear to be involved. Therefore, it was expected that the process of scarification improved the response germination by increasing the uptake and absorption of water and nutrients. This is consistent with that described by Bello-Bello *et al.* (2012) for *Pinus brutia* seeds in which mechanical scarification with an increase in the germination was performed. Hitherto, there are no reports of the use of the complete seeds to induce shoots in tamarillo, and very few for other species have been reported, as it is the case by Malik *et al.* (1993), who used intact seeds from different *Lathyrus* species, to evaluate shoots regeneration, further showing the effect of phytohormonal concentration in reducing germination and subsequent response of shoot formation.

In a more recent study, Kahia (2015) also reported the best results in leaf tissues and hypocotyl of tree tomato using high concentrations of BAP (9 mg l^{-1}) and low of TDZ (0.22 mg L^{-1}) to induce organogenesis, obtaining values of 4.67 and 3.56 shoots/explant, respectively. These averages were lower than those obtained in our work (18.4, 4.3 and 3.1 shoots/explants). Kahia (2015) obtained root formation in the organogenesis-evaluated media, with averages ranging between 0 and 3.78 roots. In this study a combination of very low concentrations of GA$_3$ (0.025 mg L^{-1}), NAA (0.025 mg L^{-1}), IAA (0.02 mg L^{-1}) and IBA (0.05 mg L^{-1}) hormones was suitable for root production and complete plantlet development (Fig. 1G and 1H). The use of NAA,

GA$_3$ (Obando and Jordan, 2001), IAA and IBA (Chacón-Cerdas *et al.*, 2013) as phytohormones has been reported for plantlet rooting obtained from shoots in tree tomato.

Since our interest was to obtain a good number of shoots by organogenesis, evaluation of the process of acclimatization was not raised, however, the adaptation of common tree tomato and other varieties to soil conditions, is relatively easy in garden soil. In our research group, a sterile substrate for this species has been established, it consists in soil:sand:poultry manure:rice husk, in a ratio of 1:1:1:1, with good results. Although it was not evaluated, some plantlets were transferred to soil conditions to verify this capacity (Fig. 1H).

Conclusions

Tree tomato organogenic responses varied between the tissues type tested; however, leaf tissue had the more regenerative capacity with 18.4 shoots/explant, using TDZ phytohormone at a concentration of 0.5 mg L^{-1}. Followed by seeds explants (4.3 shoots/explants) and finally petiole explants (3.1 shoots/explant). This regenerative capacity is enhanced insofar as they are treated with TDZ or BAP growth regulators, alone or in combination with NAA and IAA.

The tissue with the greatest potential for transformation assays is leaf, because it allows getting good amounts of shoots, in about a month, it is easy to manipulate, resists some degree of damage and it could replicate rapidly. Although seeds have a good rate of shoot induction, germination in order to obtain such response must be performed and evaluated, but high values of BAP or TDZ (2-3 mg L^{-1}) may affect the percentage of germination. In the case of petiole, the response was low; however, this tissue allows a high availability of raw material, since a single one can be fragmented in many pieces.

In addition, the results obtained in this study can be useful not only for genetic transformation processes, but also to performed works of tree tomato mass propagation, because of the advantages that offers tissue culture for obtaining high numbers plants in a short period of time and in a sustainable manner.

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