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Original Paper

Somatic embryogenesis from leaf explants of *Tagetes erecta* L.

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Abstract *Tagetes erecta* is an asteraceous plant of industrial, ornamental and medicinal importance; its inflorescences have been used as a pigment source for food coloring, mainly for poultry skin and eggs. Nevertheless, there are few reports on plant regeneration or micropropagation, because unsuccesfull results in the plant's reaction to the growth regulators, developing embryogenesis on *Tagetes erecta*. In this study, somatic embryogenesis was induced and plantlets of *Tagetes erecta* were regenerated. For induction of globular structures MS medium supplemented with 2,4-D (4.5 μM) and BAP (8.8 μM) was used; globular structures were transferred to MS medium with 45 g l^{-1} sucrose until the embryos maturation. Transmission electron microscopy showed characteristic subcellular structures of embryogenic callus. Somatic embryos were transferred to MS medium without plant growth regulators and whole plantlets were obtained. *In vitro* plants were successfully transplanted into a mixture of peat moss and vermiculite (1 : 1 v/v) under greenhouse conditions. In this study, somatic embryogenesis and plant regeneration system from foliar explants were established, an important requirement for performing genetic transformation events on *Tagetes erecta*.

Key words: asteraceae, embryo stages, *in vitro* culture, plant regeneration.

Introduction

Marigold (*Tagetes erecta* L.) is an herbaceous plant native to Mexico and belongs to the Asteraceae family which produces fragrant inflorescences whose individual flowers are called ligules. The marigold pigmentation depends on the levels of carotenoids in the ligules (Del Villar-Martínez et al. 2009). Investigations have focused on performing studies to obtain plants with higher pigment content, therefore the establishment of *in vitro* plant cultures and genetic modification are essential tools (Del Villar-Martínez et al. 2007). *T. erecta* has been used for centuries as an ornamental and medicinal plant, and researchers currently seek to increase the pigment content. There are reports about quantification of *psy*, *pds*, *lcy-β* and *lcy-ε* gene expression about *T. erecta* (Del Villar Martínez et al. 2005; Moehs et al. 2001); studies on *in vitro* tissue and cell cultures are mostly interested in analyzing the influence of medium factors on callus induction or/and regeneration. This kind of research is necessary in order to work on genetic manipulation, defining the appropiated stage and cell type for modified plants regeneration (Bespalhok and Hattori 1999; Von Arnold 2008). The aim of this study was to establish a protocol for plant tissue culture and regeneration via somatic embryogenesis of *T. erecta*, this efficient rooting and acclimatization system will be useful for genetic transformation and breeding to enhance pigment and metabolites production.

Materials and methods

Plant material and disinfection

Leaves of *T. erecta* L. var. Blanca grown under greenhouse conditions were used. The leaves were disinfected by immersion in ethanol at 100 and 70% (v/v) for 1 and 5 min respectively, then rinsed 3 times with sterile distilled water, treated with sodium hypochlorite at 2 and 1% (v/v) for 1 min each and 3 rinses with sterile distilled water between each solution (Vanegas et al. 2002).

Induction of somatic embryogenesis

Leaf explants of approximately 25 mm^2 (10 explants per Petri dish) on MS medium (Murashige and Skoog 1962) added with

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2,4-dichlorophenoxyacetic acid (2,4-D: 0, 4.5, 9.0, 22.5 μM) and benzylaminopurine (BAP: 0, 4.4, 8.8, 22.0 μM) were tested. The culture media was adjusted to pH 5.8 and autoclaved at 1.5 mm/Hg for 15 min. Cultures were incubated in the dark for 4 weeks at 25±2°C.

A Petri dish with 10 explants was used as the experimental unit, and 4 repetitions were performed for each treatment and subcultivated every 15 days. Data was analyzed statistically through one-way ANOVA. The significance of difference between means was carried out by the Tukey's multiple range test at $\alpha=0.05$ using Suite Minitab 17®. Once the growth regulators concentrations to inducing proembryos were selected and transmission electron microscopy (TEM) analysis was performed.

Maturation of somatic embryos and acclimatization

Somatic pro-embryos were placed on medium which consisted of MS medium adding different sucrose at different concentrations (30, 45, 60 and 75 g l^{-1}) without plant growth regulators, according to Freire-Seijo (2003). All cultures were maintained at 25±2°C, with a 16 h light photoperiod. Light was supplied at 30 μmol m^{-2} s^{-1} by cool-white fluorescent lamps. Subcultures were performed every 15 days and evaluated during 18 weeks. Plantlets from somatic embryos were transferred to MS medium (30 g l^{-1} sucrose) without plant growth regulators for complete development. Plantlets were transferred to *ex vitro* conditions in sterile containers with substrate composed of Peat Moss® and vermiculite (1:1, w/w), and covered with trasparent plastic material for a week to keep high humidity. During this period pots were irrigated with 50% MS medium. The Plant Forming Capacity (PFC) index was calculated according to:

$$\text{PFC index} = (\text{mean number of plant per explant}) \times (\%\ \text{explants forming embryos})/100$$

Adapted from Martínez-Pulido et al. (1992).

Table 1. *Tagetes erecta* leaf explants inducing proembryos.

2,4-D (μM)	BAP (μM)	Explants with somatic embryos (%)
0.0	0.0	0.0 d
	4.4	0.0 d
	8.8	0.0 d
	22.0	0.0 d
4.5	0.0	0.0 d
	4.4	25.0 cd
	8.8	100.0 a
	22.0	50.0 d
9.0	0.0	0.0 d
	4.4	87.0 ab
	8.8	85.0 ab
	22.0	100.0 a
22.5	0.0	0.0 d
	4.4	100.0 a
	8.8	50.0 bc
	22.0	52.5 bc

Different letters correspond to significant differences according to a $p=0.05$ and corresponds to four repetitions.

Transmission electron microscopy

Small pieces of samples were cut and fixed for 12 h in 4% formaldehyde. Post-fixation was carried out in 1% OsO_4 in cacodylate buffer for 12 h at 4°C. Tissue samples were dehydrated through graded ethanol series starting from 10% to absolute; infiltration was carried out using complete resin (EPON 812®), diluted with propylene oxide (2:1, 1:1, 1:2 w/v) for 1 h in a closed jar. A second infiltration step was done with complete undiluted resin in a rotatory shaker for 1 h at room temperature (Vanegas-Espinoza et al. 2011). After the second infiltration step, embedded samples were transferred to flat embedding molds filled with fresh undiluted resin and were polymerized in a convection oven at 60°C for 24 h. Thin sections (65 nm) were cut with a diamond knife in an ultramicrotome (Leica, Mod. Ultracut UCT, Germany) these sections were attached to a 300-mesh copper grid, contrasted with uranyl acetate (4%, p/v) and lead citrate (1%, p/v), and observed in transmission electron microscope (Jeol, Mod. JEM 1010, Akishima, Japan) hooked up to a camera (Advanced Microscopy Techniques (AMT), RX80 Woburn Massachusetts, USA).

Results and discussion

Induction and somatic embryo formation

An increase in size was initially observed in explants cultured on media with different concentrations of 2,4-D and BAP, then the formation of characteristic spherical structures of the embryogenic callus induction process was detected. Embryogenic callus was induced in all explants on 2,4-D (4.5, 9.0, 22.5 μM)/BAP (4.4. 8.8, 22.0 μM) treatments. Callus from 2,4-D (4.5 μM)/BAP (8.8 μM) treatment was selected by its friability and appearance characteristics (Table 1).

Somatic embryogenesis in *T. erecta* studies have been done with different plant growth regulators; however, only Bespalhok and Hattori (1999) reported somatic embryos induction with thidiazuron (0.02 mg l^{-1}), but germination was not achieved. Other researchers on *in vitro* culture of this species included Kothari and Chandra (1986) using NAA (2.0 mg l^{-1}), BAP (1.0 mg l^{-1}) and GA_3 (0.5 mg l^{-1}) with leaf segments as explants obtained adventitious shoots and, Belarmino et al. (1992) using NAA (0.2 mg l^{-1}) and BAP (5 mg l^{-1}) with hypocotyl portions and leaf segments as explants; plantlet regeneration from hypocotyl derived calli occurred on MS containing NAA and BAP, whereas leaf-derived calli produced mainly roots and green spots in some combinations of plant growth regulators combinations. Misra and Datta (2001) reported direct organogenesis from leaf segments on MS medium supplemented with BAP (4.44 μM) and GA (14.43 μM), and Vanegas et al. (2002) established an efficient protocol for regeneration via indirect organogenesis using IAA (17.1 μM) and BAP (13.3 μM), where the successful acclimatization and

normal plant development was reported. The induction of somatic embryos has been achieved mainly using auxins like 2,4-D, NAA, IAA, in several species such as *Swietenia macrophylla* with 2,4-D (4.0 mg l^{-1}) and kinetin (1.0 mg l^{-1}) (Collado et al. 2006), *Cymbopogon jwarancusa* with 2,4-D (18.1 μM) (Mehandru et al. 2014), *Musa acuminata* with 2,4-D, (4.5 μM), and picloram (8.2 μM) (Escobedo-GraciaMedrano et al. 2014), *Salicornia brachiata* with 2,4-D (2.0 mg l^{-1}) (Rathore et al. 2015) and *Solanum melongena* with 2,4-D (0.5 mg l^{-1}) (Ashraful-Habib et al. 2016). Transition from dedifferentiated status to embryogenic and/or organogenic status is a complex process that consists of several phases, including cell reactivation, cell division and various metabolic and developmental reprogramming steps (Pooja Mehandru et al. 2014). It has been reported that 2,4-D is the most widely used as a growth regulator for morphogenic calli induction regardless of the explant according to our results (Dey et al. 2010; Rathore et al. 2015). In this study 2,4-D in combination with BAP induced somatic embryogenesis in *T. erecta*.

Maturation and germination of somatic embryos

Somatic embryo germination on MS medium (30 g l^{-1} of sucrose) was low (5%), PFC was 0.10. The highest percentage of germination (67.5%), as well as the highest PFC (7.29) were obtained on MS medium with 45 g l^{-1} sucrose. Mature embryos were developed also on MS added with 60 g l^{-1} sucrose; and 50% of explants with mature embryos were observed (PFC=3.20). Abnormal structures were observed at 75 g l^{-1} sucrose; these structures presented broadening and merging of cotyledons unlike embryos observed in the other

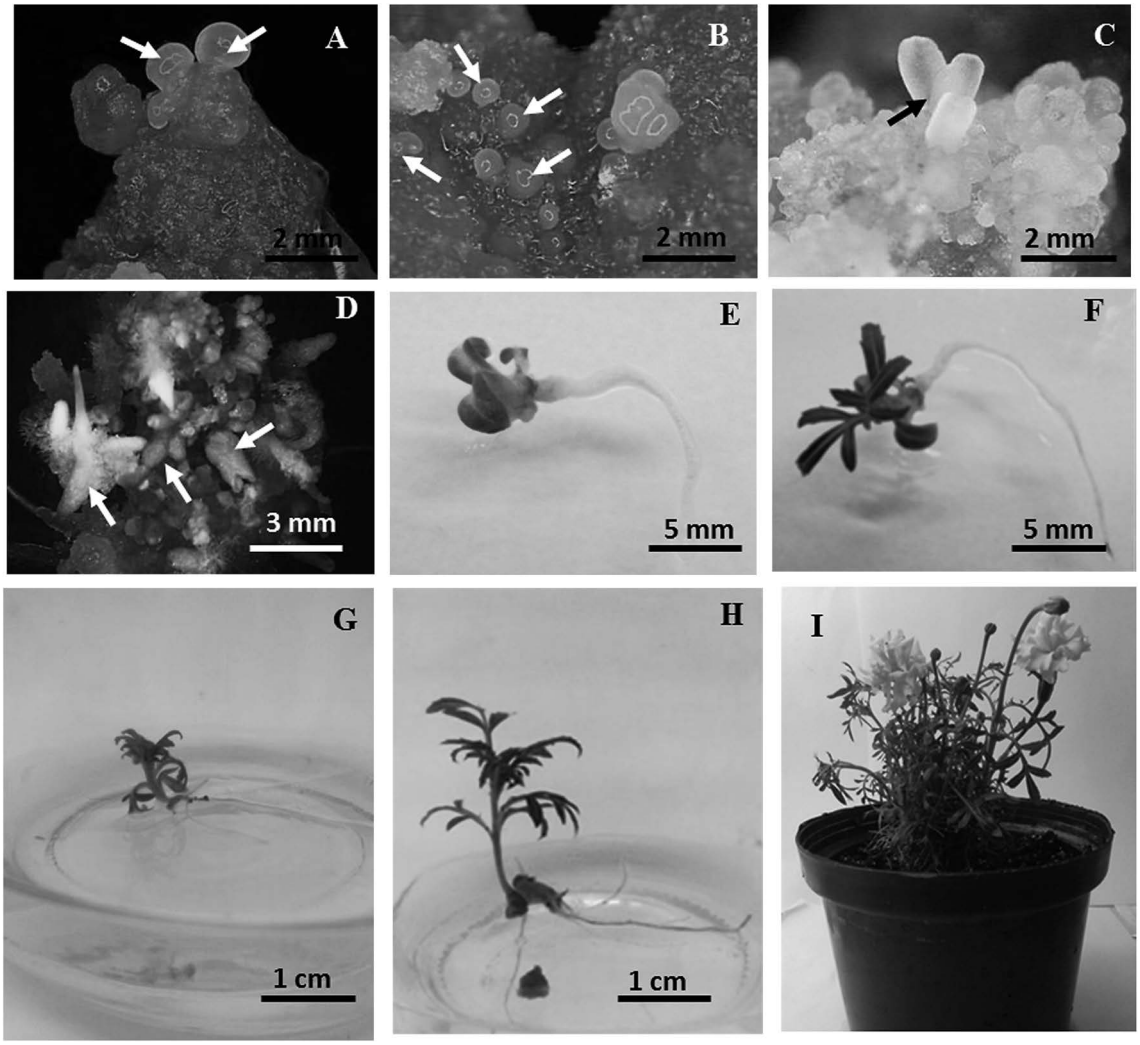

Figure 1. Plant regeneration via somatic embryogenesis of *Tagetes erecta* var Blanca. A, B) Somatic embryos (4 weeks) at globular stage on MS medium with 2,4-D (4.5 μM) and BAP (8.8 μM), C) Somatic embryos (6 weeks) at heart-shaped stage, D) Embryogenic callus on MS medium with sucrose (45 g l^{-1}) (16 weeks) showing embryos at different developmental stages, E) Germinated somatic embryo (19 weeks), F) Plantlets with first leaves (21 weeks), G) Plantlet formed from a somatic embryo (23 weeks), H) Developing plantlet with secondary roots (25 weeks) and I) Regenerated and acclimatized plant under greenhouse conditions. Arrows indicate the embryonic forms.

treatments. Gu et al. (2014) found that NLN medium containing 13% (130 g l^{-1}) sucrose and 0.1% activated charcoal was optimal for embryo formation on *Brassica nigra*. The early cotyledonary stage cultured on MS medium fortified with zeatin and indole-3-acetic acid resulted in the most efficient rates of plantlet regeneration, which was in agreement with previous studies on the culture of several *Brassica* species (Gu et al. 2004; Wang et al. 2009).

Proembryos reached maturity when plant growth regulators were eliminated from the culture medium and sucrose concentration was increased. Embryo maturation processes are under control of a number of signaling pathways, which integrate genetic, metabolic and hormonal signals (Ochatt et al. 2010). Influence of carbon source on production and growth of somatic embryos has been reported, resulting in an increase of sucrose level that improves their growth and development (Freire-Seijo 2003).

Figure 1 shows somatic embryogenesis stages. During seven days, explants growing on MS medium with 2,4-D and BAP showed increased size, about the fourth week globular structures (Figure 1A and B) were observed primarily at the edges of the explants, which were subcultured on MS medium using sucrose at concentrations above 30 g l^{-1}; at the sixth week of culture, heart-shaped structures were observed (Figure 1C) characteristic of embryogenic process. After 16 weeks of subculture on MS medium with sucrose (45 g l^{-1}) somatic embryo formation was observed, which matured asynchronously, allowing observation of somatic embryos at different stages (torpedo and cotyledon) with green coloring and elongated structure as shown in Figure 1D. After 18 weeks of subculture, embryos at the cotyledonary stage were separated and transferred to MS medium with 30 g l^{-1} sucrose. After one week of culture on this medium, the separation and expansion of the cotyledons as well as root elongation were observed (Figure 1E); after 2 weeks, development of first leaves was observed on the same medium (Figure 1F); after 2 more weeks, healthy and vigorous root system and foliage were observed (Figure 1G). The somatic embryos underwent the stages of development and plants were obtained within a period of 27 weeks. These results are consistent with those reported by Parveen and Shahzad (2014) where globular embryoids were observed with well-marked boundaries and distinction from the callus tissue; globular embryoids grew in size and became pear shaped, heart shaped embryoids. Interestingly, in this report it was not necessary the use of specific growth regulators

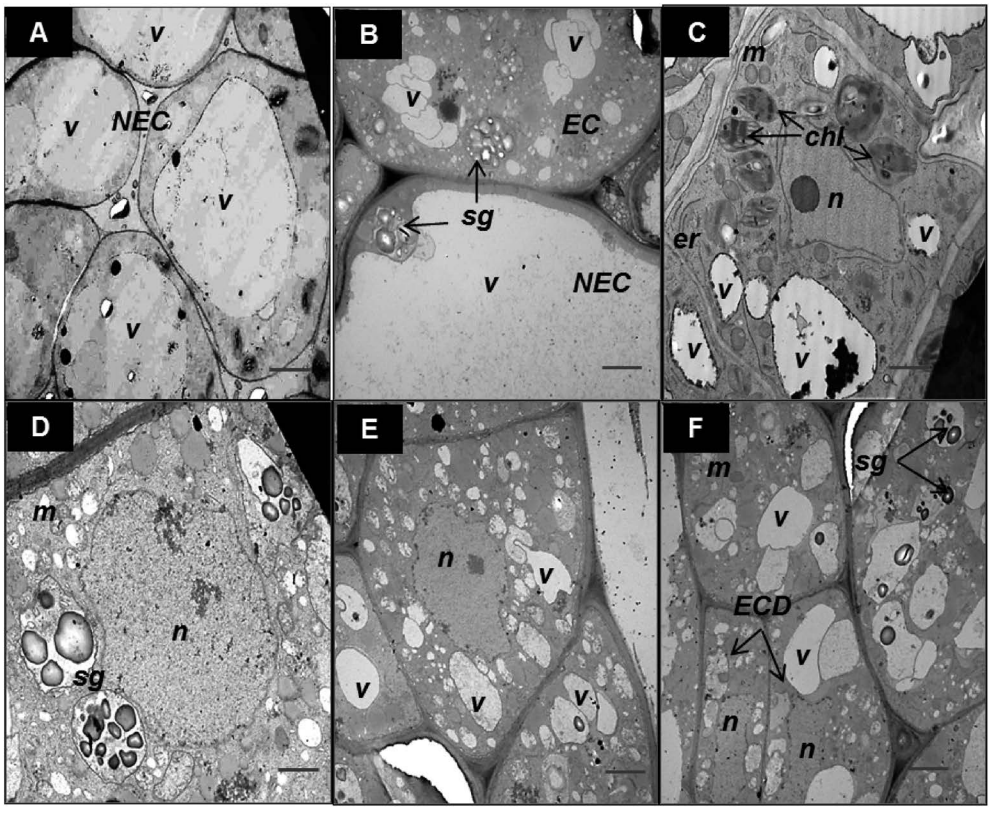

Figure 2. Histological sections of embryogenic and non embryogenic *Tagetes erecta* L. calli by TEM after 38 days old of culture. A) Non-embryogenic cells, B) Embryogenic and non-embryogenic cells, C–E) Panoramic view of whole embryogenic cells, F) Embryogenic cell in division. *EC* embryogenic cell; *NEC* non-embryogenic cell; *v* vacuole; *sg* Starch globules; *m* mitochondria; *n* nucleus; *chl* chloroplast; *er* endoplasmic reticulum; *ECD* embryogenic cell in division. bar = 2 μm.

to promote root development, unlike reported for other species (Gambhir et al. 2017).

One of the most important issues in somatic embryogenesis, is getting the embryos to reach the required level of maturity to germinate and to develop into normal plants. The correct reserve accumulation leads to an increase in mass of the somatic embryos, indicating good embryo development for subsequent germination (Fuji et al. 1990); however, in *Brassica nigra*, media with different sucrose concentrations led to either no response or very low response (Gu et al. 2014). A major problem with the induction of embryos was the presence of abnormal swelled embryos and numerous fused cotyledons; these abnormalities were observed primarily in the concentration of 75 $g\,l^{-1}$ of sucrose and have already been described in annatto (Michelangeli de Clavijo et al. 2003) cultures. Klimaszewska et al. (2007) reported that the osmotic agents (mannitol, sucrose, sorbitol, etc.) facilitate the accumulation of storage proteins and carbohydrates in the formed somatic embryos, promotting its maturity because genes induced by water stress regulate accumulation of these macromolecules (Freire-Seijo 2003; Misra et al. 1993). Our results indicate that high-sucrose culture medium promotes somatic embryos maturation.

Embryogenic cell identification by TEM

Pro-embryogenic 38 days old cells, subcultured every 15 days on the aforementioned induction medium were analyzed. Embryogenic and non-embryogenic cells are shown in Figure 2. Non-embryogenic cells showed large vacuoles (Figure 2A), whereas in embryogenic cells, many small vacuoles, mitochondria, nucleus with visible chromatin, amyloplasts with starch grains were observed (Figure 2C–F). In Figure 2F, cell division is suggested, each cell with small vacuole and nucleus with chromatin is observed. These results match with the findings of Vasilenko et al. (2000) in cells of *Dactylis glomerata* L. in which fractioned vacuoles, starch reserves, nucleus and chromatin, as well as cellular division were observed. Von Arnold (2008) reported that meristematic cells were defined as undifferentiated, small, isodiametric, with prominent nuclei and primary thin cell walls. Embryogenic cells observed agree the general pattern of the somatic cellular formation as reported by Fehér et al. (2003), which provides a model describing the embryogenic cell. On the other hand, non-embryogenic cells, showed morphological features of a callus, which are identified by the presence of vacuoles, proplastids, chloroplasts and thick cell walls (Vanegas-Espinoza et al. 2011).

Conclusions

This paper reports a protocol for *in vitro Tagetes erecta* plants regeneration via somatic embryogenesis, which will serve to expand the use of this system in applications such as genetic engineering. Our results demonstrated the ability of the somatic embryos of *T. erecta* to germinate and develop whole plants, further studies are needed in order to find other factors that may increase embryo obtaining rate, optimizing regeneration protocols. Moreover, using the histological analysis some steps relating to the process of somatic embryogenesis were determined and, differences between embryogenic and non-embryogenic cells using TEM were identified.

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