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# Marigold Biotechnology: Tissue Culture and Genetic Transformation

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## ABSTRACT

Members of the *Tagetes* genus include important floricultural (cut-flower) and ornamental (pot and garden) crops, as well as plants of medicinal and ethno-pharmacological interest. Despite the use of many of these plants in the extraction of important secondary metabolites and essential oils, the greatest biotechnological emphasis has been on their *in vitro* tissue culture and micropropagation. Few studies have been conducted on genetic transformation, with those primarily focused on increasing yield of compounds in plants. However, the application of genetic transformation methodology requires the development of efficient techniques, not only for the transfer of foreign genes into plant cells, but also for the regeneration of whole, fertile plants from the transformed cells. Thus, the development of suitable methods for regeneration is one of the main prerequisites for genetic improvement by biotechnologic means. The purpose of our review is to describe the approaches, via organogenesis or embryogenesis, that have been applied to regenerate whole marigold (*Tagetes erecta* L.) plants and the current status of targeting genes, whether via *Agrobacterium tumefaciens* or biobalistics. The advances, applications and limitations of marigold biotechnology are discussed.

**Keywords:** *Agrobacterium tumefaciens*, carotenoids, embryogenesis, glucuronidase, lutein, microparticle bombardment, organogenesis

## CONTENTS



## INTRODUCTION

The genus *Tagetes* belongs to the Asteraceae family (formerly known as Compositae). It comprises 55 species, which are largely disseminated in America. More than half of them are native to Mexico.

*Tagetes erecta* L., also called African marigold, Aztec marigold, big marigold, or cempoalxóchitl (from the nahuatl, flower with twenty petals) is an herbaceous plant, that completes its life cycle in one year. Its flowers present bright colors, ranging from yellow to deep red, due to the carotenoids present in their inflorescences, which are grouped together and thus appearing to be a single flower (Serrato-Cruz *et al.* 2000; Serrato-Cruz 2004).

It was taken to Europe in the XVI$^{th}$ century and many varieties have been developed since then, such as: Atlantis, Cortez, Discovery, Galore, Jubilée, Ladies, Piezas de Oro and Vanilla (Serrato-Cruz 2004). Given the diversity of this species in Mexico, this country has been considered its center of origin. Paradoxically given the genetic diversity present in Mexico, associated with domestication processes performed by the mesoamerican indigenous groups, there are no studies that describe their different characteristics (carotenoid levels, plant height, plant architecture, disease resistance, etc.) associated to the diverse geographical regions where they grow.

As a result, there are no documented systematic genetic improvement efforts. In fact, marigold flower production in Mexico has been diminishing in the late years, so the increased demand for this source of carotenoids in the United States and Europe is covered by crops from China, India and Peru.

The commercially exploited varieties, Sweet Cream, Inca, Antigua, Marvel and Perfection, are represented by 30-40 cm high plants with large flowers. These varieties are the result of the crossing of different lines or of *in vitro* vegetative multiplication.

No data on the genetic improvement strategies or the segregation of important characters are available in the scientific literature, so companies such as Sakata Seed Corporation, Park Seeds, Ball Horticultural Company, Goldsmith Seeds, and PanAmerican Seed among others, are covering the demands for improved seeds (Serrato-Cruz 2004).

In Mexico, *Tagetes erecta* has a very relevant role in our culture and economy. Their flowers have been used since prehispanic times in rituals associated to the celebration of death. During the "Día de Muertos" (November 1$^{st}$), graves in cemeteries and altars are adorned with these flowers, since it is believed that their bright color will illuminate the journey of the souls that come to visit their living relatives (Serrato-Cruz 2004). They are also used in traditional medicine to cure different ailments associated to bacteria and fungi. Among other uses are as green compost (Serrato-Cruz 2004), insecticide (pyrethrins), antibiotic, ne-

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maticide, fungicide (thiophenes) (Vasudevan *et al*. 1997; Romagnoli *et al*. 2005).

Though it has been utilized for pest control, marigold is the target of a multiple range of pathogens. Tarnished plant bugs provoke the appearance of distorted flowers and leaves, while leafhoppers cause cupping and in-rolling of leaf margins. Members of the genus *Alternaria* provoke the damping off in plantlets and also are responsible of the early blight in adult plants, which is characterized by the appearance of necrotic spots in leaves. Stems turn brown and shrivel at the soil line, leading to the plant's death (Gilman and Howe 1999).

*T. erecta* flowers are commercially cultivated, harvested and processed in an industrial scale as a source of carotenoid yellow-orange pigments (Sowbhagya *et al*. 2004), with values ranging from 0.17 to 5.7 grams of total carotenoids per kilogram of fresh flowers (Piccaglia *et al*. 1998). Crude flower extracts are used mainly as an ingredient for poultry food to promote a deep yellow coloration of their skin and the egg's yolk (Hencken 1992; Delgado-Vargas *et al*. 1998).

The dark orange varieties of marigold contain concentrations of carotenoids that are up to 20-fold higher than in marigold leaves, and 20 times the concentration of carotenoids found in ripe tomato fruit (Moehs *et al*. 2001). Marigold flowers are the most concentrated common source of carotenoids, with lutein, a dihydroxylated carotenoid, accounting for 85% of the total carotenoids present in the flower. Carotenoids in flowers are mostly esterified with lauric, myristic, palmitic and stearic acids in different proportions, which makes them readily soluble in hexane (Barzana *et al.* 2002).

Lutein belongs to a group of plant pigments, the xanthophylls (oxygen-containing carotenoids). Unlike other carotenoids such as β-carotene, lutein is not a vitamin A precursor; however, this bright orange-coloured phytochemical has been found to have many health beneficial effects. Diets rich in lutein have been associated to the reduction of the risk for failing eyesight due to age-related macular degeneration, the leading cause of irreversible blindness amongst senior population. It is also a potent antioxidant, more so than β-carotene and lycopene, that quenches reactive oxygen species and free radicals produced in several metabolic processes in cells, or from environmental pollutants (Edge *et al*. 1997). Lutein has also been found to protect skin from damage caused by ultraviolet light, and to prevent cardiovascular hardening caused by ageing, coronary heart disease and cancer (Wang *et al*. 2006).

Although marigold flower extracts have been used in animal feed, the potential use of marigold as a natural food colorant has not been exploited to its full extent due to the lack of information on its safety, stability and compatibility (Sowbhagya *et al*. 2004). Recently, many lutein-containing functional foods and nutraceutical products have been developed to help needy population acquire sufficient lutein intake through supplementation.

Lutein is also among the 10 phytochemicals recommended by the FDA as GRAS (generally regarded as safe) nutritional supplements (Wang *et al*. 2006). Certain carotenoids, such as β-carotene, have been found to increase the incidence of lung cancer at high dosages, specially among smokers. In contrast, a recent study with *Salmonella typhimurium* strains and Chinese hamster ovary cells, showed that lutein is non-mutagenic at all doses and has an antimutagenic effect in a dose-dependent manner. Similar results were found on chromosomal damage induced by mutagens, suggesting that it is potentially safe for lutein to be used in food supplements at high doses (Wang *et al*. 2006).

The increasing importance of carotenoids for human consumption is evident from their prices in the international markets. For example, the 1999 world market for carotenoids was US$ 750–800 million and projections estimate around US$ 1 billion in 2005, while the annual worldwide market for astaxanthin is estimated at US$ 200 million with an average price of US$ 2500/kg. It is now dominated by the synthethic form of the pigment, which is produced by BASF (Ludwigshafen, Germany) and Hoffman-La Roche (Basel, Switzerland). Natural astaxanthin is produced by *Haematoccocus pluvialis* in a two-stage culture process and its concentration can reach 1.5% to 3% of the dry weight. Given the high production costs, astaxanthin from *H. pluvialis* cannot compete commercially with the synthethic form. However, for a few particular applications, such as chicken and fish diets, natural astaxanthin is preferred due to the enhanced deposition of the natural pigment in tissues, regulatory requirements and consumer demands for natural products.

The estimated market size for natural β-carotene is 100 tons/year (Pulz *et al*. 2001), and the price of β-carotene extracts from *Dunaliella salina* for human use vary from US$ 300-3000/kg (Spolaore *et al*. 2006).

## *Tagetes erecta* tissue culture

The multiplicity of uses for *Tagetes* emphasizes its growing importance and demand for improved seeds to satisfy the market's needs. Nevertheless, this demand cannot be fulfilled by growers, given the low viability of seeds and their poor germination rates, which decreases as the seed ages.

Plants are regenerated from cell cultures via two methods, somatic embryogenesis or organogenesis. Both are controlled by plant growth regulators and other factors added to the culture medium. Somatic embyogenesis is the generation of embryos from somatic tissues, such as embryos, microspores or leaves. Organogenesis is the generation of organs, usually shoots from a variety of tissues.

In **Table 1**, an overview of the available protocols for the regeneration of *Tagetes* is presented and further details are discussed in the next sections

*Explants*. Two sources of material have been used: adult plants from botanical gardens or fields (Kothari and Chandra 1984, 1986; Misra and Datta 1999) or plantlets from seeds germinated *in vitro* (Belarmino *et al* 1992; Misra and Datta 2001; Vanegas *et al*. 2002; Miranda-Ham *et al.* 2006). In the case of grown plants, Kothari and Chandra (1984, 1986) employed unopened capitula or leaves, which were surface sterilized for 5 min in 0.1% mercuric chloride solution and washed three time in sterile distilled water; whereas Misra and Datta (1999, 2001) used shoot tips or leaves taken from plants growing in the field. These explants were

**Table 1** Regeneration studies of *Tagetes erecta* L.

| Explant | Culture media and growth regulators[a] | Morphogenetic response[b] | References |
|---|---|---|---|
| Disc florets | MS: BAP, IAA, IBA | S, P | Kothari and Chandra 1984 |
| Leaf callus | MS: BAP, IAA | S, P | Kothari and Chandra 1986 |
| Suspension culture | MS: BAP, NAA | R | |
| | KIN, 2,4-D | E | |
| Hypocotyls-derived calluses | MS: BAP, NAA | S, P | Belarmino *et al.* 1992 |
| Shoot tips (adult plants) | MS: BAP, NAA | S, P | Misra and Datta 1999 |
| Leaf | MS: BAP, $GA_3$, NAA | S, P | Misra and Datta 2001 |
| | MS: BAP, IAA | S, P | Vanegas *et al.* 2002 |
| Shoot apex-derived calluses | MSB: BAP, IAA | S, P | Miranda-Ham *et al*. 2006 |

[a] 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; $GA_3$, gibberellic acid; IAA, indole-3-acetic acid, IBA, indole-3-butyric acid, KIN, kinetin, MS, Murashige and Skoog medium (Murashige and Skoog 1962); MSB, modified Murashige and Skoog medium (Robert *et al*. 1987); NAA, α-naphthalene acetic acid

[b] E, embryo-like structures; P, plantlets; R, roots; S, shoots

washed in running tap water for 30 min, treated with 5% teepol solution, and washed with distilled water. A quick dip in 70% alcohol and then in 0.1% $HgCl_2$ solution for 1-3 min was used depending on the age and tenderness of the explants. They were thoroughly washed at least thrice at 5 min intervals each with sterilized distilled water.

When using explants from plantlets from seeds germinated *in vitro*, the desinfestation process tended to be milder, using quick washes with sodium hyplochlorite (1-2.5%), with Tween 20 as a surfactant, and ethanol (70-80%). Thorough washes with sterile distilled water followed these treatments. In all cases, the desinfested seeds were germinated on growth regulator-free MS medium (Belarmino *et al.* 1992; Vanegas *et al.* 2002) or growth regulator-free MSB medium (MS medium with a modified nitrogen source, the modification consisted of a reduction of the total nitrogen content to 28 mM with a $NO_3/NH_4$ ratio of 4.6:1; Robert *et al*. 1987) (Miranda-Ham *et al*. 2006). Three-week old seedlings were the source for the hypocotyls and leaf segments used by Belarmino *et al.* (1992) and Vanegas *et al*. (2002), respectively, while two-week-old seedling were utilized for obtaining the shoot apexes employed by Miranda-Ham *et al*. (2006).

A common problem in these types of experiments is the browning of explants, which leads to explant death or poor callus generation. To eliminate phenolic production, Belarmino *et al*. (1992) reported the dipping of explants in filter-sterilized 1% ascorbic acid for 1 min prior to putting them in the MS medium.

*Culture media and growth regulators*. Most reports used MS medium to induce the regeneration process and different combinations of 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), kinetin (KIN), 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), and gibberellic acid ($GA_3$).

For regeneration of plants from disc florets, Kothari and Chandra (1984) probed different concentrations of BAP and IAA (3, 4 and 5 mg/L); the optimal combination was 5 mg/L BAP and 3 mg/L IAA, which yielded 15-20 shoot buds. The same authors in 1986, used MS medium supplemented with two cytokinins (KIN and BAP) and four auxins (IAA, IBA, NAA and 2,4-D) to induce shoots from leaves' calluses. Results showed that the use of KIN alone or in combination with any of the four auxins could not induce regeneration, while BAP and IAA or NAA could. Adventitious shoots were obtained only with 7 mg/L BAP and 5 mg/L IAA, any other combination was unsuccessful.

When using calluses derived from hypocotyls, Belarmino *et al*. (1992) could regenerate plants shoots with the following combinations: NAA (0.2 mg/L) and BAP (2 and 5 mg/L), NAA (0.5 mg/L) and BAP (1, 2, and 5 mg/L), and NAA (1 mg/L) and BAP (0.5 and 1 mg/L). Only roots could be induced from the leaves' calluses using all tested the combinations of growth regulators.

To induce shoots from shoot tips from adult plants, MS medium and the following combinations were used: BAP (0.1-10 mg/L), KIN (0.1-1 mg/L), IAA (0.1-2 mg/L), NAA (0.1-2 mg/L), $GA_3$ (0.5-5 mg/L) and 2,4-D (1-10 mg/L). High doses of KIN or $GA_3$ or BAP induced shoots, but they showed a yellowish to brown coloration. The best condition was found to be 5 mg/L BAP for 7 days, and then transfer to 0.1 mg/L BAP, which yielded 10 shoots/explant (Misra and Datta 1999). In the case of leaves, the same authors (Misra and Datta 2001) cultured the explants with their abaxial surface on MS medium supplemented with BAP (2.2-8.8 μM), thidiazuron (2.27-9.08 μM), 2,4-D (4.52-22.62 μM), tri-iodobenzoic acid (2-10 μM), abscisic acid (ABA, 0.19-0.95 μM) and $GA_3$ (14.43-57.74 μM) to induce the direct formation of shoots without the induction of calluses. This was obtained using 14.3 μM $GA_3$ and 4.44 μM BAP (2-5 shoot buds were induced after 4 weeks). The leaf segment that resulted in the highest number of shoots was the basal petiolar end. When shoots proliferated, they were changed to a medium containing 1.1 μM BAP and 29.41 μM $AgNO_3$. These conditions yielded 15-20 healthy shoots.

Vanegas *et al*. (2002) employed the auxins NAA (0, 2.7, 5.4 and 8.1 μM) and IAA (0, 2.9, 5.7 and 17.1 μM) and the cytokinin BAP (0, 2.2, 4.4, 6.7 and 13.3 μM) to induce regeneration in leaf explants. They obtained shoots in all the tested combinations. Nevertheless the use of 8.1 μM NAA and 4.4 μM BAP resulted in the highest Bud Forming Capacity (BFC) index (0.62) (a mean of 1.6 shoots/explant). However, the combination of 17.1 μM IAA and 13.3 μM BAP, gave a BFC index of 1.39 (a mean of 2 shoots/explant), when explants were incubated by 13 days.

Shoot apices were used to obtain regeneration of *T. erecta* in MSB medium, added with different combinations of IAA (0, 10, 30 μM) and BAP (0, 30, 50 and 70 μM). Though all the tested combinations induced shoot formation, 10 μM IAA and 70 μM BAP after 10 weeks, presented a BFC index of 46.2 (66 ± 12 shoots/explant). Shoots were slightly vitrified, condition that was reversed once they were transferred to MSB medium without growth regulators for 3-4 weeks (Miranda-Ham *et al*. 2006).

*Subculturing practices*. Kothari and Chandra (1984) recommended the subculturing of shoots obtained from disc florets in MS medium with 3 mg/L BAP, 5 mg/L IAA and 5 mg/L $GA_3$ every 2 weeks, in order to preserve their morphogenetic capacity for several months. In contrast, leaf calluses lost their potential after 3 subcultures (75 days) (Kothari and Chandra 1986).

The shoots' basal portion that proliferated from shoot tips could be used to obtain more shoots in MS medium with 5 mg/L BAP and 5 mg/L adenine sulphate for at least 5-6 times. Afterwards, they saw a declination in the morphogenetic potential (Misra and Datta 1999).

Both Vanegas *et al.* (2002) and Miranda-Ham *et al.* (2006) reported that shoots derived from leaf explants or shoot apex derived calluses were transferred to medium without growth regulators, where they elongated. No loss of morphogenetic potential was mentioned after subculturing.

*Rooting*. Kothari and Chandra (1984, 1986) reported the rooting of shoots from disc florets and leaf calluses using MS medium with 5 mg/L IBA and 0.5 mg/L $GA_3$, while Misra and Datta (1999, 2001) employed a lower IBA concentration (0.05 mg/L) or NAA (0.1 mg/L). Though they had a 100% rooting response with both IBA and NAA, the latter was preferred since it did not induce callus formation. After roots began to appear, shoots were transferred to a growth regulators free medium to complete their formation.

In the case of shoots induced from leaves' explants and shoot apex derived calluses, no growth regulators were used for the rooting process, since transfer to a medium free of them yielded strong, healthy plants with a complete radicular system, which were ready for transplantation and acclimatization (Vanegas *et al*. 2002; Miranda-Ham *et al*. 2006).

*Acclimatization*. Misra and Datta (1999, 2001) reported that the plantlets were first acclimatized in Knopf's solution and then transplanted to potted soil in the nursery. The watering regime was diminished for 15 days prior to their transfer to the field. Transplant efficiency was 75%, since plants were quite sensitive to the harsh conditions outdoors. They flowered after 30-45 days.

Vanegas *et al.* (2002) mentioned that plantlets were transferred to sterile soil and were kept on a growth chamber for a week. They were watered twice during this time with half strength MS medium salts added with sucrose. After this time, plants were taken to the greenhouse, where they completed their life cycles (including flowering). They reported a survival frequency of 100%.

On the other hand, Miranda-Ham *et al*. (2006) reported that when plantlets were 4 cm tall, they were transferred to pots containing a mixture of sterile soil and vermiculite (1:1) for their acclimatization. The pots were covered with transparent polyethylene bags and kept in a greenhouse at room temperature (25 to 30°C), under natural illumination at a photon flux density of 310 μmol $m^{-2}$ $s^{-1}$. To reduce condensation, the bags had holes punched in them and after one week, they were removed. Three weeks later, the plants were transferred to the nursery, first under shading (572

μmol $m^{-2}$ $s^{-1}$), and three weeks later, exposed to open sunlight (1985 μmol $m^{-2}$ $s^{-1}$). The plants were watered daily, and supplemented with Hoagland solution every three weeks. In these conditions, survival frequency was higher than 80%.

From the revised literature regarding the regeneration protocols in *Tagetes*, it is evident that the bottleneck is the number of shoots obtained per explant, since it is critical for determining the efficiency of the regeneration system employed. Evidence points out that the type of explant is the main variable to control: 15-20 shoots/disc florets (Kothari and Chandra 1984), 12 shoots/3.65 g of leaves-derived callus (Kothari and Chandra 1986), 10 shoots/shoot tips from adult plant (Misra and Datta 1999), 15-20 shoots/ leaves (Misra and Datta 2001), 5.2 shoot/leaves (Vanegas *et al*. 2002) and 66.12 shoots/shoot apex-derived calluses (Miranda-Ham *et al.* 2006).

There are other systems that have not been explored yet for *T. erecta*, which could enhance the number of shoots per explant. One of them is micropropagation *via* thin cell layer (TCL). Thin cell layer is a simple but effective system that relies on a small size explant derived from a limited cell number of homogenous tissues. TCL is a model system, which will find applications in higher plant tissue and organ culture and genetic transformation (Teixeira da Silva 2005).

Another system to explore could be the induction of direct somatic embryogenesis, since embryo-like structures from leaf callus cultures has been obtained (Kothari and Chandra 1986; Bespalhok and Hattori 1998).

The development of an efficient *in vitro* regeneration protocol, via organogenesis or embryogenesis, is a prerequisite for genetic improvement through biotechnological means, since the use of genetic engineering techniques *per se* decreases shoot and embryos formation in any culture.

For plant genetic transformation, somatic embryogenesis may be more suitable than organogenesis, since in most cases, somatic embryos have a single-celled origin and chimeric transgenic plants are less likely to develop. However, the use of embryogenic tissue can present some limitations: it can be labor intensive to establish and maintain the culture and the recovery of plants can take a long time, with the risk of encountering morphological abnormalities and sterility. This system also requires a constant source of material to initiate new embryogenic cultures. The advantage of the organogenesis protocol is that shoots can usually form roots readily. Sometimes tissues can acquire a translucent and brittle appearance with a high water content (hyperhydricity). The occurrence of this hyperhydric state can be avoided or reduced by modifying the sugar source, the calcium concentration or by use of antivitrifying agents, such as phloridzin (Hansen and Wright 1999).

The extent of the use of *Tagetes* has not been circumveted to *in vitro* regeneration; it has also been utilized for studies on differentiation and thiophene presence in leaf callus cultures (Ketel 1986), the coupling of thiophene synthesis to root regeneration from stem calluses (Croes *et al*. 1989), the histological dissection of embryogenic calluses from cotyledons (Bespalhok and Hattori 1999), and the determination of pyrethrins in calluses (Sarin 2004).

## GENETIC TRANSFORMATION OF *TAGETES ERECTA*

The production of transgenic plants with stable and predictable gene expression patterns has long been a major goal for researchers. In the last decade, great efforts have been directed towards obtaining transgenic plants with agronomically important traits, such as resistance to pests and diseases, increased tolerance to environmental stresses (temperature, water and saline soils), herbicide tolerance, modifications in fruit ripening and fruit pigmentation patterns, the improvement of nutritional composition (vitamin A and iron) or the production of pharmaceutical compounds (edible vaccines production). Shape, height, fenology, longevity are suitable candidates for genetic manipulation. The use of *Agrobacterium tumefaciens*, microparticle bombardment (biolistics) or any other gene transfer technique (protoplast transformation), would confer the ability to transform economically important medicinal and aromatic varieties. Though *A. tumefaciens* has been used as the vector for genetic transformation of diverse dicotyledonous (Zupan *et al*. 2000; Gelvin 2003) and for monocotyledonous especies (Cheng *et al*. 2004), only recently has biolistics been turned into a powerful technique to introduce foreign DNA into plant cells, regardless of being monocotyledonous and dicotyledonous (Taylor and Fauquet 2002).

There are only two reports on the genetic transformation of *Tagetes erecta* (**Table 2**). Godoy-Hernández *et al*. (2006) reported transient transformation with the GUS reporter gene in four types of explants (shoot tip, leaf primordium, hypocotyl and radicle) from *in vitro* germinated plantlets, using *A. tumefaciens* strain LBA4404 and binary vector pCAMBIA 2301. The plasmid contained the kanamycin-resistant marker gene *nptII* within T-DNA borders for bacterial selection. In order to prevent interference of bacterial GUS activity in *A. tumefaciens* cells, the gene contained an intron sequence of catalase. Acetosyringone was added to the bacteria prior to use to facilitate transformation. For the transformation experiments, 10-day old plantlets were sectioned and the different explants (hypocotyls, radicles (10 mm length), leaf sections (0.25 $cm^2$ approx.) and shoot tips) were superficially wounded by making longitudinal scraps and then infected by vacuum infiltration. Explants were placed on MS medium, supplemented with 1 μM IAA, 5 μM BAP, 100 mg/L cefotaxime and 7.5 mg/L kanamycin (pH 5.7), for further development. Transient expression of GUS was histochemically assayed on the 3rd day after infection, by staining the explants with X-GLUC. Before assessing the number of blue spots in the explants, they were washed in methanol:acetone (3:1, v/v). Each blue spot was considered as one transient GUS-expression focus (Godoy-Hernández *et al.* 2006).

On the other hand, Vanegas *et al*. (2006) reported the stable transformation of leaf explants with *uidA* via microparticle bombardment. For the bombarding, they employed leaf explants (0.025 $cm^2$), which were incubated in the rege-

**Table 2** Studies involving genetic transformation of *Tagetes erecta* L.

| Variables | *A. tumefaciens*[a] | Biolistics[b] |
|---|---|---|
| Binary vector | pCAMBIA 2301 | pBI426 |
| Promotor | 35 S CaMV single | 35 S CaMV double |
| Reporter gene | GUS | GUS |
| Selective agent | Kanamycin (7.5 mg/L) | Kanamycin (100 mg/L) |
| Agent to kill bacteria | Cefotaxime (100 mg/L) | - |
| Culture medium | MS | MSB |
| Plant growth regulators | IAA (1 μM), BAP (5 μM) | IAA (17.1 μM), BAP (13.3 μM) |
| Transformation type | Transient | Transient and stable |
| Demonstration | GUS assay | GUS assay, PCR (*nptII*) and Southern blot analysis |
| Plant regeneration | - | From 9 PCR positive plantlets, 5 were Southern blot positive |

[a] Godoy-Hernández *et al*. 2006
[b] Vanegas *et al.* 2006
35S CaMV, 35S cauliflower mosaic virus, GUS, β-glucuronidase (*uidA* gene), MS, Murashige and Skoog medium (Murashige and Skoog 1962), MSB, Modified Murashige and Skoog medium (Robert *et al.* 1987), IAA, indole-3-acetic acid, BAP, 6-benzylaminopurine

neration medium (MS plus 17.1 µM IAA and 13.3 µM BAP), supplemented with sorbitol and/or mannitol, for 2, 4 or 6 hours. Tissues were bombarded using four different pressures (413.7, 551.6, 689.5 or 827.4 kPa) and distances (7.5, 10.5, 13.5 or 16.5 cm). The particles were liberated using a pulse of 50 ms in a vacuum chamber (3.73 kPa). The best results were obtained with a distance of 10.5 cm and a pressure of 551.6 kPa, producing 25.7 foci per event.

The bombarded explants were put in the regeneration medium with 100 mg/L kanamycin. Twenty-five kanamycin resistant plants were isolated after 160 bombardment events, which represented an efficiency of 16%. After a second round of selection in 200 mg/L kanamycin, only 15 plantlets (60%) were selected, a final 9% of the total population.

They also employed the development of blue plaques as an indication of transient cell transformation. In order to verify the presence of the transgene in regenerated plantlets on selective medium, the *nptII* gene was amplified by PCR. To confirm *nptII* gene integration and determine its copy number, they analyzed the 9 PCR positive plantlets by Southern blot analysis using the gene as a probe. From 9 PCR-positive plantlets, 5 were Southern blot positive (4 plantlets with one copy of the *nptII* gene and 1 with at least two copies), with a final efficiency of 3%. No reference was made to the possibility of transgene silencing, to the physiological stability of the transgenic plants in the greenhouse or to the characteristic of the progeny. They mentioned that further studies were needed to optimize their transformation efficiency (Vanegas *et al.* 2006).

### *Tagetes erecta* hairy roots

Hairy roots, in contrast to the non-tranformed counterparts, grow rapidly, show plagiotropic growth and are highly branched on phytohormone-free media. The transformed roots are highly differentiated and can produce in an intensive and stable manner, a lot of valuable secondary metabolites, in comparison to other plant cell cultures (Hu and Du 2006).

Transgenic root systems offer a tremendous potential for introducing additional genes along with the Ri T-DNA genes for the engineering of metabolic pathways and the production of compounds of interest (Giri and Narasu 2000). Axenic hairy root cultures growing under controlled conditions are a convenient model to study the root metabolism and offer a detailed view of contacts between roots and symbiotic or parasitic microorganisms.

*Tagetes* spp. produce aromatic sulfur-containing compounds known as thiophenes in their roots, which are toxic to nematodes when ingested. Hairy roots of *T. erecta*, obtained through transformation with *A. rhizogenes* strain TR105, produce the same thiophene profile as normal root cultures and roots of the intact plant (Mukudan and Hjortso 1990). In contrast, transgenic hairy root lines, product of the infection with *A. rhizogenes* LBA 9402, showed a different spectrum of thiophenes compared to wild-types plants (Jacobs *et al*. 1995)

There are very few studies on the colonization and responsiveness to inoculation by different arbuscular mycorrhizal fungi (*Glomus intraradices*, *G. mosseae* and *G. deserticola*) in *T. erecta* (Linderman and Davis 2004).

Contrastingly, most studies on hairy root cultures have been done using *Tagetes patula*. Hairy root cultures of *T. patula* have been used to study the effect of exogenously applied indole-3-acetic acid (IAA) on root morphology and secondary metabolism (Arroo *et al*. 1995), to study the regulation of thiophene biosynthesis under a limited supply of sulphate (Arroo *et al*. 1997), the effect of aqueous extracts of green alga (*Haematococcus pluvialis*) and blue green alga (*Spirulina platensis*) to induce thiophene accumulation (Ramachandra Rao *et al*. 2001), to record growth and thiophene production in an acoustic mist bioreactor (Suresh *et al*. 2005), and also to study the biosynthesis of benzofuran derivatives (Margl *et al*. 2005).

## CONCLUSIONS

To the authors' knowledge, it is evident that there are just two reports on genetic transformation in marigold, via *A. tumefaciens* (Godoy-Hernández *et al*. 2006) or microparticle bombardment (Vanegas *et al*. 2006). Nevertheless, both studies show that these systems can be applicable to transform *T. erecta* with genes of interest, using the *in vitro* regeneration protocols established with shoot apex-derived calluses (Miranda-Ham *et al.* 2006) and leaves (Vanegas *et al*. 2002). The transformation strategy to employ will depend on the technical facilities and experience of each research group, even though the *A. tumefaciens* method will consistently be more cost-friendly.

Regarding the ongoing debate on the ingestion of compounds derived from transgenic plants or products manufactured with such compounds, based on the use of antibiotic resistance genes as selective markers, there is ground for hope, given the use of other genes, such as the phosphomannose isomerase (Joersbo *et al*. 1998), or no markers at all (Daley *et al*. 1998).

Another concern has been that of the ecological impact due to genetic contamination of other varieties via transgenic pollen, which has also been circunveted by the new approach of chloroplast transformation (Bogorad 2000; Daniell *et al*. 2002; Maliga 2004).

## PERSPECTIVES

Carotenoid contents among marigold varieties can present 100-fold differences, making them an excellent system to examine the regulation of fluxes through this pathway (Moehs *et al*. 2001). There is interest of some research groups to enhance astaxanthin production (a more valuable carotenoid than lutein) through transformation with the *crtW* gene, which codes for a ketolase from *Agrobacterium aurantiacum*, using biobalistics.

Another area of interest is the transformation of marigold with two genes from the plastidic MEP (methyl-D-erythritol 4-phosphate) pathway: *cla1*, which codes for 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (Mandel *et al.* 1996) and the *isph* gene, that codes for the 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase, both from *A. thaliana* (Guevara-García *et al*. 2005). These enzymes control key regulatory points in this pathway and thus, the aim is to increase the flux towards the synthesis of plastidic isoprenoids (monoterpenes gibberellins, carotenoids, chlorophylls, tocopheroles, plastoquinones, phylloquinones).

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