Marigold Biotechnology: Tissue Culture and Genetic Transformation

Gregorio Godoy-Hernández • María de Lourdes Miranda-Ham

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 #130, Chuburná de Hidalgo, Mérida, Yucatán, 97200, México
Corresponding author: ggodoy@cicy.mx

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

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 brilliant colors, ranging from yellow to deep red, due to the carotenoids present in their inflorescences, which 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 1st), 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-
micate, 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 for 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 (Gilm 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 synthetic form of the pigment, which is produced by BASF (Ludwigshafen, Germany) and Hoffman-La Roche (Basel, Switzerland). Natural astaxanthin is produced by *Haeomatococcus 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 synthetic 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 embryogenesis 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.

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

<table>
<thead>
<tr>
<th>Explant</th>
<th>Culture media and growth regulators</th>
<th>Morphogenetic response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disc florets</td>
<td>MS: BAP, IAA, IBA</td>
<td>S, P</td>
<td>Kothari and Chandra 1984</td>
</tr>
<tr>
<td>Leaf callus</td>
<td>MS: BAP, IAA</td>
<td>S, P</td>
<td>Kothari and Chandra 1986</td>
</tr>
<tr>
<td>Suspension culture</td>
<td>MS: BAP, NAA</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Hypocotyls-derived calluses</td>
<td>KIN, 2,4-D</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>Shoot tips (adult plants)</td>
<td>MS: BAP, NAA</td>
<td>S, P</td>
<td>Belarmino et al. 1992</td>
</tr>
<tr>
<td>Leaf</td>
<td>MS: BAP, GA, NAA</td>
<td>S, P</td>
<td>Misra and Datta 1999</td>
</tr>
<tr>
<td>Shoot apex-derived calluses</td>
<td>MS: BAP, IAA</td>
<td>S, P</td>
<td>Vanegas et al. 2002</td>
</tr>
<tr>
<td></td>
<td>MBA: MS, BAP</td>
<td>S, P</td>
<td>Miranda-Ham et al. 2006</td>
</tr>
</tbody>
</table>

* 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; GA₃, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid, KIN, kinetin; MS, Murashige and Skoog medium (Murashige and Skoog 1962); MBA, modified Murashige and Skoog medium (Robert et al. 1987); NAA, α-naphthyl acetic acid
* A, embryo-like structures; P, plantlets; R, roots; S, shoots

*2007 Global Science Books*
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% HgCl₂ 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, usually requiring washing with sodium hypochlorite (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 NO₃/NO₂ ratio of 8:1; Robertson 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 apaxes 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 formation. 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 (GA₃).

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), GA₃ (10 μM), tri-iodobenzoic acid (2-10 μM), abscisic acid (ABA, 0.1-10 μM), GA₃ (0.1-10 mg/L), KIN (0.1-1 mg/L), IAA (0.1-2 mg/L), NAA (0.5 mg/L) and BAP (1, 2, and 5 mg/L), and 2,4-D (0.1-10 mg/L). High doses of KIN or GA₃ 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. 8.1 mg/L BAP), IAA (0.5-5.5 mg/L), IBA (0.5-5.5 mg/L), KIN (0.08-0.8 mg/L), GA₃ (14.43-57.74 μM) to induce the direct formation of shoots without the induction of calluses. This was obtained using 14.3 μM GA₃ 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 mg/L BAP and 29.41 μM AgNO₃. These conditions yielded 15-20 healthy shoots.

Vanegas et al. (2002) reported 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 induced 1 shoot from 1.2 mg/L IAA (2 shoots/explant), when explants were incubated by 13 days.

Shoot apices used to be obtained by using 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 GA₃ 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 decline 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 GA₃, 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 radiculogenetic potential (Misra and Datta 1999).

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 (0-30°C, 1000 lux and 70 μE/m² s⁻¹).

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 \textit{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/disk 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 (Misra and Datta 2004).

There are other systems that have not been explored yet for \textit{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 hyperhydrdic 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 \textit{Tagetes} has not been cummulated to in vitro regeneration; it has also been utilized for studies on differentiation and thiphene presence in leaf callus cultures (Ketel 1986); the coupling of thiophene synthesis to root regeneration from stem callus (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).

**Table 2** Studies involving genetic transformation of \textit{Tagetes erecta} L.

<table>
<thead>
<tr>
<th>Variables</th>
<th>\textit{A. tumefaciens}⁴</th>
<th>Biologies⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binary vector</td>
<td>pCAMBIA 2301</td>
<td>pBH26</td>
</tr>
<tr>
<td>Promotor</td>
<td>35 S CaMV single</td>
<td>35 S CaMV double</td>
</tr>
<tr>
<td>Reporter gene</td>
<td>GUS</td>
<td>GUS</td>
</tr>
<tr>
<td>Selective agent</td>
<td>35S  uidA (7.5 mg/L)</td>
<td>Kanamycin (100 mg/L)</td>
</tr>
<tr>
<td>Agent to kill bacteria</td>
<td>Cefotaxime (100 mg/L)</td>
<td>-</td>
</tr>
<tr>
<td>Culture medium</td>
<td>MS</td>
<td>MSB</td>
</tr>
<tr>
<td>Plant growth regulators</td>
<td>IAA (1 μM), BAP (5 μM)</td>
<td>IAA (17.1 μM), BAP (13.3 μM)</td>
</tr>
<tr>
<td>Transformation type</td>
<td>Transient</td>
<td>Transient and stable</td>
</tr>
<tr>
<td>Demonstration</td>
<td>GUS assay</td>
<td>GUS assay, PCR (nptII) and Southern blot analysis</td>
</tr>
<tr>
<td>Plant regeneration</td>
<td>From 9 PCR positive plantlets, 5 were Southern blot positive</td>
<td></td>
</tr>
</tbody>
</table>

⁴Godoy-Hernández et al. 2006  
⁵Vanegas 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

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 agriculturally 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 \textit{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 \textit{A. tumefaciens} has been used as the vector for genetic transformation of diverse dicotyledonous (Zupan et al. 2000; Gelvin 2003) and monocotyledonous species (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 Faquett 2002).

There are only two reports on the genetic transformation of \textit{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 \textit{in vitro} germinated plantlets, using \textit{A. tumefaciens} strain LBA4404 and binary vector pCAMBIA 2301. The plasmid contained the kanamyacin-resistant marker gene nptII within T-DNA borders for bacterial selection. In order to prevent interference of bacterial GUS activity in \textit{A. tumefaciens} cells, the gene contained an intron sequence of catalase. Acetylsyringone 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 cm² 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 \textit{uidA} via microparticle bombardment. For the bombarding, they employed leaf explants (0.025 cm²), which were incubated in the rege-
eration 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 (+) 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 (*Gliomus intraradices, G. mosseae* and *G. decorticulat*) 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 bio-reactor (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 circumvented by the new approach of chloroplast transformation (Bogorad 2000; Danell 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-Garcia 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, phytloquinones).

**REFERENCES**


In Vitro Cellular and Developmental Biology – Plant 40, 31-45


174