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## *In vitro* regeneration and *Agrobacterium*-mediated transformation of male-sterile marigold (*Tagetes erecta* L.)

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**Abstract** In this study, we devised a method for the *in vitro* regeneration and subsequent genetic transformation of male sterile marigold. To our knowledge, this is the first report of generation of transgenic plants with a single genotype of marigold via *Agrobacterium*-mediated transformation. We obtained four transgenic lines from two independent experiments with 496 leaf explants, which were inoculated by an *Agrobacterium* strain LBA4404 harboring the plasmid, pIG121-Hm. Although the efficiency of the transformation in our system was low, stable expression of *uidA* gene in adventitious shoots and compound leaves could be detected in  $\beta$ -glucuronidase histochemical analysis. This protocol contributes to the progress of genetic studies and molecular breeding of this species.

**Key words:** *Agrobacterium*, genetic transformation, male sterility, meropenem, *Tagetes erecta*.

Marigold (*Tagetes erecta* L.) belongs to the Asteraceae family and is an important agronomical, medicinal, and ornamental plant. Marigold contains potentially useful compounds, such as antioxidants, fungicides, insecticides, bactericides, and nematocides in flowers and other organs (Vasudevan et al. 1997). The marigold flower is a major source of plant pigments such as lutein, which is an oxygen-containing carotenoid (Piccaglia et al. 1998). This orange compound has many beneficial effects on human health (Granado et al. 1998). Furthermore, cultivation of marigold for one or more seasons effectively reduces the number of nematodes in soil and suppresses plant diseases caused by nematodes (Vasudevan et al. 1997). In addition, marigold has a potential application in phytoremediation of heavy metals, such as arsenic and cadmium (Chintakovid et al. 2008).

Despite the importance of marigold, successful genetic transformations of this species have been described only in a few reports. Vanegas et al. (2006) reported transformation of marigold by particle bombardment with DNA containing the *nptII* and *uidA* genes, resulting in a transformation efficiency

of 3%. Hibberd et al. (1998) and Kim et al. (2007) also reported successful transient and stable transformation of marigold by particle bombardment, respectively. In addition, Godoy-Hernández et al. (2006) reported *Agrobacterium*-mediated transient transformation of marigold. They used explants of four different tissues, such as leaf primordium and shoot tips, as recipients of *A. tumefaciens* and performed histochemical detection of  $\beta$ -glucuronidase (GUS) activity in explants, 3 days after inoculation with *Agrobacterium*. Recently, Gupta and Rahman (2015) reported stable *Agrobacterium*-mediated transformation of *T. erecta* and histochemical detection of GUS expression in transgenic leaves. All previous studies on transgenic marigold used different parts of seedlings and subsequent young plantlets, such as cotyledons and young leaves, as explants for *Agrobacterium* inoculation. Although these young starting materials are useful for efficient transformation, the genotypes of the resultant transgenic plants differ because they are mostly derived from different embryos.

In this study, we developed techniques for the *in vitro* regeneration of clonally propagated marigold and subsequent genetic transformation by *A. tumefaciens*. In

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Abbreviations: BAP, 6-benzyladenopurine; GUS,  $\beta$ -glucuronidase; IAA, indole-3-acetic acid; MS, Murashige and Skoog; *nptII*, gene for neomycin phosphotransferase; SIM, shoot-induction medium; *uidA*,  $\beta$ -glucuronidase gene; X-Gluc, 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide.

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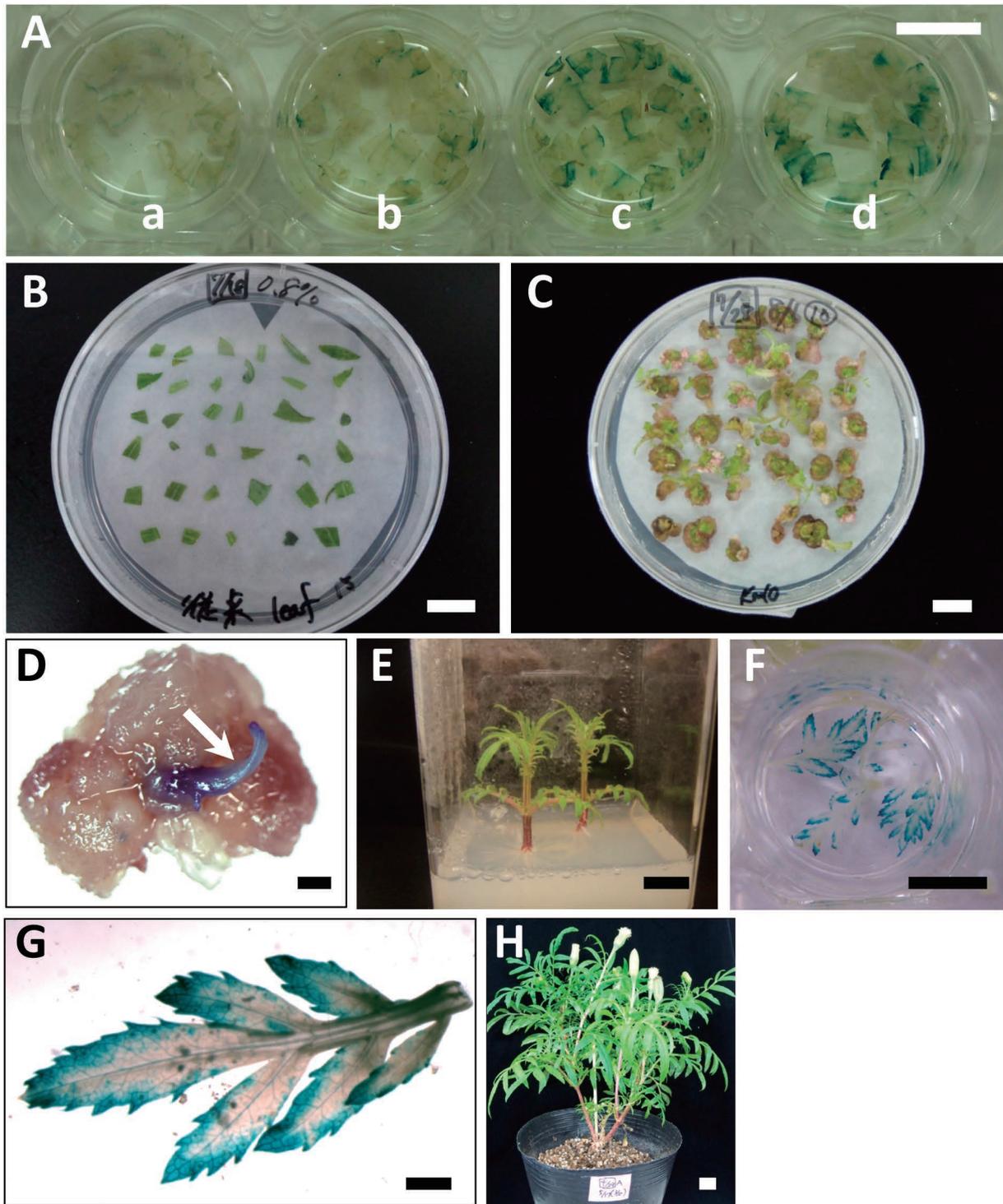


Figure 1. Phenotypic characteristics of transgenic marigold. A: Transient expression of *uidA* gene in *Agrobacterium*-inoculated leaf explants. Leaf explants in a suspension of *A. tumefaciens* cells were subjected to vacuum infiltration (b), vacuum infiltration following sonication for 1 min (c) and vacuum infiltration following sonication for 5 min (d). Untreated controls are marked as (a). Treatments were performed prior to co-cultivation of leaf explants and bacterial cells on MS medium and the assays were performed after co-cultivation for 2 days. B: Leaf explants that had been subjected to sonication for 5 min after 2-day co-cultivation on MS medium. Filter paper was placed on the MS medium to prevent vitrification of regenerated shoots. C: Adventitious shoots emerged from inoculated leaf explants subjected to sonication for 5 min after 1-week incubation on SIM without kanamycin and a subsequent 2-week incubation on SIM with kanamycin (10 mg/L). D: An adventitious shoot (arrow) with positive expression of *uidA* gene, regenerated from an inoculated leaf explant. E: Young transgenic plantlets after cultivation on MS medium supplemented with kanamycin (10 mg/L) and meropenem (12.5 mg/L). F: Assay for expression of *uidA* gene in young compound leaves from transgenic plantlet #1A. G: High-magnification view of one of the leaves shown in Figure 1F. H: Transgenic plantlet #1A with male-sterile flowers, after growth on soil. Bars=1 cm in A, B, C, E, F, and H; 1 mm in D and G.

this system, all transgenic plants had the same genotype, except for the introduced genes, which is, to some extent, advantageous for genetic studies of this species. In addition, the marigold line used in this study exhibited stable male sterility. Transgenic technology based on the male-sterile line could partially restrict the transgene flow via pollen to the natural environment and eliminate crosses with wild relatives.

The plantlets of male-sterile marigold (*T. erecta* vanilla, line #39-7) used in this study were established from shoot primordia of greenhouse-grown plants. This line was established as a derivative in the course of developing the new variety of marigold "REMEDIPEARL" from their parent variety (registration no. 20197 in the variety list of Ministry of Agriculture, Forestry and Fisheries of Japan). The plantlets of the line were easily propagated on solidified phytohormone-free Murashige-Shoog (MS) medium with 3% sucrose and 0.8% agar. We propagated the plantlets clonally from cuttings, under sterile

conditions *in vitro* in plastic box (60×60×100 mm). Each leaf was cut from the growing plants in the box and then further cut into two or three pieces as shown in Figure 1B. The resultant leaf explants were used as starting materials for tissue culture and *Agrobacterium*-mediated transformation. To determine the optimum hormonal conditions for the regeneration of shoots from the leaf explants, we incubated the leaf explants on MS medium containing indole-3-acetic acid (IAA) and 6-benzyladenopurine (BAP) at various concentrations (Table 1). The explants were incubated at 25°C with 16 h of light daily in a culture room or in growth chambers. At relatively high levels of IAA (above 1 mg/L) and BAP (above 3 mg/L), we observed the regeneration of adventitious shoots from leaf explants on the medium after 2 to 3 weeks of incubation. Regeneration efficiencies exceeded 70% under two sets of conditions: 1) IAA at 3 mg/L plus BAP at 3 mg/L and 2) IAA at 3 mg/L plus BAP at 5 mg/L. Therefore, we used the latter hormonal combination for shoot induction medium (SIM) in further experiments.

To obtain transgenic plants through *Agrobacterium*-mediated transformation, the *Agrobacteria* should be suppressed and eliminated via the addition of antibiotics to minimize their interference on the growth and regeneration of the transgenic tissues and organs. However,  $\beta$ -lactam antibiotics, the most commonly used antibiotics for this purpose, negatively affect plant regeneration (Ogawa and Mii 2005). In our preliminary experiment, carbenicillin, a typical antibiotic for the *Agrobacterium* elimination, inhibited apparently adventitious shoot formation in leaf explants (data not shown). Therefore, we used another  $\beta$ -lactam antibiotic, meropenem, for the *Agrobacterium* elimination. We simultaneously tested the bacterial growth and the shoot regeneration of leaf explants that were co-cultivated with the *Agrobacteria* on SIM (IAA at 3 mg/L plus BAP at 5 mg/L) with 4 different concentrations of meropenem. As indicated in Table 2, the bacterial growth was efficiently inhibited by the antibiotic even at a low concentration. In contrast, regeneration efficiency of adventitious shoots from *Agrobacterium*-inoculated leaf explants on the medium seems to depend on the meropenem concentration in the medium. The

Table 1. The combination of two phytohormones for induction of adventitious shoots from leaf explants.

BAP/IAA concentration (mg/L)	Leaf explants with regenerated adventitious shoots <sup>a</sup>
1/0.1	0
1/0.5	0
1/1	0
1/3	0
1/5	0
3/0.1	0
3/0.5	0
3/1	2
3/3	14
3/5	10
5/0.1	0
5/0.5	0
5/1	0
5/3	15
5/5	8
10/0.1	0
10/0.5	0
10/1	2
10/3	0
10/5	7

<sup>a</sup>Twenty leaf explant were used as the starting material under each condition. The number of leaf explants with adventitious shoot regeneration was counted after a 3-week cultivation period.

Table 2. Induction of adventitious shoot and inhibition of bacterial growth on the medium with different concentrations of meropenem.

Concentration of meropenem (mg/L)	Leaf explants with regenerated adventitious shoots <sup>a</sup>			Bacterial growth on the medium <sup>b</sup>		
	2 weeks	3 weeks	4 weeks	2 weeks	3 weeks	4 weeks
0	10	17	19	–	+	+
6.25	10	14	15	–	–	–
12.5	17	24	26	–	–	–
25.0	12	14	16	–	–	–

<sup>a</sup>Number of inoculated leaf explants from which shoots were regenerated after 2, 3 or 4 weeks of cultivation. Thirty leaf explants each were used for SIM with different concentrations of meropenem. The medium did not contain any antibiotics for selection of transgenic shoots. <sup>b</sup>Visible growth of *A. tumefaciens* LBA4404 on SIM after 2, 3 or 4 weeks of cultivation. +, Apparent bacterial growth; –, Inhibition of bacterial growth

highest efficiency of the regeneration was observed with 12.5 mg/L of the antibiotic. The adventitious shoot regeneration was observed in 87% of leaf explants tested (26 out of 30 explants) under this treatment. Thus, the specific concentration of meropenem (12.5 mg/L) was used for *Agrobacterium* elimination after the co-cultivation procedure.

To further screen for the successfully transformed plants, we wanted to determine the appropriate concentration of kanamycin to be used. Two independent experiments were performed at different concentrations of the antibiotic (Table 3). After the inoculation of *A. tumefaciens* strain LBA4404 harboring pIG121-Hm (Ohta et al. 1990), the leaf explants were cultured on SIM and then the regenerated shoots were stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-Gluc) for visualization of the transgenic cells (Jefferson et al. 1987). The male-sterile marigold used in our study is more sensitive to kanamycin than those used in previous studies (50–400 mg/L kanamycin has been used for selection in the previous studies; Gupta and Rahman 2015; Kim et al. 2007; Vanegas et al. 2006). In our case, most of the regenerated shoots showed necrosis after 3 to 4 weeks of cultivation on SIM even with low kanamycin concentration (20 mg/L). As indicated in Table 3, we failed to recover GUS-positive shoots in the first trial, but a few blue-stained shoots could be observed under the condition with 10 and 20 mg/L of kanamycin in the second trial. No GUS-positive shoots were obtained with less than 10 mg of kanamycin in the both trials. In view of these results, we chose the following condition of SIM for the selection of transgenic plants: MS basal salts with vitamins, 3 mg/L IAA, 5 mg/L BAP, 12.5 mg/L meropenem, and 10 mg/L kanamycin.

To increase the transformation efficiency, we used sonication treatment of leaf explants and subsequent vacuum infiltration of *Agrobacterium*. With the combination of these treatments, the GUS-positive cells were apparent over wide areas of the inoculated leaf explants two days following the *Agrobacterium* inoculation (c and d in Figure 1A), whereas such blue-stained cells were only observed around the cut edge

of each explant after the vacuum-infiltration treatment alone (b in Figure 1A). Therefore, in order to achieve efficient transformation, we used a 5-min sonication treatment of before vacuum infiltration of *Agrobacterium*.

To obtain stable transgenic plants, leaf explants were inoculated with *A. tumefaciens* LBA4404 that harbored pIG121-Hm, in two independent experiments (78 and 418 of the leaf explants were used in the first and second trials, respectively). Leaf explants for *Agrobacterium* inoculation were prepared from plants subcultured in hormone-free MS agar medium every three weeks. The leaf was cut into two or three pieces and then the explants were sonicated by an ultrasonic machine (Branson 1200, YAMATO SCIENTIFIC Co., Ltd., Tokyo) for 5 min in the hormone-free MS liquid medium. The resultant explants were transferred to an *Agrobacterium* suspension (O.D. 0.4 at 660 nm) and were then vacuumed for a few minutes in a plastic desiccator attached with an aspirator (EYELA A-3S, Tokyo Rikakikai Co., Ltd., Tokyo). Following the vacuum infiltration, the explants were left standing; this procedure, including the vacuum infiltration, was completed in 15 min. The excess amount of *Agrobacterium* suspension on the explants was removed on the filter papers and the inoculated leaf explants were transferred to a hormone-free MS medium. After 2 days of co-cultivation with *A. tumefaciens* in the dark at 28°C, these leaf explants were cultured for 1 week on the SIM without kanamycin and then were transferred to SIM containing kanamycin at 25°C. To avoid vitrification of the regenerated shoots, we placed filter paper on the medium, as shown in Figure 1B and 1C. After 3 weeks, we observed the regeneration of adventitious shoots from 50% of the first set of explants and from 52% of the second set, as shown in Figure 1C. As indicated in Figure 1D, some of the adventitious shoots were positive for the expression of *uidA* gene. After further cultivation on SIM with 10 mg/L kanamycin and 25 mg/L meropenem, normal shoots extended from 36% and 12% of the explants that showed shoot generation in the first and second experiments, respectively. Unfortunately, most of the regenerated shoots were difficult to root on

Table 3. Two independent trials for preliminary selection of transgenic shoots on the medium with different concentrations of kanamycin.

Trial	Kanamycin concentration (mg/L)	Leaf explants <sup>a</sup>	Leaf explants with regenerated adventitious shoots <sup>b</sup>	No. of shoot with GUS positive <sup>c</sup>
1	0	168	35	0
	5	168	14	0
	10	168	1	0
2	0	72	24	0
	5	108	28	0
	10	108	10	2
	20	72	3	1

<sup>a</sup>Number of leaf explants inoculated by *A. tumefaciens* LBA4404 harboring pIG121-Hm. <sup>b</sup>Number of leaf explants from which adventitious shoots were regenerated after a 4-week cultivation on SIM. The medium contains 12.5 mg/L of meropenem. <sup>c</sup>Number of adventitious shoot stained with blue color in X-Gluc solution.

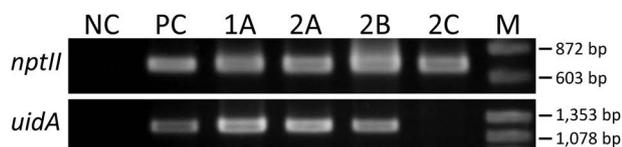


Figure 2. Detection of transgenes by PCR. After isolation of total DNA from leaves of each candidate transgenic plantlet, PCR was performed with this DNA as template and primers specific for the *nptII* and *uidA* genes. Binary vector pIG121-Hm was also used as template for a control experiment. M, DNA size marker (HaeIII-digested  $\phi$ X174); NC, wild-type plantlet; PC, pIG121-Hm; 1A, 2A, 2B, and 2C represent lines described in the text.

hormone-free MS medium supplemented with 10 mg/L of kanamycin. These results suggest that these unrooted shoots are escapes. Finally, we obtained one and three independent candidate plantlets for transgenic marigold from the first and second trials, respectively, which showed rooting on hormone-free MS medium with meropenem and kanamycin (Figure 1E). We designated these candidates #1A, #2A, #2B, and #2C, respectively.

To verify the presence of the *nptII* and *uidA* transgenes under the control of the nopaline synthase promoter and cauliflower mosaic virus 35S promoter, respectively, in the candidate plantlets, part of each gene was amplified by PCR with total DNA isolated from each candidate as described by Ogawa and Mii (2007). Figure 2 shows that amplified DNA corresponding to *nptII* (expected size, 700 bp) was detected in the analysis of all of the four candidates. By contrast, DNA corresponding to *uidA* (expected size, 1,203 bp) was detected in three candidates (#1A, #2A and #2B) but not in #2C. To confirm the expression of *uidA* in the transgenic plantlets #1A, #2A and #2B, we performed histochemical staining with X-Gluc as substrate, using leaves from each transgenic plant. In this analysis, we observed blue coloration derived from positive cells only in leaves from transgenic lines, #1A and #2A. In both cases, blue-colored areas were located along the edges of compound leaves (Figures 1F and 1G). Previously, Gupta and Rahman (2015) also reported the uneven distribution of staining in parts of transgenic leaves of marigold in GUS histochemical analysis.

We also detected weak staining in the roots of plantlet #1A, but not in stem tissue of the same plantlet (data not shown). Our results suggest that, in our transgenic marigold, the *uidA* gene might be expressed strongly in parts of leaves but not in the stems and roots. In future studies, we should identify a promoter that results in the

high expression in stems and roots to allow the practical application of transgenic marigolds in phytoremediation and production of useful compounds.

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### References

- Chintakovid W, Visoottiviset P, Khokiattiwong S, Lauengsophonkul S (2008) Potential of the hybrid marigolds for arsenic phytoremediation and income generation of remediators in Ron Phibun District, Thailand. *Chemosphere* 70: 1532–1537
- Godoy-Hernández G, Berzunza EA, Concha LC, de Lourdes Miranda-Ham M (2006) *Agrobacterium*-mediated transient transformation of marigold (*Tagetes erecta*). *Plant Cell Tissue Organ Cult* 84: 365–368
- Granado F, Olmedilla B, Gil-Martinez E, Blanco I (1998) Lutein ester in serum after lutein supplementation in human subjects. *Br J Nutr* 80: 445–449
- Gupta V, ur Rahman L (2015) An efficient plant regeneration and *Agrobacterium*-mediated genetic transformation of *Tagetes erecta*. *Protoplasma* 252: 1061–1070
- Hibberd JM, Linley PJ, Khan MS, Gray JC (1998) Transient expression of green fluorescent protein in various plastid types following microprojectile bombardment. *Plant J* 16: 627–632
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions- $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher-plants. *EMBO J* 6: 3901–3907
- Kim HJ, Hong SB, Mo SY, Kim DH (2007) Particle bombardment-mediated transformation and regeneration of marigold (*Tagetes erecta*). *Hort Environ Biotechnol* 48: 197–200
- Ogawa Y, Mii M (2005) Evaluation of 12  $\beta$ -lactam antibiotics for *Agrobacterium*-mediated transformation through in planta antibacterial activities and phytotoxicities. *Plant Cell Rep* 23: 736–743
- Ogawa Y, Mii M (2007) Meropenem and moxalactam: Novel  $\beta$ -lactam antibiotics for efficient *Agrobacterium*-mediated transformation. *Plant Sci* 172: 564–572
- Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a  $\beta$ -glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol* 31: 805–813
- Piccaglia R, Marotti M, Grandi S (1998) Lutein and lutein ester content in different types of *Tagetes patula* and *T. erecta*. *Ind Crops Prod* 8: 45–51
- Vanegas PE, Valdez-Morales M, Valverde ME, Cruz-Hernandez A, Paredes-Lopez O (2006) Particle bombardment, a method for gene transfer in marigold. *Plant Cell Tissue Organ Cult* 84: 359–363
- Vasudevan P, Kashyap S, Sharma S (1997) *Tagetes*: A multipurpose plant. *Bioresour Technol* 62: 29–35