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An integrated in planta infection and in vitro selection strategy for Agrobacterium-mediated transformation of *Tagetes erecta*

Strategi terintegrasi infeksi *in planta* dan seleksi *in vitro* dalam transformasi *Tagetes erecta* yang dimediasi Agrobacterium

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ABSTRACT
Marigold (*Tagetes erecta*) is an important ornamental plant valued for its diverse flower colors and morphological variation. Enhancing the genetic variability of this species can be achieved through biotechnological approaches, particularly genetic transformation mediated by *Agrobacterium tumefaciens*. This study aimed to evaluate an integrated transformation strategy that combines *in planta* infection with in vitro selection and regeneration in *T. erecta*. Gene transfer was initiated through *in planta* infection, followed by hygromycin-based selection and shoot regeneration under in vitro conditions. This approach resulted in a transformation efficiency of 20.93% and a regeneration efficiency of 2.32%, calculated based on the number of hygromycin-resistant explants and regenerated shoots relative to the total number of treated seeds. PCR-based molecular analysis detected the presence of the target gene fragment in putative transformants, suggesting that gene transfer events may have occurred during the transformation process. However, PCR detection alone cannot confirm stable genomic integration or inheritance of the introduced gene. Despite the relatively low regeneration efficiency, the integration of *in planta* transformation with in vitro selection demonstrates potential as a preliminary strategy for further optimization of transformation protocols in *T. erecta*, and may support the development of more practical transformation approaches in ornamental plant breeding.

ABSTRAK
Marigold (*Tagetes erecta*) merupakan tanaman hias penting yang memiliki nilai estetika tinggi karena keragaman warna dan morfologi bunganya. Peningkatan keragaman genetik tanaman ini dapat dilakukan melalui pendekatan bioteknologi, khususnya transformasi genetik yang dimediasi oleh *Agrobacterium tumefaciens*. Penelitian ini bertujuan mengevaluasi strategi transformasi terintegrasi yang mengombinasikan infeksi *in planta* dengan seleksi dan regenerasi *in vitro* pada *T. erecta*. Transformasi dilakukan melalui infeksi *in planta*, diikuti seleksi menggunakan higromisin serta regenerasi tunas pada kondisi *in vitro*. Pendekatan ini menghasilkan efisiensi transformasi sebesar 20.93% dan efisiensi regenerasi sebesar 2.32%, yang dihitung berdasarkan jumlah eksplan resisten higromisin dan tunas yang berhasil diregenerasikan dibandingkan dengan total benih yang diberi perlakuan. Analisis molekuler berbasis PCR mendeteksi keberadaan fragmen gen target pada transforman dugaan (*putative transformants*), yang menunjukkan bahwa peristiwa transfer gen kemungkinan terjadi selama proses transformasi. Namun demikian, deteksi PCR saja belum dapat memastikan integrasi genom yang stabil maupun pewarisan gen yang diperkenalkan. Meskipun efisiensi regenerasi masih relatif rendah, integrasi metode transformasi *in planta* dengan seleksi *in vitro* menunjukkan potensi sebagai pendekatan awal untuk pengembangan dan optimalisasi protokol transformasi pada *T. erecta*, serta dapat mendukung pengembangan strategi transformasi yang lebih praktis dalam pemuliaan tanaman hias.

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INTRODUCTION

Tagetes erecta L. is an ornamental plant widely cultivated for its diverse flower colors and morphological variations, making it valuable for landscaping, ornamental horticulture, and cultural ceremonies. The species originates from Mexico and Central America but is now widely cultivated in tropical and subtropical regions (Karjee, 2017). In Indonesia, marigold flowers are commonly used for decorative purposes, medicinal applications, and traditional rituals, particularly in Bali where the demand for flowers is reported to be substantial (Sanjawani, 2017; Beti, 2020). The economic and ornamental value of marigolds strongly depends on the development of cultivars with improved flower traits and adaptability. Therefore, breeding strategies aimed at enhancing genetic diversity are essential for improving ornamental plant quality (Anjelita et al., 2025).

Genetic improvement of *T. erecta* has been pursued through conventional breeding, mutation breeding, and modern biotechnological approaches. However, conventional breeding often encounters several constraints, including limited seed viability and the emergence of undesirable traits due to the random nature of hybridization (Zhang et al., 2019). Mutation breeding has also been explored to increase genetic variability; however, such approaches frequently generate untargeted genetic alterations and unpredictable phenotypic outcomes (Lenawaty et al., 2022). Consequently, plant biotechnology, particularly genetic transformation, has become an important strategy for introducing desirable traits into plant genomes in a more targeted manner (Bhuyan et al., 2023)

Among the available transformation methods, *Agrobacterium tumefaciens*-mediated transformation remains one of the most widely used gene delivery systems in plants. This bacterium naturally transfers T-DNA segments containing genes of interest into plant cells through a mechanism regulated by virulence (Vir) genes (Azizi-Dargahlou & Pouresmaeil, 2024). Despite its broad application, the production of transgenic plants generally requires tissue culture-based regeneration systems. In many plant species, regeneration through in vitro culture represents a major bottleneck due to genotype dependency and low regeneration efficiency (Moniruzzaman et al., 2021). In *T. erecta*, previous studies have also reported relatively low transformation efficiency, indicating that improvements in transformation protocols are still required (Narushima et al., 2017).

To address the limitations associated with tissue culture-dependent transformation systems, the in planta transformation approach has been proposed as an alternative method. This technique allows gene transfer directly into plant tissues without extensive tissue culture regeneration steps. Various target organs such as seeds, seedlings, shoots, buds, flowers, and fruits have been successfully used for in planta transformation in several plant species (Zhang et al., 2017; Mei et al., 2024). Nevertheless, the method requires efficient selection strategies to identify transformed cells. Antibiotic-based selection systems are commonly employed to eliminate non-transformed cells while allowing cells carrying antibiotic resistance genes to survive and regenerate (Ontiveros-Cisneros et al., 2022)

Integrating in planta infection with in vitro selection may combine the advantages of both transformation approaches. Such a strategy may reduce dependence on complex tissue culture procedures while maintaining the ability to selectively regenerate transformed tissues. Previous studies have demonstrated that combining transformation strategies can improve efficiency and reduce labor and operational costs in plant genetic engineering (Hanana et al., 2018; Karthik et al., 2018; Prem Kumar et al., 2021). However, the application of integrated in planta and in vitro transformation strategies in *T. erecta* remains limited, and optimization of transformation systems for this species is still required.

Therefore, this study aimed to evaluate an integrated transformation strategy combining in planta infection with in vitro selection and regeneration for *A. tumefaciens*-mediated transformation of *T. erecta*. This approach is expected to provide preliminary insights into developing a more practical and efficient transformation system for ornamental plant improvement.

MATERIALS & METHODS

Preparation of Agrobacterium tumefaciens

The *Agrobacterium tumefaciens* strain EHA105 carrying the T-DNA construct was used for transformation. The construct contained the gene of interest located within the right border (RB) region under the control of a transcriptional promoter and the nopaline synthase terminator (tNOS), while the hygromycin resistance gene (Hyg) was positioned within the left border (LB) region driven by the nopaline synthase promoter (pNOS).

The *A. tumefaciens* culture was maintained in yeast extract peptone (YEP) liquid medium and stored as glycerol stock at −80 °C. For bacterial activation, 50 μL of glycerol stock was inoculated into 2 mL of YEP medium supplemented with rifampicin (15 mg L^{-1}) and spectinomycin (50 mg L^{-1}), and incubated on a rotary shaker (120 rpm) at 28 °C for 24 h, following previously described protocols for *Agrobacterium* culture preparation (Amal et al., 2020).

The bacterial suspension was then streaked onto solid YEP agar plates containing the same antibiotics and incubated at 28 °C for three days. Colonies were used as templates for colony PCR to confirm the presence of the target construct. Confirmed colonies were subsequently inoculated into 20 mL of liquid YEP medium and cultured until the optical density reached OD_{600} ≈ 0.4. The bacterial cells were pelleted by centrifugation and resuspended in liquid Murashige and Skoog (MS) medium supplemented with 100 μM acetosyringone (Sigma-Aldrich, USA).

Hygromycin concentration toxicity assessment

Hygromycin was used as a selection agent to distinguish transformed from non-transformed tissues. Preliminary experiments were conducted to determine the optimal hygromycin concentration for selection using four levels (0, 10, 20, and 30 mg L^{-1}) following the procedure described by Sjahril et al. (2018). A total of 45 seedling explants were evaluated for each concentration treatment. The explants were cultured on MS medium supplemented with hygromycin under controlled light conditions for four weeks. The observed variables included the time to explant mortality, the percentage of dead explants, and explant coloration recorded weekly. Hygromycin B (Sigma-Aldrich, USA) was used as the selection antibiotic.

In planta transformation of T. erecta seeds

In planta transformation of *T. erecta* seeds was performed using a modified protocol based on Hanana et al. (2018). Seeds were surface-sterilized using 10 % sodium hypochlorite for 10 min followed by 5 % sodium hypochlorite for 5 min and rinsed several times with sterile distilled water before being placed on Murashige and Skoog (MS) medium. Germinated seeds with emerging radicle and plumule were used for transformation.

The seeds were immersed in an *A. tumefaciens* suspension containing 100 μM acetosyringone for 60 min. Three independent biological replicates were performed, each consisting of 14–15 seeds. Prior to inoculation, the seed surface and radicle were gently punctured using a sterile needle to facilitate bacterial infection. The inoculated seeds were incubated in darkness at 28 °C for three days for co-cultivation.

Selection and regeneration

After co-cultivation, the seeds were washed five times with 200 mg L^{-1} cefotaxime (Duchefa Biochemie, Netherlands) alternating with sterile water to eliminate residual bacteria. The seedlings were transferred to MS selection medium containing hygromycin (20 mg L^{-1}) and timentin (50 mg L^{-1}) (Duchefa Biochemie, Netherlands). Cultures were maintained under a 16 h photoperiod for 21 days with weekly subculturing. Regenerated plantlets showing green cotyledons and active apical shoots were transferred to ex vitro conditions. Prior to transplantation, the seedlings were rinsed twice with a 200 mg L^{-1} cefotaxime antibiotic solution and subsequently washed with sterile water before being planted in a medium composed of an equal mixture of soil and rice husk charcoal (1:1).

In the experiment, the transformation efficiency and regeneration efficiency were assessed by calculations.

$$\text{Transformation efficiency (\%)} = \frac{\textit{Number of putative transformants}}{\textit{Total number of treated plants}} \times 100\% \quad (1)$$

Identification of genetically modified plants

Plants that survived hygromycin selection and exhibited normal shoot development were considered putative transformants prior to molecular confirmation. The identification of putative transformant plants was performed to verify the presence of the target transgene using PCR-based molecular analysis. Genomic DNA was extracted from leaf tissues of presumed transformants using a modified CTAB method based on Doyle and Doyle (1987). PCR amplification was carried out to detect the presence of the *InMYB-CCD4a* gene. The primer sequences used were as follows: forward primer (F) 5′GCCATCAAATTTGCAACCAA3′ and reverse primer (R) 5′CGGCAACAGGATTCAATCTT3′. The primers were designed to amplify a 1252 bp fragment of the target gene. Each PCR reaction was performed in a total volume of 10 µl containing 1 µl of genomic DNA template, 5 µl of 2× PCR master mix (Promega, USA), 0.25 µl of forward primer (10 µM), 0.25 µl of reverse primer (10 µM), and 3.5 µl of ddH_2O.

The amplification program consisted of an initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; with a final extension at 72°C for 7 min. PCR products were separated on a 1% agarose gel prepared in 1× sodium borate buffer and stained with GelRed (Biotium, USA). Electrophoresis was conducted at 100 V for 28–30 min. The amplified fragments were visualized under UV illumination and compared with a 1 kb DNA ladder (Geneaid, Taiwan). Amplification was performed using a thermal cycler (Bio-Rad T100, USA). Plasmid DNA containing the target gene was used as a positive control, while genomic DNA from wild-type marigold served as a negative control.

RESULTS & DISCUSSION

Transformation of *Tagetes erecta* was performed using an integrated approach combining in planta seed infection with subsequent in vitro selection and regeneration. Seeds were inoculated in planta with *Agrobacterium tumefaciens* and subsequently subjected to antibiotic selection using tissue culture media.

The results of seed transformation using *Agrobacterium* inoculation showed a transformation efficiency of 20.93%, calculated from the number of hygromycin-resistant explants relative to the total number of treated seeds. Seed wounding through the pricking procedure was applied to facilitate bacterial infection during inoculation and co-cultivation. This treatment likely enhanced *Agrobacterium* infection and facilitated T-DNA transfer into the meristematic tissues of actively developing seedlings (Figure 1). The addition of acetosyringone to the infection and co-cultivation media induced the expression of virulence (*vir*) genes, which are essential for T-DNA transfer from *Agrobacterium* to plant cells (Gelvin, 2021). Transformation efficiency was influenced by the duration of bacterial infection. Infection periods that were too short or excessively prolonged reduced transformation efficiency (Sisharmini et al. 2018).

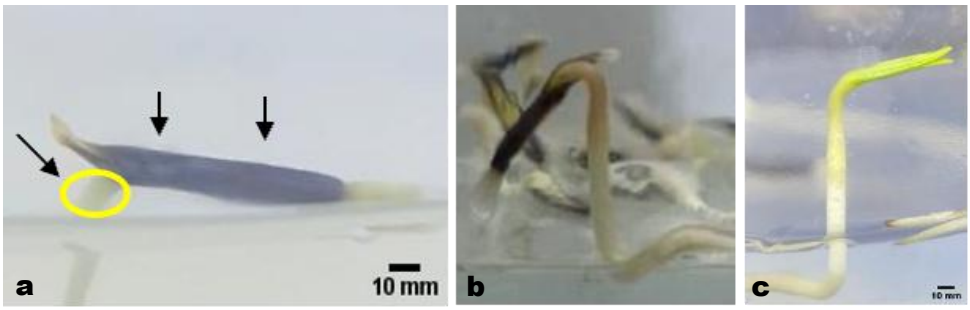

Figure 1. Overview of seed preparation and selection process: (a) preparation of seeds, (b) seedlings on co-cultivation media, (c) seedlings on selection media, ↓ = position of injured seed

The co-cultivation phase and the pathogenicity of the infecting genes contributed to the efficacy of the transformation. The duration of explant co-cultivation is highly prone to overgrowth by *A. tumefaciens*, which can impact explant development. Karthik et al. (2018) asserted that the optimal co-cultivation duration is two days in darkness at 28°C.

Assessing the susceptibility of explants to the antibiotic hygromycin evaluates the efficacy of eradicating non-transformed plant cells and cultivating cells that are successfully gene-integrated. The fatal dose of the selection agent may differ among plant species and genotypes (Sisharmini et al. 2018). The test results indicated a reduction in the proportion of viable explants in non-transgenic samples cultivated in media augmented with the antibiotic hygromycin. This data is illustrated in Table 1.

Table 1. Proportion of seedling mortality

Explant	Hygromycin (mg L^{-1})	Weekly percentage of viable explants (%)				Hygromycin-resistant explants (seedling)
		7	14	21	28	
Seed	0	100	100	0.0	100	0
	10	100	100	46.0	0.0	0
	20	100	25.58	18.60	18.60	8
	30	100	4.65	2.33	2.33	1

Based on these observations, 20 mg L^{-1} hygromycin was selected as the optimal concentration for transformation experiments because it effectively suppressed non-transformed explants while allowing the recovery of hygromycin-resistant seedlings. The treated seeds cultured in MS medium supplemented with hygromycin at concentrations of 10 mg L^{-1}, 20 mg L^{-1}, and 30 mg L^{-1}, exhibited decreased survival, as seen by browning of the seedling and absence of growth (Figure 2). Seedling survival markedly decreased after 21 days of culture in media containing 20 mg L^{-1} and 30 mg L^{-1} hygromycin. Hygromycin at 10 mg L^{-1} inhibited non-transformed cell growth, although the selection effect occurred more slowly. Hygromycin functions as a selection agent by inhibiting the growth of non-transformed plant cells rather than eliminating Agrobacterium contamination.

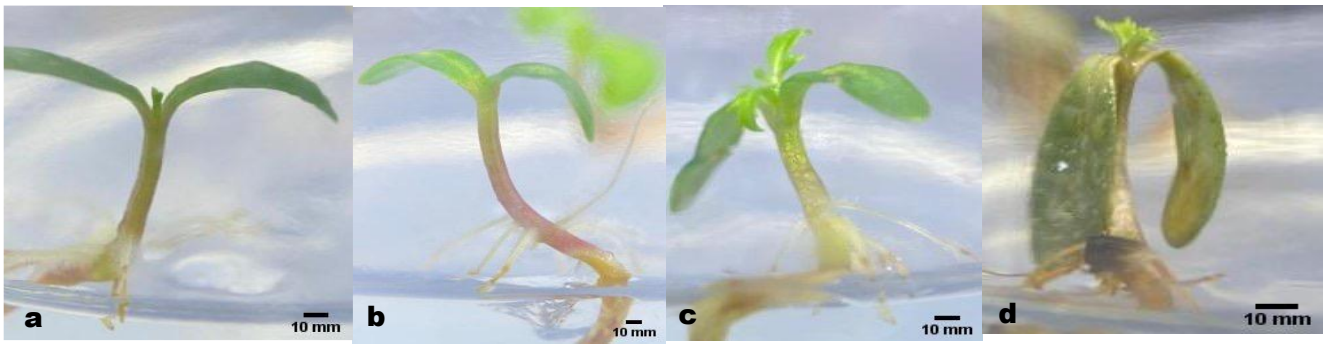

Figure 2. Response seedlings on selection media. (a) seedlings aged 1 month after culture; (b) seedlings 2 weeks after planting; (c) seedlings 3 months old resistant to hygromycin; (d) seedlings aged 3 months not resistant to hygromycin.

In induction media devoid of hygromycin, plant explants demonstrated typical shoot development. Cells deficient in the *Hpt* gene, responsible for conferring resistance to hygromycin, were incapable of proliferating on the selective media. The *Hpt* gene incorporated into plant cells encodes amino phosphotransferase molecules that disrupt the structure and inhibit the activity of the antibiotic hygromycin in the growth media (Patra et al., 2021). The application of hygromycin at concentrations of 20-50 mg L^{-1} as a selection agent in various plant species did not impact the regeneration and fertility of transgenic plants (Sjahril et al. 2018). The treated seeds response to 20 mg L^{-1} hygromycin was adequate to select plant

cells lacking the gene for hygromycin antibiotic resistance. Hygromycin-resistant plants exhibited vigorous seedling morphology, green cotyledons, the development of apical shoots and were devoid of *Agrobacterium* infection (Figure 2c).

Seed transformation in planta was performed to accelerate the recovery and regeneration of transgenic cells. To mitigate the risk of *Agrobacterium* contaminating the environment, we executed the transformation by integrating in vitro and ex vitro techniques, which ensured that the selection and eradication of *A. tumefaciens* were maintained. Furthermore, prior to the exvitro transplantation of the hygromycin-resistant seedlings, they were subjected to three washes with the antibiotic cefotaxime at a concentration of 200 mg L^{-1} to ensure the absence of *Agrobacterium* in the seedlings designated for transplantation. The results of transforming 43 seeds in planta showed a regeneration efficiency of 2.32% based on the number of successfully acclimatized shoots relative to the total treated seeds (Table 2). Although the regeneration efficiency was relatively low (2.32%), similar limitations in regeneration frequency have been reported in previous transformation studies of *Tagetes* species and other ornamental plants (Narushima et al., 2017; Wang et al., 2025).

Table 2. Efficiency transformation of seed *T. erecta* through *A. tumefaciens*

Method	[A]	[B]	Transformation Efficiency (%) [B/A]	[D]	Regeneration efficiency (%) [D/A]
In planta	43	9	20.93	1	2.32

Note: [A] number of treated seeds; [B] hygromycin-resistant explant; [D] regenerated shoots.

Figure 3. Transformation in planta on seeds: (a) antibiotic resistant seedlings post-transformation, (b) transplanted 21 days in antibiotic medium, (c) non-transgenic seedlings, (d) non-transformed plantlet (after six weeks), (e) four weeks post acclimatization, (f) putative transformant plantlet. 1;2;3 = sample DNA isolation

Nine seedlings cultivated on the selection medium exhibited normal growth and satisfied the criteria for ex vitro transplanting. The low regeneration rate observed under ex vitro conditions may be attributed to environmental differences between in vitro and ex vitro environments, which can reduce plantlet survival during acclimatization.

Transplanting in vitro explants to ex-vitro conditions requires a hardening phase to improve the vigour and readiness of the plantlets for adapting to the natural environment. Moreover, in vitro explants undergo significant physiological stress upon transition to field conditions (Grzelak et al., 2024). The seedlings remain in the recovery phase following bacterial inoculation and selection on an antibiotic medium. The injury applied to the seed surface likely facilitated bacterial access to meristematic tissues, thereby increasing the probability of T-DNA delivery during early seedling development. Seedlings that failed to develop new organs gradually ceased growth and eventually died (Figure 2d).

Molecular analysis was performed on putative transformant plants grown after six weeks post-acclimatization. DNA isolation was performed on leaves from different plant leaf positions (Figure 3f). PCR amplification detected the expected 1252 bp fragment of the target gene in one of the analyzed samples, indicating the presence of the introduced construct (Figure 4). This result indicates that the presence of the target gene fragment was detected only in a limited portion of the analyzed plant tissue. Similar observations have been reported in transformation systems where gene transfer occurs in somatic tissues, resulting in uneven detection of the target gene among regenerated plant organs (Prem Kumar et al., 2021). However, PCR detection only confirms the presence of the target gene fragment and does not necessarily indicate stable genomic integration or heritable transformation events. Additional molecular analyses would be required to further verify integration stability and inheritance of the transgene.

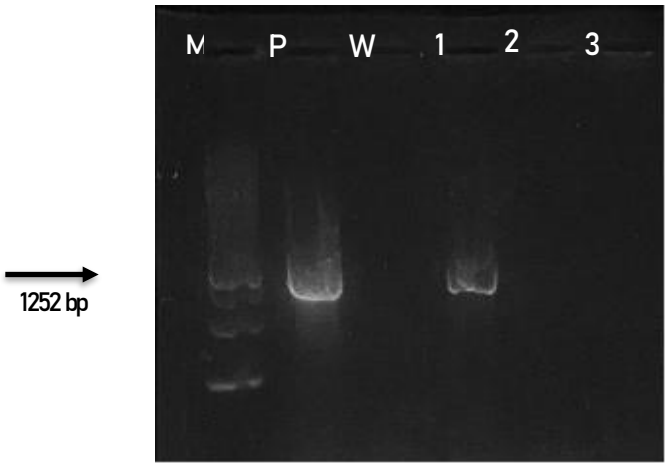

Figure 4. PCR amplification of the target gene fragment from putative transformant plants after acclimatization. M: 1 kb DNA ladder; P: plasmid positive control; WT: wild-type plant; T1–T3: putative transformant samples; arrow indicates the expected 1252 bp fragment.

Despite these limitations, the integrated in planta infection and in vitro selection strategy demonstrated in this study provides a practical framework for developing simplified transformation systems in *T. erecta* and other ornamental species. Putatively transformed tissues can be further propagated through embryogenesis or organogenesis under in vitro conditions to increase the likelihood of recovering more uniform transgenic lines.

CONCLUSIONS

This study demonstrates the feasibility of an integrated transformation strategy combining in planta seed infection with in vitro antibiotic selection for genetic transformation of marigold (*T. erecta*). The approach produced a transformation efficiency of 20.93% and a regeneration efficiency of 2.32% based on hygromycin-resistant explants and successfully acclimatized shoots. PCR analysis detected the target gene fragment in putative transformants, although PCR detection alone cannot confirm stable genomic integration or inheritance of the introduced gene. Therefore, additional molecular analyses are required to further verify the stability of transgene integration. Despite the relatively low regeneration efficiency, the integrated in planta and in vitro approach provides a practical basis for further optimization of

transformation systems in ornamental plant breeding. This strategy may facilitate the development of efficient transformation systems for genetic improvement of ornamental plants

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