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INDIRECT SHOOTS ORGANOGENESIS AND AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF *InMYB1-CCD4A* GENE IN MARIGOLD (*TAGETES ERECTA* L.)

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SUMMARY

The marigold (*Tagetes erecta* L.) is an ornamental plant with high economic value. The generation of the white flower marigold phenotype could proceed through genetic transformation with the *InMYB1-CCD4a* gene. This study aimed to evaluate a) the regeneration of marigold leaf through indirect organogenesis, b) the transformation of callus with the *InMYB1-CCD4a* gene, and c) analyze the *InMYB1-CCD4a* gene presence in transgenic plantlets. Testing of shoot formation from callus continued in MS medium containing indole-3-acetic acid (IAA, 0.5 mg L^{-1}) with the addition of different levels of Thidiazuron-TDZ (0.25, 0.5, 0.75, and 1 mg L^{-1}), and 6-Benzylaminopurine, or BAP (2.5, 5, and 7 mg L^{-1}). BAP (7 mgL^{-1}) produced the highest number of shoots (six shoots per explant). In genetic transformation with *A. tumefaciens* harboring *InMYB1-CCD4a*, the explants entailed pre-culturing in a bacterial suspension for one hour, co-cultivating in MS medium containing acetosyringone for 2–3 days, washing, and eliminating the Agrobacterium. Afterward, transferring explants to resting media ensued before selecting transgenic callus in the medium containing hygromycin (20 mg L^{-1}). The results showed the PCR analysis confirmed six plants regenerated from 24 calluses passed antibiotic selection (2.5%) and harbored the *InMYB1*-CCD4a gene.

Keywords: Marigold (*T. erecta* L.), 6**-**Benzylaminopurine (BAP), callus, cytokinins, hygromycin, leaf explants, Thidiazuron (TDZ), transformation efficiency

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Key findings: In marigold (*T. erecta* L.), the optimum BAP concentration (7 mg L^{-1}) medium for indirect shoot organogenesis through callus of marigold leaf explants produced the highest number of shoots with shooting explant (100%) and rooting explant (11.1%). Genetic transformation protocol by using Agrobacterium was successful in establishing with the 4% of transformation efficiency at the antibiotic selection and 1% at the PCR confirmation.

INTRODUCTION

The marigold (*Tagetes erecta* L.), locally known as 'gemitir,' 'kenikir,' 'randakencana,' and 'tahikotok,' originates from Central America, specifically from Mexico (Kumar *et al.,* 2019). The marigold plant has long been utilized for various purposes, such as garden bed plants, potted ornamental plants, and as refugia plants to prevent pests on various crops and vegetables. In Bali, Indonesia, marigold flowers are ornamental plants that have the highest economic value because of their use as the main flower in religious ceremonies and daily offerings. The marigold flower business in Bali, Indonesia, is growing, with an estimated USD 6 to 12 million per year on a flower requirement of eight tons per day (Isnawati *et al*., 2023).

Regeneration and propagation of *Tagetes* spp. through tissue culture is essential for plant genetic transformation, in vitro mutations, and induction of somaclonal variations to improve plant genetic diversity. In vitro cultures are the suitable solution to meet expectations using callus culture, somatic embryogenesis, protoplast culture, and organogenesis (Mehbub *et al*., 2022). An organogenesis process is forming organs, such as shoots and roots, directly through callus formation or not. In the media, treatment with plant growth regulators is also necessary for tissue formation capable of developing callus and regenerating in creating plant organs (Shin *et al*., 2020).

The marigold plant commonly cultivated in Indonesia is *T. erecta,* which has vanilla white, orange, and yellow flower color variants. In marigold plants, three main groups of pigments include betalains, flavonoids, and carotenoids (Liu *et al.,* 2019). Carotenoids are the most widely distributed pigments in plants, including marigolds, ranging from yellow and orange to dark red. However, until now, no pure white marigold flower exists; therefore, the genetic transformation with genes that can degrade carotenoids in flowers has the potential to produce white marigolds.

Genetic transformation has reached wide application to plants, both directly and indirectly (Su *et al.,* 2023). Direct genetic transformation does not require the vectors to insert target genes into plants; however, it may cause chaotic DNA integration events (Anami *et al.,* 2013) and plant tissue damage, rendering an inefficient regeneration (Demirer *et al.,* 2019). The indirect transformation method uses an intermediary vector such as *Agrobacterium tumefaciens*. The *A. tumefaciens* is a gram-negative bacterium that contains a Ti-plasmid comprising genes encoding virulence factors and T-DNA. Plant transformation with *Agrobacterium* has the advantage because it does not require special equipment and can take place with simple laboratory equipment. A single gene insertion has a high probability compared with direct transformation; however, it has its limitations with species genotype and explant type (Altpeter *et al.,* 2016).

Genetic engineering can be applicable to modify flower color by introducing the carotenoid cleavage dioxygenase 4a (*CCD4a*) gene, which can control the accumulation of carotenoids in flower petals by degrading carotenoids into colorless apocarotenoid compounds combined with the *InMYB1* promoter in flowers. The occurrence of carotenoid degradation in flower-specific promoters is capable of accumulating carotenoids, which only occur in flower tissue through regulation of carotenoid genes. Several past studies have shown that *CmCCD4a*, especially expressed in yellow flowers, can produce white flowers due to carotenoid degradation, as observed in *Chrysanthemums* (Yoshioka *et al*., 2012; Imai *et al*., 2013).

Carotenoids are an important pigment in leaves, involved in photosynthesis and photoprotection of chlorophyll (Maoka, 2020). Carotenoid degradation in leaves and other plant parts may cause physiological defects in plants. In the genetic transformation approach, using a specific promoter in gene construction can regulate the gene expression in the target organ. The petal-specific promoter has been prevalent in some plants, such as *Ipomea nil* MYB (*InMYB*) (Azuma *et al.,* 2016). It may help in the petal-specific expression of *Ccd4a* genes to degrade carotenoids in petals and produce petals with white color. The consequent research aimed to develop a regeneration and genetic transformation protocol on leaf explants of the marigold cultivar 'Sudamala Oranye-1.'

MATERIALS AND METHODS

Plant material and gene construction

This research on marigold (*T. erecta* L.) plant transpired in the tissue culture laboratory of the IPB University, Bogor, Indonesia. The research material used was leaf explants of *T. erecta* var. Sudamala Oranye-1. The *A. tumefaciens* strain EHA 105 harboring the *InMYB1-CCD4a* gene came from Ming-Tsair Chan's Lab, Academia Sinica Biotechnology Center, Southern Taiwan.

Organogenesis regeneration response of leaf explants to cytokinins

The experimental stages began with planting *T. erecta* leaf explants from in vitro plantlets in organogenesis induction media. The composition of each induction media using Murashige and Skoog (MS) basic media had the addition of the Thidiazuron (TDZ) hormone (0.25, 0.5, 0.75, and 1 mg L^{-1}, respectively) in the first experiment, and 6-Benzylaminopurine (BAP) hormone (2.5, 5, and 7 mg L^{-1}) in the second experiment. In both experiments, the media received the Indole Acetic Acid (IAA) 0.5 mg L^{-1}. Organogenesis induction continued for four weeks under full light conditions with variables observed as shoot percentage, root percentage, and the number of shoots and the number of roots formed. Data compilation for each experiment used Minitab at the 5% test level, and further comparison continued with the least significant difference ($LSD_{0.05}$) test.

Explant preparation for transformation

Obtaining explants of leaves came from in vitro-grown shoots. The in vitro shoots resulted from apical shoots originating from seed germination, routinely propagated in the laboratory. The leaf explants incurred excision into 1 cm segments before pre-culturing in the MSO medium for 24 hours before transformation. The leaves selected from the in vitro shoots were those that had fully developed and reached their maximum size.

Agrobacterium tumefaciens preparation

The *A. tumefaciens* strain EHA 105 culture harboring the *InMYB1-CCD4a* construct underwent culturing in 50 µl of liquid yeast extract peptone (YEP) medium, supplemented with spectinomycin 50 mg L^{-1} and 15 mg L^{-1} rifampicin antibiotics. Shaking the bacterial culture was at 130 rpm in the dark with a temperature of 28 °C for 24 hours. Then, taking the bacteria used a loop needle and streaked on solid AB media with the addition of spectinomycin 50 mg L^{-1} and 15 mg L^{-1} rifampicin antibiotics. Then, its storage in the dark had a temperature of 28 °C for three to four days before being transferred to a liquid MSO, with the optical density (OD) optimized until the density bacterial population was equivalent to OD = 0.3. After the bacteria reached an OD of 0.3, adding 150 mg L^{-1} acetosyringone ensued, and leaving it again for one hour in the dark.

Agrobacterium tumefaciens-mediated transformation and transformant selection

The infection stage commenced by immersing the pre-cultured explants in Agrobacterial suspension for an hour. The *Agrobacterium* suspension received an adjustment with the optical density (OD_{600}) of 0.3–0.4. Next,

planting the explants proceeded in co-cultivation media (MS and acetosyringone 100 mg L^{-1}) for 3 days in the dark. The co-cultivated tissue then incurred washing using sterile distilled water for 5 min three times and using the cefotaxime antibiotic 100 mg L^{-1} to eliminate bacteria for 15 min twice. The transformed explants succeeded in their transfer to resting media (IAA 0.5 mg L^{-1} + BAP 7 mg L^{-1}) with the addition of the timentin antibiotic 100 mg L^{-1} in full light conditions for three weeks. Afterward, their transfer to antibiotic selection media (hygromycin 20 mg L^{-1}) ensued every 10 days for three times. Observations continued on the number of live and regenerated calluses. Transformation and regeneration efficiency entailed calculations using the following formula:

$$\text{Transformation efficiency (\%)} = \frac{\textit{Number of hygromycin resistant callus}}{\textit{number of explants infected with A.tumefaciens}} \; x\; 100\%$$

$$\text{Regeneration efficiency} = \frac{\textit{Number of regenerant plantlets (T0)}}{\textit{number of hygromycin-resistant callus}} \; x\; 100\%$$

DNA extraction for PCR confirmation

Total DNA extraction took place from plantlets that passed antibiotic selection. A shoot sample of 100 mg bore grinding using a mortar and received 700 µL of cetyl trimethyl ammonium bromide (CTAB), polyvinyl pyrrolidone (PVP), and 50 µL of mercapto-ethanol. Then, the sample, upon being transferred to a 2 ml tube and vortexed, proceeded with the tube containing the sample to incubation in a water bath at 65 °C for 30 min (turned back and forth every 10 min). Afterward, the sample sustained centrifugation at 8,500 rpm for 15 min before taking the upper phase. Then, adding chloroform isoamyl alcohol (CIA) was according to the volume of the upper phase, being centrifuged again at 8,500 rpm for 15 min. Taking again the upper phase, the addition of isopropanol had the amount of 0.8 × the amount of the upper phase. The samples underwent inversion and incubation in the freezer for three hours. After three hours, its centrifugation again at 8,500 rpm occurred for 15 min, discarding the supernatant in the tube and leaving the pellet at the bottom of the tube. The pellet's washing used 300 µL of 70% ethanol, turning it back and forth. The sample centrifugation happened again at the speed of 8,500 rpm for 15 min. Afterward, discarding the supernatant preceded the pellet obtained for air-drying. The pellet then entailed dissolving using TE 1 x as much as 100 µL before storing in the -20 °C freezer until the use of the DNA.

Gene amplification by PCR and visualization by electrophoresis

In PCR amplification, the specific primers used for the target gene had each reaction set as follows: pre-start at 95 °C for 10 min, denaturation at 95 °C for 3 s, annealing at 50 °C for 30 s, and synthesis at 72 °C for 1 min before stopping at 72 °C for 5 min. The amount of each reaction mixture was 10 µL consisting of 5 µL Master Mix, 0.5 µL (0.25 µL) of each specific primer for the *InMYB1-CCD4a* F gene: 5'- GCC ATC AAA TTT GCA ACC AA -3' (20 bases) and R: 5'- CGG CAA CAG GAT TCA ATC TT -3' (26 bases), 1 µL DNA, and 3.5 µL ddH_2O, with the reaction run for 33 cycles using a PCR machine. The PCR amplification results entailed electrophoresis on a 0.8% agarose gel for 70 min at 50 V in 1 x TAE buffer. Then the results on the gel succeeded in their documentation using a UV transilluminator.

RESULTS AND DISCUSSION

Indirect shoot organogenesis response to the TDZ hormone

Shoot organogenesis on leaf explants in vitro is de novo organogenesis due to the absence of a shoot meristem on this explant. De novo regeneration of shoots involved two steps, such as a) induction of cell division and callus formation, which need auxin-rich callus-inducing medium, and b) tissue differentiation to grow shoots and roots. These processes require a different ratio of auxin and cytokinin

(Sugimoto *et al.,* 2010). Plant cell cycle regulation and other developmental processes had the general management of cytokinins (Schmulling, 2013). Thidiazuron (TDZ) hormone has reached wide usage *in vitro* to induce shoot formation. It encourages shoot proliferation and other propagation processes. The TDZ is a synthetic cytokinin used as a plant growth regulator. It is potentially more effective in micropropagation, somatic embryogenesis, callus induction, and shoot organogenesis than other cytokinins (Deepa *et al.,* 2018). However, the optimal TDZ concentration can gain influences from the plant species and its genetic makeup, as well as the type of explant used.

In this study, in the first experiment, the entire medium for callus induction and shoot regeneration contained 0.5 mg L^{-1} IAA and different levels of TDZ concentrations (0.25, 0.50, 0.75, and 1.00 mg L^{-1}). The results showed the addition of the TDZ hormone caused a decrease in the percentage of the number of shoots per explant of the marigold (*T. erecta* L.) (Table 1). The explant in the media containing the lowest concentration of TDZ (0.25 mg L^{-1}) produced the most shoots, averaging four shoots per explant with a percentage of 73.3%. Meanwhile, the higher the TDZ concentrations (0.50, 0.75, and 1.00 mg L^{-1}) only produced three, two, and one shoots, respectively, while the shoot formation percentage lowered to 53.3%, 46.7%, and 26.7%, respectively. The results detailed that TDZ proved more effective at a lower concentration for shoot formation in marigold explants. A report of a similar result came from Mehraj *et al.* (2024), who showed TDZ was more effective at lower concentrations and below 0.5 mg L^{-1} on marigold plants' tissue culture. Besides, the higher TDZ concentration delayed the time of shoot formation. Wang *et al*. (2020) also reported the milestone yellow marigold obtained the best medium in the combination of MS medium supplemented with TDZ (0.2 mg L^{-1}) and IBA (0.5 mg L^{-1}), which resulted in 70% of plant regeneration.

The TDZ showed a considerable pressure in root formation on the explants (Table 1). The outcomes revealed only 11.1% of explants produced roots at the lowest concentration of TDZ (0.25 mg L^{-1}), while increasing TDZ concentrations decreased the percentage of explants forming roots and root number. The TDZ at the concentrations of 0.5, 0.75, and 1.00 mg L^{-1} inhibited root development on explants, with even no root forming on the explants. Thus, TDZ is a good hormone for suppressing root growth. These findings aligned with several studies conducted by Guo *et al.* (2011). They noted that TDZ was the most active cytokinin for sprouting; however, this hormone can also inhibit the root system to some extent. High concentrations of TDZ reduced the number of roots. Although TDZ emerged as more effective than the adenine type of cytokinin in inducing adventitious shoots (Nowakowska *et al.,* 2019). Unfortunately, a high TDZ concentration produced abnormal leaf shapes, short and compact shoots, and inhibited shoot elongation, causing deformation and shoot hyperhydricity (Deepa *et al.*, 2018). It implies that TDZ can induce morphological abnormalities of shoots.

Table 1. Effect of single factor TDZ hormone on marigold organogenesis.

Levels	Explants forming shoots (%)	Explants forming roots (%)	Shoot number	Root number
0.25 mg L^{-1}	73.3a	11.1	4.0a	0.6
0.50 mg L^{-1}	53.3ab	8.9	3.0ab	0.4
0.75 mg L^{-1}	46.7ab	0.0	2.0ab	0.0
1.00 mg L^{-1}	26.7b	6.7	1.0b	0.3
F test	*	ns	*	ns

The numbers followed by the same letters in the same column were not significantly different based on the BNJ test with α = 0.05. * = significant difference; ns = the data is not significantly different.

Table 2. Effect of single factor BAP hormone on marigold organogenesis.

Levels	Explants forming shoots (%)	Explants forming roots (%)	Shoot number	Root number
2.5 mg L^{-1}	77.8b	26.7a	4.0b	3.0a
5.0 mg L^{-1}	77.8b	31.3a	4.0b	6.0a
7.0 mg L^{-1}	89.0a	11.1a	6.0a	1.0a
F Test	*	ns	**	ns

The numbers followed by the same letters in the same column were not significantly different based on the BNJ test with α = 0.05. ns = the data is not significantly different.

Indirect shoot organogenesis response to the BAP hormone

The leaf explants' response in different concentrations of BAP appears in Table 2. The highest percentage of explant forming shoots (89.0%) resulted in the medium containing higher concentration of BAP (7.0 mg L^{-1}). It showed significantly different with the medium with lower concentrations of BAP (2.5 and 5.0 mg L^{-1}) (77.8%). The BAP concentration level considerably affected the shoot number. However, it did not substantially altered the percentage of explant forming roots. The highest shoot number (6.0 shoots) was evident in BAP at 7.0 mg L^{-1}, while BAP lower concentrations (2.5 and 5.0 mg L^{-1}) only produced four shoots. The results also revealed the root number had no significant variation among the three concentration levels of BAP, highlighting the light-mediated inhibitory role of BAP in root initiation. Kumar *et al*. (2019) reported the medium supplemented with BAP (2.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) was effective for direct shoot regeneration from leaf explants of French marigold, with a high regeneration efficiency of 89.7%. For African marigold, the medium supplemented with BAP (0.5 mg L^{-1}) and NAA (0.25 mg L^{-1}) produced the maximum regeneration rate of 66.66%. BAP is a synthetic cytokinin, included in the adenine type of cytokinin. It has a wide usage in in vitro plant shoot propagation. It considerably increases cell division and enlargement in tissue culture cells, which can stimulate the growth and development of shoots (Arafah *et al.,* 2021).

Cytokinins, including BAP, in plant tissue culture medium undergo absorption through passive diffusion across the cell wall and plasma membrane. Then, specific cytokinin transporters actively transport them into the cytoplasm, where they bind to membrane-localized histidine kinase receptors to trigger signaling cascades (Kaur *et al.* 2022). According to Skoog and Miller (1957), media with a high ratio of cytokinin to auxin were able to accelerate cell division and cell formation and eventually led to optimal shoot growth. Using BAP is common because it is more effective and cheaper than other types of cytokinins in producing shoots. The increasing number of shoots was due to the addition of exogenous cytokinin, which accumulates well (Fitrahtunnisa and Aisah, 2021).

The results showed BAP (7 mg L^{-1}) induced the callus, the most shoots, and the least roots from the callus. Kothari and Chandra (1986) reported in their study that leaf callus cultured on MS medium + BAP (7.0 mg L^{-1}) + IAA (5.0 mg L^{-1}) regenerated shoots and adventitious roots, while lower concentrations of BAP (0.5 mg L^{-1} and 5.0 mg L^{-1}) only showed rhizogenesis. The findings further revealed the higher levels of cytokinin in the media have an effect on meristem shoot formation, thereby increasing the number of shoots. The more shoots than roots were due to the addition of exogenous cytokinin, which accumulates better in shoot formation than in roots (Thirupathi *et al.,* 2013). This gained reinforcement from the opinion of Ramassamy *et al.* (2014) that BAP with higher concentrations blocked auxin activity and inhibited the root growth; thus, roots formed at the higher concentrations of BAP grow at low rates.

Transformation of the *InMYB1-CCD4α* gene to marigold

Plant genetic transformation is a complex process involving the insertion of a foreign gene into plant genomes. The source of genes may originate from plants, animals, and microbes. The genes entail an introduction to modify the specific plant traits, thereby producing genotypes with superior characteristics than their parental lines. The common target traits include vigorous growth, higher biomass accumulation, and resilience against biotic and abiotic stress conditions (Su *et al.,* 2023). However, the success rate of genetic transformation remains a challenge in various plant species. Genetic transformation on male sterile marigold had given reports from Nuoendagula *et al*. (2017), who obtained four transgenic plants from 496 leaf explants, corresponding to 0.4% of transformation efficiency.

In this presented experiment, the transformation method received improvements compared with previous methods by Nuoendagula *et al*. (2017) and Apriliani *et al*. (2022) by using an AB minimum medium for *Agrobacterium* as explained in the Materials and Methods section. Besides, before entering the antibiotic selection media, leaf explants first underwent planting in resting media containing the antibiotic cefotaxime to stop the growth of *Agrobacterium* for three weeks without selecting antibiotics for transgenic explants. This method proved successful in making 89% of leaf explants infected with *Agrobacterium* to form callus (Table 3). This phase allowed the explant to rest from shocks and damage that occur during the transformation process, thereby increasing the scope of more explants surviving. Apart from that, this rest phase also provides an opportunity for small responding cells to grow, hence increasing the tolerance of transgenic tissue, deemed as a selective agent. The results were in accordance with research by Matheka *et al.* (2019), who also carried out a rest phase during the transformation stage for 30 days.

In our research, transformation repetition occurred three times with 200 transformed leaf explants and 40 control leaf explants per repetition. Overall, not all the explants were able to pass the antibiotic selection, both control explants and explants soaked in *Agrobacterium*, revealing that the antibiotics (Hygromycin 20 mg L^{-1}) used in the media were effective in selecting the transformed explants (Table 3). According to Tang *et al.* (2004), the antibiotics function to eliminate *Agrobacterium* in the transformation process but can also inhibit the shoot and root induction and regeneration percentage. Hygromycin-resistant callus were able to grow normally on the selection medium, characterized by a shiny yellow and crumbly appearance, with no growth of *A. tumefaciens* bacteria on the surface of the explant and media. Supplementing the antibiotic timentin 150 mg L^{-1} in the selection medium suppressed *Agrobacterium* growth in transformed explants without inhibiting the ability of cells to proliferate and regenerate (Tamosiune *et al.,* 2017).

Table 3. Number of explants that survived in the antibiotic selection medium after transformation.

Replications	Number of explants		LSA1		LSA2		LSA3	
	K	T	K	T	K	T	K	T
1	40	200	27	100	7	20	0	10
2	40	200	29	80	12	21	0	4
3	40	200	15	50	0	16	0	10

K = control (untransformed explants), T = transformed explants, LSA1 = number of survived explants 1 selection, LSA2 = number of survived explants 2 selection, and LSA3 = number of survived explants 3 selection.

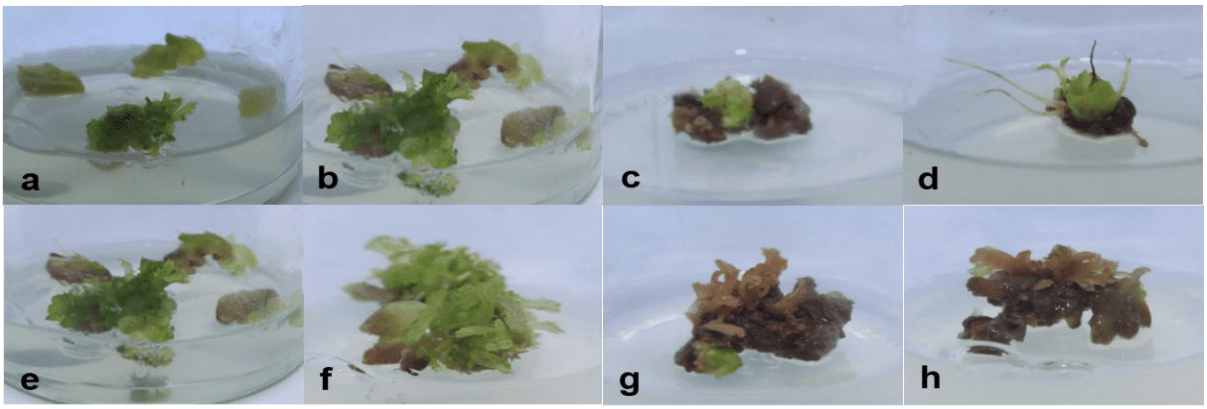

Figure 1. Explants' condition after three subcultures in hygromycin antibiotic selection media. a) transformed callus after resting phase, b-d) transformed callus on hygromycin antibiotic selection phase 1, phase 2, and phase 3, e) callus control (untransformed explants) after resting phase, and f-g) callus control on hygromycin antibiotic selection phase 1, phase 2, and phase 3.

Table 4. Efficiency of transformation and regeneration of marigold.

Observations	Number / percentage
Inoculated explants (A)	600 explants
Explants formed callus (B)	534 explants
Calluses (B/A %)	534/600 = 89%
Higromycin-resistances calluses (C)	24 calluses
Transformation efficiency (C/A %)	24/600 = 4%
Regenerated calluses (D)	7 calluses
Regeneration (D/C %)	7/24 = 29.2%
InMYB1-CCD4a gene PCR positive plantlet (E)	6 plantlets
Transformants (E/A%)	6/600 explants = 1.0%

The explants' condition after three subcultures on the hygromycin antibiotic selection medium is available in Figure 1. The top images (a–d) were transformed callus clumps, while the bottom images (e–h) were untransformed callus clumps after resting and phases 1, 2, and 3 in hygromycin-antibiotic selection media. The callus performance showed a gradual symptom of browning from phases 1 to 3 in the hygromycin-antibiotic selection media. The results revealed that hygromycin (20 mg L^{-1}) can select the transgenic callus clump after three phases of selection.

The transformation efficiency, from 600 explants inoculated, 24 callus clumps passed the hygromycin-antibiotic selection, corresponding to 4% transformation efficiency (Table 4). However, from the 24 putative transgenic callus clumps, only seven callus clumps generated shoots, with a regeneration efficiency of 29.17%. The low regeneration capacity of transgenic cells or callus may refer to genetic factors, such as explant type, *Agrobacterium* strain applied for inoculation, and the antibiotic selection agent in the culture medium (Sabbadini *et al*., 2019). Each cell has totipotent properties during in vitro propagation; however, some meristematic cell tissues were incapable of forming organs (Fehér, 2019).

An analysis of transgenic plants sought to identify the presence of the *InMYB1-CCD4a* gene integrated into the plant genome through PCR-based molecular analysis. The PCR analysis proceeded using DNA isolated from the putative transformant plant organs. In transformed plants, detecting the presence of

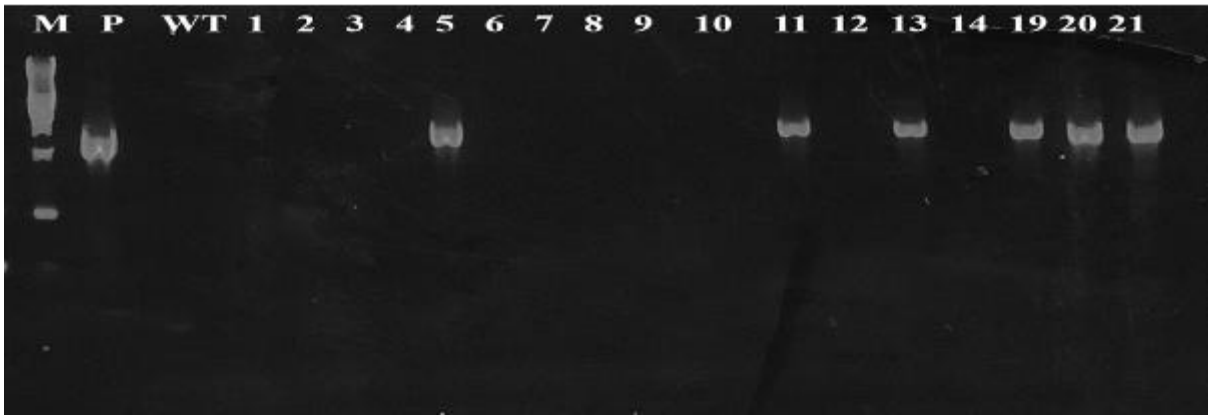

Figure 2. Electropherogram results of the *InMYB1-CCD4a* gene PCR based. M = DNA ladder 1 kb; P = DNA plasmid; WT = wild type plants; 1, 2, 3, 4, 6, 7, 8, 9, 10, 12, 14 = non transformants; and 5, 11, 13, 19, 20, 21 = transformants.

specific genes can occur via DNA amplification using specific primers for the *InMYB1* promoter. Out of 24 plants that passed the antibiotic selection, 17 plants (71%) have undergone testing by PCR. The PCR test revealed six plants (35%) of the transgenic plant candidates were positive for the presence of the *InMYB1* promoter sequence. The remaining 11 plants (65%) that passed through the selection medium did not contain the target gene, indicating the occurrence of escapes during the selection process.

The visualization results showed a specific PCR product with the expected amplicon size of 1200 bp. The six out of 17 putative transformants that had amplicon products showed positive results (Figure 2). This revealed the success of the *InMYB-CCD4a* gene insertion in the plant genome and confirmed the presence of the transgene in the plants. Adversely, the control and wild-type plants have no detectable amplification product. The morphological characters of the regenerated plants disclosed differences between the wild-type plants and putative transformant plants, which can be visible through smaller leaves with slightly paler color and longer plant growth (Figure 2). This can be because of the disruption of the plastid function, where the plastid disruption in question was the undifferentiated plastids in plants, thereby inhibiting the process of thylakoid formation. In crop plants, the minimal amounts of thylakoids were indicative of a morphological appearance that was paler (Rudella *et al*., 2006).

Generally, the experiment of in vitro organogenesis revealing the best treatment for indirect shoot organogenesis of leaf explants is the MS medium containing IAA (0.5 mg L^{-1}) and BAP (7.0 mg L^{-1}), producing about six shoots per explant. The TDZ concentration above 0.25 mg L^{-1} will decrease the number of shoots. Root formation also had a significant reduction at elevated concentrations of TDZ and BAP. The protocol of leaf explant transformation proved effective to increase the genetic transformation efficiency compared with previous methods reported in marigold (*T. erecta*).

CONCLUSIONS

This study successfully identified the optimal BAP (7.0 mg L^{-1}) concentration for efficient shoot regeneration and established a transformation protocol with the *InMYB1-CCD4a* gene integration in marigold (*T. erecta* L.). A total of 24 out of 600 explants passed through antibiotic selection, corresponding to 4% transformation efficiency. Shoot regeneration rate from 24 putative transformants was 6/24 (29.17%). The PCR analysis confirmed that six out of 17 putative

transformants carried the promoter of the *InMYB1-CCD4α* gene.

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