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# Engineering of high production of salicylic acid in transgenic hairy roots of Marigold *Calendula officinalis* L. by *Agrobacterium rhizogenes* ATCC 13332

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**This study is directed to engineering the high production of salicylic acid from both genetically transformed hairy roots and callus cultures of Marigold, *Calendula officinalis* L., by pRi-DNA plasmid of the wild type *Agrobacterium rhizogenes* ATCC 13332, via "Direct injection" and "Co-cultivation" techniques. High frequency of transformed hairy roots was recorded as leaves were inoculated by mixture of pRi-DNA and PEG. Also, incubation of Marigold leaves with pRi-DNA plasmid in the presence of PEG, enhanced hairy roots formation up to 60%. Genomic DNA was isolated from these hairy roots and the derived callus. Amplification of *rol* C gene was done by polymerase chain reaction (sPCR) carried on T-DNA of Ri plasmid. These genes were expected to be inserted in Marigold DNA. Electrophoresis results were a decisive proof that point out from the transfer of *rol* C genes. The latter separated from agarose coincide with molecular weights of the specific primers. Subsequently, HPLC data confirmed the availability of salicylic acid in both transformed hairy roots and callus that recorded 45 and 42-fold, respectively more than SA content in the natural field plants. This finding indicates that this increase was due to the incidence of transgenesis of these tissues.**

**Key words:** Transgenic plant, *Agrobacterium rhizogenes, Calendula officinalis* L., salicylic acid.

## INTRODUCTION

Transgenic tissues obtained from transformed plant cells, by *Agrobacterium rhizogenes*, can synthesize the natural products characteristic of native plants (Zhou et al*.,* 2011). *A. rhizogenes* was specified as a natural vector with Ri-plasmid DNA. This Ri (root-inducing) plasmid can be transferred from the bacterial vector to plant cells producing hairy roots (Chilton et al*.,* 1982; Gelvin, 2009). Bacterial vectors are considered successful systems for genetic transformation and the production of genetically transformed plants due to their ability to donate Ri plasmid or part of its "T-DNA" fragment that integrate with genome of plant cell producing genetically modified hairy roots (Al-Nema and Al-Mallah, 2018). Gene transfer process involves four sequential events, chemo-attraction, release of this single-stranded T-DNA region from the plasmid Ri and translocation to plant cells, followed by nuclear targeting of T-DNA, its integration into the plant genome, and finally expression of loaded genes. At sites of infection, in the host plant, which usually appears after 1 to 4 weeks, is an evidence of their altered phenotypes (Zhou and Wu, 2006). These hairy roots are considered a unique pattern in their genetic and

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biological stability, as well as their role in increasing industrial plant products that are useful in many pharmaceutical and food industries (Hom-utai, 2009). The success of genetic transformation in plant cells and tissues can be inferred by several methods represented by observing a group of phenotypic differences that appear either on genetically modified plants or in some molecular genetics tests (Davey and Anthony, 2010). This is to detect the inclusion of T-DNA genes in the host genome, indicating their transmission and expression (Daniell, 2004). The gene expression of T-DNA genes is phenotypically expressed by the formation of hairy roots from regions inoculated with the bacterial vector carrying the Ri plasmid (Sretenovic et al., 2006). This specific molecular evidence for detection of transgenesis and T-DNA conservation in the plant cell genome is polymerase chain reaction (PCR) test. The *rol* genes are settled in the LT-DNA of the Ri plasmid. Success of their expression in the genome of plant cells results in a change in some phenotypic traits in the plant, such as production of hairy roots (Palazón et al., 1998). The *rol* C genes may be responsible for changes in secondary metabolism and increase by-products in transgenic plant tissues (Shkryl et al., 2008). Some studies reported that the expression of *rol* C genes succeeded in crossing from the bacterial vector *A. rhizogenes* ATCC18534 to hairy roots formed in *Calendula officinalis* L. leaves, and their role in increasing the secondary metabolites (Alsoufi et al., 2021). Marigold, *C. officinalis* L., is a winter annual plant species (Asteraseae), including 20 species of medicinal and ornamental plants of dual use of a high economically importance (Dole and Wilkins, 2004). Its importance is due to the abundance and diversity of its secondary metabolites, such as tannins, coumarins, sterols, steroids, terpenes, a large group of vitamins, quinones (aromatic compounds), amino acids, resins, saponins, and essential oils (Andersen et al., 2010). Also, carotenoids and flavonoids are natural pigments, as well as phenolic acids, such as, para-hydroxybenzoic, vanillic, caffeic and salicylic acids (Rigane et al., 2013). Generally, phenolic compounds are of a great importance for human health (Martins et al., 2016). Salicylic acid (SA) compound is found in trace amounts in Marigold plants (Andersen et al., 2010). Moreover, salicylic acid (orthohydroxybenzoic acid) synthesized by plants, that possess an aromatic ring and a hydroxyl group (Klessig et al., 2018) probably present in all plants. SA is as a free phenolic acid and as a conjugate form in plants, which is an essential ingredient in the manufacture of aspirin drug and cosmetics (Andersen et al., 2010). To date, SA biosynthesis can be created from two freelance and compartmentalized pathways, (1) isochorismate (*IC*) pathway localized into the plastids, and (2) phenylalanine ammonia-lyase (*PAL*) pathway, that takes place in the cytosol. Generally, SA is produced in the chloroplast, and afterward exported to the cytosol (Maruri-López et al., 2019). The aim of this work is to investigate the role of *rol* C genes in engineering the high yield of salicylic acid in transgenic hairy roots and calli of *C. officinalis* L.

## MATERIALS AND METHODS

### Plant

Healthy and fully-expanded leaves of Marigold, *C. officinalis* L. (Hortus-Calendula Doppia IN MIXED, Italy) were excised from 12-weeks-old plants grown in the nurseries of the main campus of Mosul University. Leaves were soaked in solution consisting of distilled water and bleaching solution (FAS, Babylon Comp. for detergents, Baghdad) 3:1 v:v ratio for 25 min on rotary shaker. Then they were washed with sterilized water three times for 3 min each (Al-Abasi et al., 2018). Leaf explants were cultured on agar-solidified MS (Murashige and Skoog, 1962) medium provided with sucrose 32 $gL^{-1}$ and supplemented with 0.1 $mgL^{-1}$ 6-benzylaminopurine (BAP). Induced callus was sub-cultured on MS medium supplemented with 1.0 $mgL^{-1}$ naphthaleneacetic acid (NAA) and 0.1 $mgL^{-1}$ BAP for growth.

### *A. rhizogenes* strain and pRi-DNA isolation

Wild type *A. rhizogenes* strain ATCC 13332, containing pRi-DNA was supplied by Leibniz Institute (DSMz- German Collection of Microorganisms and Cell Cultures, Germany). Single colony of the bacterial vector was grown in 25 ml of nutrient broth (NB) medium at 6.9 pH, in the dark at 28°C. The plasmid pRi-DNA was isolated (Brinboim and Doly, 1979), and then migrated on agarose layer to confirm its presence.

### Induction of hairy roots

Fifteen microliters of isolated pRi-DNA suspension were mixed with 20% PEG of M.Wt 4000 sterile solution (PEG+). Similar volume of pRi-DNA suspension was not mixed with PEG solution (PEG-). Each mixture was used for inoculation sterilized leave segments 2.5 cm, by direct injection method, in which these segments were wounded at the midrib, using a sterile needle (Insulin Syringe U-100-29G1/2-0.33×13 mm 1CC), loaded with plasmid suspension (Christen, 2002). Also, co-cultivation method, where segments were incubated in 10 ml of plasmid suspension in 9.0 cm diameter plastic Petri dishes (tightly closed) for 30 min, with gentle shaking, in dark was also done. These two methods were applied once with plasmid suspension alone (Abou Rayya et al., 2010), and with the presence of 20% PEG solution at ratio 1:1 (V:V). Control segments were inoculated with sterile distilled water, and with PEG solution. After co-cultivation, explants were dried with sterile filter paper, as well as the control. All samples were transferred to the surface of agar-solidified WP free medium (Al-Mahdawe et al., 2013).

Hairy roots developed from leaf inoculated with pRi-DNA plasmid in the presence of 20% PEG solution were transferred to the surface of 25 ml of solidified MS medium supplemented with 0.1 mg $L^{-1}$ BAP (Al-Abasi et al., 2018). Subsequently, callus formed from hairy roots was transferred to the MS medium supplemented with 1.0 mg $L^{-1}$ BAP and 0.1 mg $L^{-1}$ IBA. Cultures were maintained in culture room conditions.

### Establishment of hairy roots cultures

As hairy roots formed at site of inoculation of Marigold leaves, 2 to 3 cm in length hairy roots tufts with a small part of the leaf midrib were excised and cultured on the surface of solidified WP medium

supplemented with 0.5 mg $L^{-1}$ NAA in 9.0 cm diameter plastic. Petri dishes were kept in darkness at 22±2°C. Data was recorded after 5, 10, 15, 20 and 30 days of inoculation.

**Extraction of DNA**

DNA was extracted from each hairy roots, transformed callus, normal callus and leaf of field plant (negative control). pRi-DNA was isolated from the vector *A. rhizogenes* ATCC 13332, CTAB extraction solution was used to extract DNA (Weigand et al., 1993), 0.5 g of each sample was taken, placed in the (pre-cooled) mortar in the presence of 0.5 ml of the extraction solution containing 0.25 ml of 2-Mercaptoethanol. Each sample was ground, then transferred to 2.0 ml Eppendorf tube, all tubes were tightly closed, and placed in a water bath at 55°C for 1 h, 0.5 ml of the mixture consisting of chloroform/iso-amyl alcohol 1:24 v:v was added, centrifuged at 16000×*g* for 10 min, the supernatant was taken into a new 2.0 ml Eppendorf tube, 0.028 ml of ammonium acetate $CH_3COO^-$ $NH_4^+$ was added, this was followed by addition of 0.204 ml of (cooled) isopropanol, the produced solutions was well mixed, and kept for an hour at -20°C. The mixture was centrifuged at 16,000 ×*g* for 5 min. The supernatant was discarded, and 0.7 ml of cooled 70% ethanol was added, sample was mixed, centrifuged at the same speed for 5 min. Then, the supernatant was discarded, and 0.7 ml of 95% cooled ethanol was added to the precipitate, mixed by hand. It was centrifuged at the same speed for 3 min. The entire supernatants were carefully taken and discarded. The tubes were left open for 10 min. As alcohol had evaporated, 0.03 ml of TE-buffer was added to the precipitate, and kept at -20°C until use.

**sPCR analysis**

Each sPCR reaction was carried out by using total volume of 9.0 µl premixed solution ($MgCl_2$ buffer 10X + KCl + Tris-HCl + dNTPs + Taq DNA polymerase), 4.0 µl of 50 ng $µl^{-1}$ Template DNA, and 10 µl $Bicamol^{-1}$ of each one of forward and reverse specific primer. Possible transfer and integration of *rol* C genes into genome of these tissues were detected the using specific primers for *rol* C gene, as positive control.

**Determination of concentration and purity of the isolated DNA**

The concentration and purity of each DNA were measured at wavelengths of 260 and 280 nm, respectively, 1.0 µl of DNA samples isolated from plant tissue and pRi-T-DNA isolated from *A. rhizogenes* ATCC 13332 were each placed on a nanodrop (Nanodrop, BioDrop-England) for measuring the absorbance, the concentration and purity of DNA (Dhahi et al., 2011).

**Quantification of salicylic acid**

This test involved three steps to obtain free salicylic acid (Al-Abasi et al., 2020), as the following.

***Preparation of samples***

The stock solution of standard SA was prepared carefully by weighing 100 mg of salicylic acid (BDH-England), dissolved in 100 mL of distilled water. Four hundred milligrams of each transformed hairy roots and callus were each transferred to a separate pre-cold pestle and mortar, as well as normal callus, field plant leaf as a control samples. These samples were ground in the presence of liquid nitrogen; the obtained powder was kept in 2.0 ml Eppendorf tube. To the latter 1.6 ml of 70% ethanol was added. Specimens were carefully vortexed for 1.0 min, centrifuged at 10000 ×*g* for 10 min at room temperature. Each supernatant was transferred to 15 ml falcon tubes. Again 1.6 ml of 90% methanol was added to the pellet, re-vortexed, for 1.0 min for re-extraction. The produced mixture was centrifuged again under the same conditions. The supernatant was added to the stock supernatant in 15 ml falcon tube. The pooled and clear supernatant solution contains free SA and conjugate SA (Allasia et al., 2018).

**Free salicylic acid**

Two milligrams of each supernatant were placed in 2.0 ml microcentrifuge tubes to evaporate EtOH and MeOH by air currents for 2.0 h. The remaining supernatants were transferred to 2.0 ml Eppendorf tubes and concentrated up to approximately 600 µl. To this aqueous solution, 65 µl of 20% of aqueous trichloroacetic acid (TCA, w/v) was added to each solution, and 650 µl of ethyl acetate and cyclohexane 1:1 v/v solution was added, vortexed for 30 s, centrifuged for 2.0 min at 10,000 ×*g* for phase separation. Transfer the upper organic phase to 2.0 ml eppendorf tube, re-extract the aqueous phase again with 650 µl of ethyl acetate-cyclohexane mixture. Then, centrifuged for 2.0 min at 10,000 ×*g* for phase separation. Evaporate the solvent to dryness for 30 to 45 min, solubilize the dry residue in 100 µl of 10% aqueous methanol (v/v) containing 0.1% aqueous trifluoracetic acid TFA (v/v), and vortex for 1 .0 min. The samples are ready to be assessed by HPLC.

**Quantification of SA in plant tissue**

Twenty microliters of each sample were separately injected in high-performance liquid chromatography (Sykman-2014-Germany). Separation conditions were carried out by C18 column (250 × 4.6 mm, 5 µm) at 30°C and flow rate (1.0 ml/min) of aqueous MeOH gradient from 10% (v/v) was used as linear fluorometric detection (excitation at 305 nm; emission at 407 nm). Salicylic acid concentrations were quantified by comparing peak area of the assessed samples with peak area of standard sample under the same conditions using the standard equation (Kimura and Amaya, 2002).

## RESULTS

### Production of hairy roots via direct injection

Data (Table 1) demonstrate efficient stimulation and development of hairy roots on Marigold leaf inoculated by different number of nicks with needle tip immersed in the mixture of plasmid suspension with 20% PEG solution. These hairy roots involved 10 days to emerge on leaves segments grown on WPM medium containing 5.0 mg $L^{-1}$ NAA, with dense of root hairs compared to their numbers induced using plasmid suspension alone. They required 20 days to emerge.

### Five replicates/treatment of hairy roots

Also, data refer to the superiority of injection method with plasmid-PEG mixture in induction of hairy roots, and its high density at the injection sites, as well as, a few of

**Table 1.** Induction of transformed hairy roots on *Calendula officinalis* L. leaf segments inoculated by direct injection with pRi-DNA and/or pRi-DNA-PEG mixture of *Agrobacterium rhizogenes* ATCC 13332.

| No. of Nicks | PRi-DNA + PEG | | | PRi-DNA | | |
|---|---|---|---|---|---|---|
| | Leaves response | Response (%) | No. of H.R | Leaves response | Response (%) | No. of H.R |
| 1 | 26 | 14 | 53 | 12 | 24 | 17 |
| 2 | 38 | 76 | 83 | 23 | 46 | 52 |
| 3 | 40 | 80 | 86 | 23 | 46 | 38 |
| 5 | **47** | **94** | **196** | 34 | 68 | 73 |
| Dist. Water(Cont.) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| PEG(Cont.) | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

Source: Author


**Figure 1.** Tranformed hairy roots formation from leaves of *Calendula officinalis* L. inoculated with pRi-DNA plasmid of *A. rhizogenes* ATCC 13332 + PEG mixture grown on agar-solidified WP medium + 0.5 mg $L^{-1}$ NAA. A: Hairy roots after 10 days of inoculation at the nicking sites, note two types of hairy roots type- rich with root hairs (double arrows), type- without root hairs (single arrows), and callus was formed (black arrows). B: Development of hairy roots in (A) after being transferred to agar-solidified WP medium, note the numerous roots and formation of callus. C: Development of hairy roots excised from (B) after 50 days of transferring to agar-solidified WP medium. The active growth of hairy roots and the formation of micro-clumps of callus.
Source: Author

these hairy roots were developed in the neighboring tissues. This was not observed with leaves segments inoculated with plasmid alone. The direct injection method by five Pricks with 20% PEG and plasmid mixture was more efficient in inducing hairy roots than single prick injections (Table 1).

Marigold leaves segments inoculated with the mixture of pRi-DNA plasmid and PEG stimulate formation of hairy roots at the midrib of leaves inoculated with 5 nicks. Transfer of inoculated leaves to solid WP medium led to the emergence of hairy roots after 10 days of inoculation (Figure 1A). The results observed two types of hairy roots, the first was rich with root hairs and the other was without root hairs. As callus primordia formed hairy roots were transferred to agar solidified WP medium supplemented with 0.5 mg $L^{-1}$ NAA that sustained growth of hairy roots (Figure 1B), excised hairy roots that cultured forming hairy roots cultures of abundant branching (Figure 1C).

## Induction of hairy roots by co-cultivation

Data show that incubating leaf segments of Marigold with pRi-DNA plasmids of *A. rhizogenes* ATCC 13332 with an equal volume of 20% PEG solution supported the formation of hairy roots on leaf at submerged sites, as compared with incubation with plasmid alone or with distilled water alone, as control treatments (Table 2).

## Twenty replicates/treatment

Again, results of co-cultivation methods of Marigold leaf segments with pRi-DNA plasmid suspension in the presence of PEG solution for 30 min express a clear stimulation of hairy roots on the leaf bases after 20 days of culture on agar-solidified WP medium supplemented with 0.5 mg $L^{-1}$ NAA. They were characterized by rapid emergence at the cutting ends (Figure 2A). Additionally, roots were elongated with frequent branching from the center of the tuft (Figure 2B).

## Production of transgenic callus from hairy roots

The results indicate the ease of producing callus from transformed hairy roots. The latter was of compact

**Table 2.** Formation of transformed hairy roots by co-cultivation of *Calendula officinalis* L. leaves with pRi-DNA plasmid of *Agrobacterium rhizogenes* ATCC 13332 in presence or absence of 20 % (W/V) PEG solution.

| Co-cultivation | Leaves response | No. of hairy roots | Response (%) |
|---|---|---|---|
| Leaves + pRi-DNA-PEG | **15** | **61** | **60** |
| Leaves + pRi-DNA | 3 | 11 | 12 |
| Leaves + PEG (Cont.) | 0.0 | 0.0 | 0.0 |
| Leaves + Dist. Water (Cont.) | 0.0 | 0.0 | 0.0 |

Source: Author

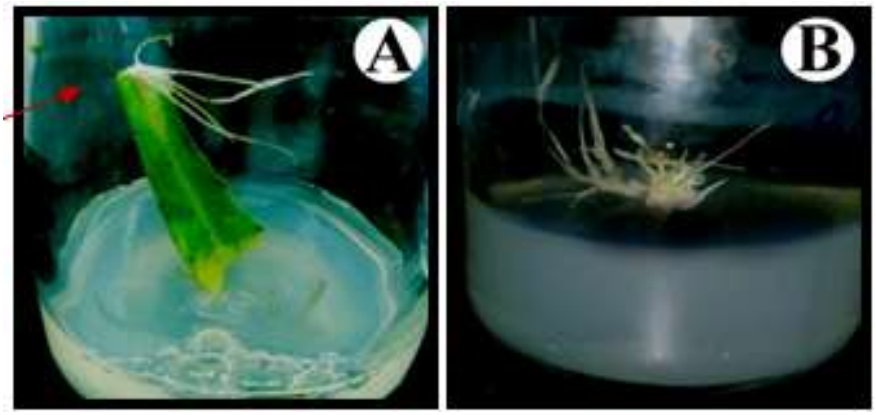


**Figure 2.** Induction of transformed hairy roots by co-cultivation of *Calendula officinalis* L. with *A. rhizogenes* leaves with pRi-DNA of *A. rhizogenes* ATCC 13332 in the presence of 20% PEG solustion, grown on agar-solidified WP medium + 0.5 mg $L^{-1}$ NAA. A: Formation of transformed hairy roots after 20 days of incubating leaves with pRi-DNA + PEG, note the hairy roots at the basal sites of the leaf segments (arrowed). B: A tuft of transformed hairy roots excised from (A) 30 days old grown in fresh WP medium. Note the development of hairy roots from the center of the culture.
Source: Author

texture and was characterized by its yellowish-green color (Figure 3A). Callus also was observed at the sites of inoculation of leaves segments with pRi-DNA plasmids of *A. rhizogenes* ATCC 13332 in the presence of 20% PEG solution. This type of callus was distinguished by its friable appearance and creamy white color (Figure 3B).

## sPCR of pRi-DNA plasmid of *A. rhizogenes* ATCC 13332

Specific polymerase chain reaction of each sample of DNA isolated from leaves of field plant, hairy roots, transgenic callus and normal callus, as well as the pRi-DNA isolated from the bacterial vector *A. rhizogenes* ATCC 13332. Electrophoresis results demonstrated the separation of bands from both DNA of hairy roots and callus formed at sites of inoculation with pRi-DNA, as well as pRi-DNA. The bands molecular weights matched with the molecular weights of the specialized primers was 545 bp of the *rol* C gene (Figure 4). This evidence confirms its genetic transformation conclusively as a result of the transfer of a piece of T-DNA from the plasmid and expression of *rol* C genes in the genomic DNA of plant cells. The absence of DNA bands from other control samples submits a strong evidence of the absence of these genes in their genomic DNA (Figure 4).

## Presence of salicylic acid in transgenic Marigold tissues

HPLC data (Table 3) exhibit that leaves of Marigold field plants and their normal callus contained salicylic acid at low concentrations, whereas, each of the transformed hairy roots and transgenic calli samples recorded a high content of salicylic acid reaching about 45-fold.

In other words, data refers to the low levels of salicylic acid in the leaves of field plant, and conclusively revealed an increase of salicylic acid concentrations in transgenic hairy root samples. This data confirmed that transgenesis, achieved in these samples, supports the engineering of the high production of salicylic acid in transgenic calli and hairy roots.

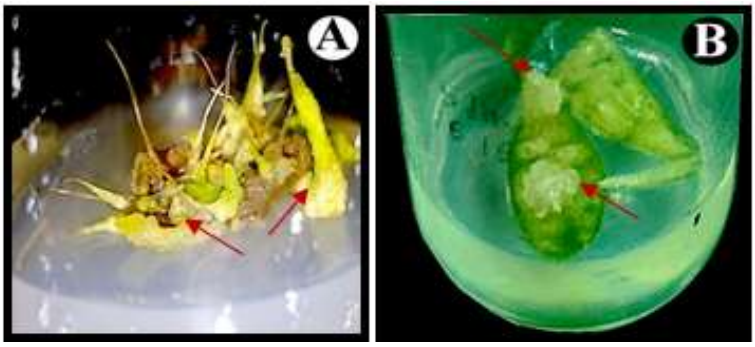


**Figure 3.** Transgenic callus induction from transformed hairy roots induced on leaf of *Calendula officinalis* L. inoculated with pRi-DNA plasmid of *A. rhizogenes* ATCC 13332 in the presence of PEG grown on agar-sloidified MS + 0.1 mg $L^{-1}$ BA medium. A: Callus formation from hairy roots (arrows). Note the beginning of callus appearance at the sites where hairy roots in touch with medium. B: Genetically transformed callus formed at inoculation sites (arrowed) with pRi-DNA + PEG mixture.
Source: Author

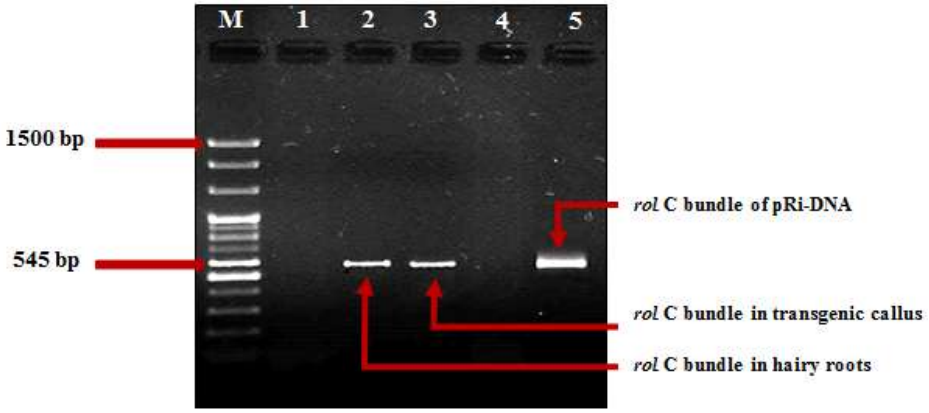


**Figure 4.** Amplified *rol* C gene transferred from plasmid DNA vector of *A. rhizogenes* ATCC13332 to genomic DNA of *Calendula officinalis* L. Lane M: Volumetric Directory (DNA Ladder). Lane 1: Amplified genomic DNA of field leaves. Lane 2: Amplified genomic DNA of transformed hairy roots. Lane 3: Amplified genomic DNA isolated from a transgenic callus. Lane 4: Amplified genomic DNA of a normal callus. Lane 5: Ri-Plasmid DNA isolated from the bacterial victor *A. rhizogenes* ATCC13332.
Source: Author

## DISCUSSION

In this investigation, transgenic hairy roots production is attributed to the successful interaction between this plant species and the plasmid DNA of the bacterial vector *A. rhizogenes* (Patel and Krishnamurthy, 2013). Studies indicated that hairy roots are considered the first signs of genetic transformation (Zupan et al., 2000). Many reports confirmed that wounding plant tissues was an important step for the attachment of bacterial vector to cells and stimulating the formation of hairy roots. This explains the high response achieved by direct injection method compared to the co-cultivation method. The effect of PEG solution when interfering with the bacterial plasmid DNA in inoculation was expected to be due to the nature of PEG as a hydrophilic polymer, and to its carbonyl group (Al-Mallah et al., 1990). Development of hairy roots in infection locus of plasmid may be attributed to a group of physiological and morphological changes as a result of the gene expression of T-DNA fragment that carry *iaaH* and *iaaM* genes that encode to the biosynthesis of cytokinins and auxins, especially IAA (Takei et al., 2001). This causes a disturbance in the hormone balance that stimulates cell divisions to produce a unique pattern of hairy roots (Meyer et al., 2000). This may support the emergence of minute masses of callus besides hairy roots in their growth medium and their development into large biomass. The formation of hairy roots is due to the

**Table 3.** Salicylic acid Contents in transgenic tissues of *Calendula officinalis* L. genetically transformed by *Agrobacterium rhizogenes* ATCC13332.

| Sample | Peak area | SA conc. (µg/g) |
|---|---|---|
| Standard SA (cont.) | 7101.385 | 100 |
| Transformed hairy roots | 6751.079 | **94.46** |
| Transgenic callus | 6304.240 | **88.21** |
| Normal callus | 359.327 | 5.02 |
| Field plant leaves | 147.861 | 2.069 |

Source: Author

fact that the RiT-DNA genes contributed to directing cellular aggregates to hairy roots as a result of hormonal balance loss between auxin and cytokinin (Hom-utai, 2009), or perhaps due to the contribution of *vir*-genes in the formation of hairy roots (Fu et al., 2005). It can be said that co-cultivation technique is an efficient protocol in the processes of genetic transformation, specifically when using pRi-DNA as a substitute for bacteria. Also, the ease of implementation and detection of the interference of these plasmids with the genome of plant recipient cells (Fu et al., 2005), *Onco*-genes group represents the most important stable genes in the T-DNA segment carrying the *rol* A, *rol* B, *rol* C and *rol* D genes, which (all or some of them) are described as easily expressed in the plant genome (Bulgakov, 2008). Also, the have effect on cell growth and differentiation, as well as play role in synthesizing secondary metabolites in transgenic cells of plants (Bulgakov, 2008), in particular *rol* C genes (Tzfira and Citovsky, 2008). However, if *rol* A and *rol* B co-exist together, they stimulate the activity of hairy root cultures (Pavlova et al., 2014). In this study, hairy roots production or their transgenic callus mediated by the plasmid vector interfering with the Marigold plant, represents evidence that the genomic DNA of the transgenic tissues retains the *rol* C genes, according to the sPCR data. This gave an explanation and evidence for the expression of *rol* C in the plant genome (Zupan et al., 2000). The *rol* C gene provides a signal that activates secondary metabolic processes. It is likely that *rol* C may confer a wider spectrum of defense reactions in addition to secondary metabolite stimulation. Interestingly, *rol* C gene encodes cytokinin glucosidase and stimulates the production of many secondary compounds in various plants (Dilshad et al., 2015). Phenols accumulation in hairy roots was more than its concentration in field plants. This may be due to the effectiveness of hairy roots in increasing the content of secondary metabolites, perhaps because the high expression activity of *rol* C gene (Wang et al., 2006).

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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