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Propagation of *Rosa hybrida* L. cv. Coolwater Under Tissue Culture and Transformation of the *RhAA* Gene via *Agrobacterium tumefaciens*

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ABSTRACT

Rose is the most favorite cut flowers all over the world. Production of high-quality flowers, prevention, and delay of flower senescence, is a major goal in floriculture. Now a day, biotechnological approaches have been used to improve ornamental attributes. Tissue culture and genetic transformation appear to offer valuable advancements for operating floral characteristics. In this study, after optimizing sterilization for the first step of tissue culture, lateral buds of *Rosa hybrida* cv. Coolwater were cultured on MS medium supplemented with different concentrations and combinations of BA (Benzyladenine). The results indicated that the highest growth rate and establishment were 80% on 1.5 mg l^{-1} BA. In the second stage, explants were transferred to MS medium containing various concentrations of BA, NAA (Naphthaleneacetic acid) and IAA (Indole acetic acid) hormones. The maximum number of shoots per each explant (8.00 ± 0.18) belonged to 3 mg l^{-1} BA with 0.5 mg l^{-1} NAA. Explants were also transferred to the rooting medium induction with different concentration of IBA (Indole butyric acid) and Phl (Phloroglucinol). The best rooting induction was selected in MS/2 with 3 mg l^{-1} IBA (62.22%). In order to the production of transgenic plants, the lateral buds and *Agrobacterium tumefaciens* LBA 4404 were used for transformation. Two parameters affecting *Agrobacterium* infection efficiency were investigated, including inoculation, media. The optimum time for infection was 10 minutes. The effects demonstrated that the best medium in inoculation was sucrose 3% and the percentage of transgenic was 10%. Transgenic plants were confirmed by PCR (Polymerase chain reaction). The appearance of the 470bp band revealed that plants were transgenic with *RhAA*. According to studies, it seems that the gene in the roses affects the vase life of the flower.

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Introduction

One of the best potential tools that have used in latest agriculture perusals is plant tissue culture that allows a perfect plant to be grown from a single plant cell and a specific district that the most commonly used lateral buds and the tip of stems (Baccara *et al*., 2013). The results have shown that tissue culture of rose is an *in vitro* modern technique for rapid multiplication and mass propagation in cultivars with production disease-free plants (EL-Tarras *et al*., 2017). The senescence is the latest stage of plant development that exhibits many of the structural changes with combination interaction that is kind of programmed cell death (Sillanpa *et al*., 2012; Wu *et al*., 2016). The results of these events are the death of the floral organs (Hajizadeh *et al*., 2011). One of the significant problems about post-harvest vase life of roses goes back to leaf faded and poor quality that recognizing of these events is very useful (Zakizadeh *et al*., 2013; Ali *et al*., 2012). Gene transformation is a new method for changing morphological and physiological characteristic in plants (Niazian *et al*., 2017). One of the section that can be used in gene transformation goes back to

lateral and shoot apex buds (Ming *et al*., 2007; Hajizadeh *et al*., 2011) identified the expression pattern of 6 genes differentially displayed in *Rosa hybrida* petals during flowers innocence. In miniature potted Rose (*Rosa hybrida* L. cv.linda), P_{SAG}-*ipt* gene was transferred and thus flowers were resistant to ethylene and senescence (Zakizadeh *et al*., 2013).

In a previous study Hajizadeh *et al*. (2011) isolated and characterized some senescence inducible genes by cDNA-AFLP technique, which one of them was *RhAA*.

The purpose of this project about investigated *in vitro* tissue culture of *Rosa hybrid* cv. Coolwater and other part was the investigation of *RhAA* gene effect probably dependent on flower vase life.

Materials and Methods

Plant material

Rosa hybrid cultivar Coolwater was grown under 27±1°C and photoperiod in 16h of light in Ornamental Plants Research Center in Mahallat, Markazi, Iran.

Establishment of a regeneration system

The stem lateral buds of the Coolwater genotype were used for the development of the micropropagation system. After washing with water (45 min), nodal explant sterilized with 2 treatments: i) Immersion in ethanol 70% (30s) + Plunging and shake in 2.5% solution of sodium hypochlorite with 3% tween 80 (20min). ii) The second treatment was used 0.1% solution mercuric chloride (8min).

After discarding sterile treatments, explants were washed three times with sterilized distilled water and were moved in culture medium MS (Murasnige and Skoog, 1962) with different concentration of BA (0.2, 0.5, 1, 1.5 mg l^{-1}) for establishment. The pH of the medium was regulated in 5.8 and solidified by 8 gr l^{-1} Agar. It was auto calved at 121°C and a pressure of 1 kg cm^{-2} for 20 minutes.

For shoot propagation subsequent subcultures, BA (1, 2, 3, 4 mg l^{-1}) combination with NAA (0.3, 0.5 mg l^{-1}) or IAA (0.3, 0.5 mg l^{-1}) were added to the basal medium for induction of numerous shoots. Each treatment consisted of 3 replications in which 5-7 explants were used. Explants were subcultured every 4 weeks in fresh medium and the number of shoots in every explant was recorded.

Then, single shoots were transferred to root induction medium with 2 medium culture containing MS, MS/2 with (1, 2, 3 mg l^{-1}) IBA in combination with (0, 0.2) mg l^{-1} Phl to achieve root proliferation. All cultures were incubated and grown under 25±1°C and a photoperiod 16h light provided by cool -white fluorescent light. Also, all data analyzed using ANOVA a means were compared with F-test using the SPSS23 at the 5% probability level.

Agrobacterium-mediated transformation

In the next stage, *Agrobacterium tumefaciens* LB4404 strain harboring the plasmid pBII21, coding for *RhAA* gene (Hajizadeh *et al*., 2014), was used for transformation of lateral buds. To prepare a solid LB medium, 1% Bacto-peptone, 0.5% Bacto-yeast extract, 10% NaCl, and 1.5% agar with distilled water to a volume of 1L. After adjusting the pH to 5.2, the resulting solution was autoclaved at 121 °C for 15 minutes.

In this study, cefotaxime as an antibiotic was used to remove *Agrobacterium* in selected media. In order to determine the best concentration that, concentrations of 150, 200, 250 mg l^{-1} antibiotics were used and the best concentration was examined in selected media. Also, 3 concentrations (15, 20, 25 mg l^{-1}) of kanamycin were selected to obtain the best concentration and to be used to continue the process for selecting transgenic plants.

Agrobacterium culture and explants

An Agrobacterium colony in 5 ml of LB medium containing 50 mg l^{-1} kanamycin and 50 mg $l^{-1}$of rifampicin in sterile falcon was cultured overnight at 28°C to reach OD_{600} = 0.5-1. Then, centrifuged at 3500 rpm for 10 minutes, and the supernatant was discarded, and the remaining deposition was used in the next steps. The precipitate was mixed with a medium that was prepared for bacterial culture (water and sucrose 3%) and complete MS medium, and for contamination of different explants were used after one hour.

Lateral buds used for infection and inoculation times were 5, 7, 9 and 10 minutes. After creating scratching, the surface of the exterior of meristems and the immersion with bacterial suspension, explants were transferred to co-

culture medium with 0.45 mg l^{-1} BA for 3 days, after this time were grown in selective shoot culture with optimal cefotaxime and kanamycin. Every 2 weeks, explants were transferred to new medium culture.

DNA extraction

Transgenic plants were resistant to kanamycin and remained green, and non-transgenic plants DNA extraction was carried out. To extract DNA from control and transgenic explants CTAB (Cetyltrimethylammonium bromide) method was used.
CTAB method was conducted according to the classical method(Stefanova *et al*., 2013) with some modifications in order to achieve higher concentration and better purity of the extracts. Each sample, as well as the reference material (200 mg), was transferred to a 2 ml sterile reaction tube and mixed with 400 µL of deionized water, followed by the addition of 1000 µL of CTAB extraction buffer (20 g CTAB; 1.4 mol, NaCl; 0.1 mol, Tris-HCl; 20 mmol, $EDTA_{Na2}$) and 0.001 mg l^{-1} of Proteinase K (20 mg). The mixture was vortexed and incubated at 65 °C for 60 min. Further, 20 mg l^{-1} of RNase A (10 mg) was added and the samples were homogenized, incubated at 65 °C for 10 min and centrifuged at 16000×g for 10 min. The supernatant (1000 µL) was transferred to a new 2 µL sterile reaction tube, mixed with 800 µL of chloroform, vortexed vigorously and centrifuged at 16 000×g for 10 min. This step was repeated. After that, the upper phase was mixed with a double volume of CTAB precipitation buffer (5 g l^{-1} CTAB, 0.04 mol l^{-1} NaCl) and incubated for 60 min at room temperature. After centrifugation at 16000×g for 10 min, the supernatant was discarded, and the precipitate was dissolved in 700 µL of 1.2 mol l^{-1} NaCl and extracted with an equal volume of chloroform. The mixture was centrifuged at 16000×g for 10 min and 0.6 volume parts of isopropanol were added to the upper phase. After incubation for 10 min at room temperature, the mixture was centrifuged at 16000×g for 10 min and the supernatant was discarded. The pellet was washed with 1000 µL of 70 % ethanol. After centrifugation at 16000×g for 10 min, the supernatant was discarded and the pellet was dried at 37 °C for 30 min. The dry pellet was dissolved in 100 µL of deionized water and stored at -20 °C.

PCR analysis

PCR reaction performed in 50 µl final volumes with a mixture of 25 ng of genomic DNA, 5µl 10X PCR buffer, 0.5 mM mix dNTP, 1.5mM $MgCl_2$, 0.5mM of each forward and reverse of *RhAA* specific primers and 0.2U of *Taq* DNA polymerase. The amplification figure consisted of an initial denaturation for 5 minutes at 94°C followed by 35 cycles of 1 minute at 94°C, 30 seconds at the annealing temperature 58°C, 1 minute for elongation at 72°C, and a final extension step of 5 minutes at 72°C.
The PCR products were analyzed by 1% agarose gel electrophoresis and stained by Red Safe™ (Intron Co. Korea). The PCR products were electrophoresed on 8% polyacrylamide gel (30.8 % acrylamide bisacryl amide) in TBE buffer (25 mM Tris, 25 mM Boric acid, 50 mM EDTA, pH 8.0) at 180 V for 2-3hours, depending on the fragment sizes. Then, the polyacrylamide gel stained by DNA silver staining (AgNO3) methods. All of the electrophoresis materials provided by the Merck Company.

Results and Discussion

Optimization of sterile methods was shown that usage of Sodium hypochlorite was more effective (100%) than mercuric chloride (75.83%). After the sterile, explants transferred to the establishment medium. Based on the observations obtained in the present study, it seems that immersion with ethanol and sodium hypochlorite have effects on sterilization *Rosa hybrida* cv. Coolwater lateral buds explants. Although the dependence on species is not affected, the studies have shown that sodium chloride influence most of the cellular components including lipids, proteins, and DNA (Nizamani *et al*., 2016). MS medium tissue culture was used in this study that is the most common and was confirmed with other studies[22]. In addition, in the medium culture influence of different hormones in the explants establishment cannot be ignored. After 4 weeks, buds on nodal explants growth on MS medium supplemented by different concentrations of BA that the highest frequency obtained from 1, 1.5 mg l^{-1} BA (60%) (Fig. 1A) in compare with 33.33% from MS medium consist of 0.2, 0.5 mg l^{-1} BA. The propagation of shoots step, the combination of 3 mg l^{-1} BA and 0.5 mg l^{-1} NAA was

confirmed as the optimal treatment. The simultaneous use of these two hormones in tissue culture of *Rosa hybrid* L. was confirmed.

In this study, different concentrations of BA were used that the maximum establishment 80% was observed. The last results demonstrated BA single induce buds growth[23]. Studies have demonstrated that cytokinins have a positive effect on cell division stimulation and lateral buds growth (Dawande and Gurav, 2015). In order that shoot propagation, we used MS medium with different concentration of BA, IAA or NAA. The results were shown that the best amount of shoots obtained from 2 mg l^{-1} BA with 0.3 mg l^{-1} IAA (5.66 ± 0.28), and in combination of BA and NAA, the maximum rate of shoot formation get from 3 mg l^{-1} BA with 0.5 mg l^{-1} NAA with 8.00 ± 0.18 for any explants (Fig. 1B) (Table 1). Compare of two combination hormones was shown that BA interaction with NAA was the best result for shoot multiplication in rose cultivar Coolwater.

Root induction medium was optimized by using of different concentration of IBA and Phl in MS and half of MS salt combination (MS/2) media (Table 2). Most of the root induction was obtained from MS/2 with 3 mg l^{-1} IBA. Apply to phl improved root formation, but no significant differences between them (Fig. 1C). In rooting step, the highest root number was observed in MS/2 with 3 mg l^{-1} IBA. Ruedell *et al*. (2013) have offered IBA hormone can increase the amount of Carbohydrate in the root and it increases root growth. On the other hand, studies have shown that root initiation and the amount of phenol are increased immediately after IBA treatment, then IAA-oxidase and peroxide being to increase. When Phenol is increased, induction of root will begin and with IAA-oxidase and Peroxidase, the number of roots will be increased (Nag *et al*., 2001; Emam & Assareh, 2015).

RhAA gene transformation

For gene transformation, optimal media and hormone combination was selected. Also, the optimum time for infection was 15 minutes that had the best inoculation and the lowest bacterial contamination for inhibition of Agrobacterium 250 mg l^{-1} cefotaxime were applied. For the selection of transforming shoots, kanamycin was used as a selectable marker. The transformed shoots containing NPTII gene had a normal green color that could grow in selection medium supplemented with 10 mg l^{-1} kanamycin.

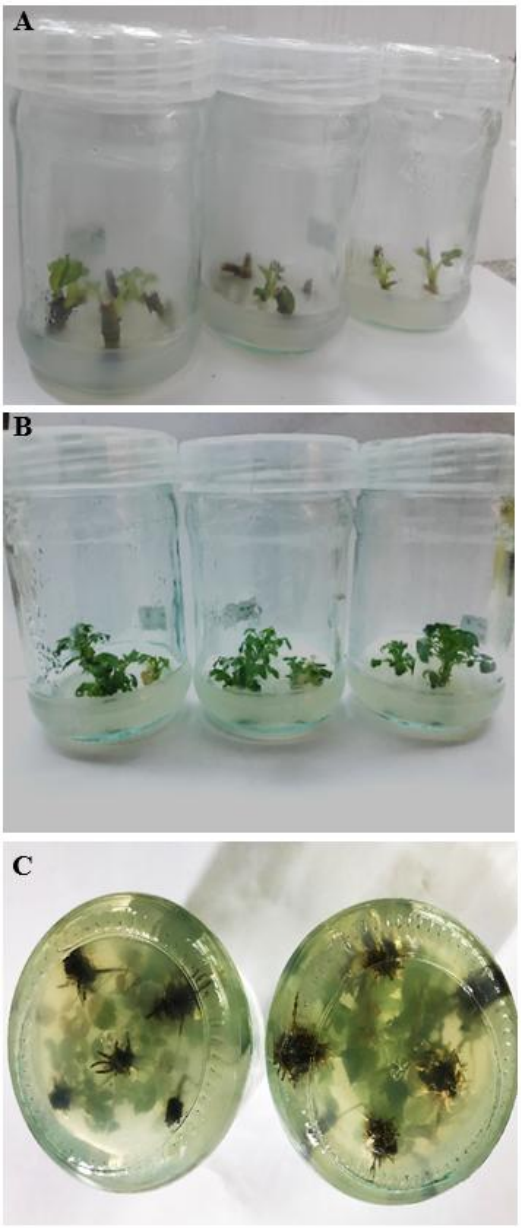

Fig. 1. Micropropagation in *Rosa hybrida* cultivar Cool water: A) shoot growth from lateral buds on stem explants in establishment media containing 1 mg l^{-1} and BA; B) Shoot propagation in MS medium with 3 mg l^{-1} BA and 0.5 mg l^{-1} NAA; C) Root induction in MS/2 supplemented with 3 mg l^{-1} IBA.

Table 1. Effect of different BA combination with different IAA or NAA concentrations on shoot induction in rose cultivar Coolwater*

MS Medium		Shoot number	MS Medium		Shoot number
BA (mg l^{-1})	IAA (mg l^{-1})		BA (mg l^{-1})	NAA (mg l^{-1})	
1	0.3	4.66 ± 0.13 bc	1	0.3	5.66 ± 0.28 b
1	0.5	4.33 ± 0.11 cd	1	0.5	4.66 ± 0.18 bc
2	0.3	5.66 ± 0.28 a	2	0.3	4.66 ± 0.15 bc
2	0.5	3.66 ± 0.11 e	2	0.5	5.00 ± 0.12 bc
3	0.3	3.66± 0.11 e	3	0.3	5.00 ± 0.12 bc
3	0.5	4.00 ± 0.14 d	3	0.5	8.00 ± 0.18 a
4	0.3	5.00 ± 0.18 a	4	0.3	4.66 ± 0.15 bc
4	0.5	5.00 ± 0.19 b	4	0.5	5.00 ± 0.12 bc

*Value of mean number of shoot induction not having a superscript in common differs significantly using Duncan's multiple range test at $p \leq 0.001$.

Table 2. Effect of different concentration of IBA and Phloroglucinol in MS or MS/2 salts medium on root induction in rose cultivar Coolwater*

MS		Rate of root induction (%)	MS/2		Rate of root induction (%)
IBA (mg l^{-1})	Phl (mg l^{-1})		IBA (mg l^{-1})	Phl (mg l^{-1})	
1	0	20 b	1	0	46.66 b
2	0	26.66 a	2	0	43.33 c
3	0	20 b	3	0	63.33 a
0	0.2	20 b	0	0.2	44.44 b
1	0.2	13.33 b	1	0.2	26.66 b
2	0.2	33.33 a	2	0.2	53.33 a
3	0.2	13.33 b	3	0.2	53.33 a

*Value of mean number of shoot induction not having a superscript in common differ significantly using Duncan's multiple range test at $p \leq 0.001$

Transgenic plants were transformed to root induction medium contained 50 mg l^{-1} km and could produce vigorous root. Improving the genetic structure of plants is one of the effective methods in the economic situation in every country. The latest goal of this present study was the optimization of genomic DNA extraction technique by modifying the CTAB method. Before that, the best concentrations of cefotaxime and kanamycin were confirmed that were 250 mg l^{-1} and 10 mg l^{-1} respectively. Also, the optimum time for infection was solicited 10 minutes that had the best inoculation and the lowest bacterial contamination. After the DNA extraction, quality of the isolated DNA was analyzed by PCR technique. In order to convince the transfer and the presence of the *RhAA* gene into transgenic plants that were grown on selection medium with kanamycin, PCR was achieved. The advent of the bands of 470bp in transgenic plants indicated the presence of the *RhAA* gene, which was similar to the band created in the positive control sample from transgenic bacteria containing this gene (Fig. 2).

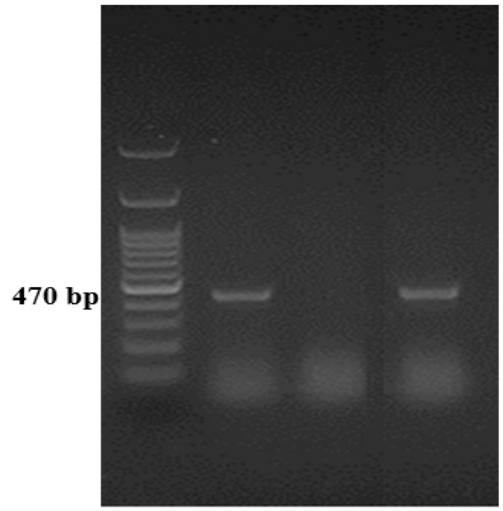

Fig. 2. Determination of *RhAA* gene amplification by PCR: 2.5 % agarose gel was used for separation of PCR products; The lanes are as follows: Lane1. Molecular size marker 100bp Ladder; Lane 2. PCR analysis of kanamycin-resistant plants that showing amplification of 470bp DNA fragment of *RhAA* gene; Lane 3. A negative control containing *RhAA* gene (Wild type); Lane 4. Positive control (Transgenic bacteria).

In the latest level of the present study, lateral buds and *Agrobacterium tumefaciens* were used for the *RhAA* gene transfer and the appearance of the 470bp band was confirmed on the verification of the test. Ming *et al*. (2007) demonstrated lateral buds are more effective than leaflets in GUS gene transfer to *Rosa hybrida* L. cv. Nikita. Also**,** the results showed that 10% of the explants were transgenic. Hajizadeh *et al.* (2014) reported that there is a highly significant similarity to proteins involved in the senescence of *Picea abies*. Thus it seems *RhAA* gene can throw an important influence on senescence induce in rose and cutting of this gene delayed senescence. According to the importance of transferring the gene in Rose to produce desirable characteristics and low transgenic percentages in this plant in different methods, this method can be optimally introduced.

Conclusion

Our results demonstrated tissue culture is a good way for the multiplication of *Rosa hybrid* L. cv. Coolwater and the using of lateral buds are really effective for transformation in this flower. It is also possible that the expression of the *RhAA* gene in the plant accelerates the senescence of the flower.

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