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Original Article

Induction of hairy roots and plant regeneration from the medicinal plant *Pogostemon Cablin*

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ABSTRACT

An efficient transformation system for the medicinal and aromatic plant, *Pogostemon cablin* Benth was developed by using *Agrobacterium rhizogenes* ATCC15834 and C58C1. Hairy roots formed directly from the cut edges of leaf explants after infection for 2 days. The highest frequency of leaf explant transformation by *A. rhizogenes* ATCC15834 and C58C1 were 83.3% and 80.5% after pre culture about 2 days and infection by the bacterium containing 15 mg l^{-1} acetosyringone about 25 min. The PCR amplification showed that rolB genes of Ri plasmid of *A. rhizogenes* were integrated and expressed into the genome of transformed hairy roots. The optimum medium for callus induction of hairy roots consisted of 2.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA while optimum medium for adventitious shoot regeneration from these cultures consisted of 0.1 mg l^{-1} BA and 0.1 mg l^{-1} NAA. Adventitious shoots could be rooted on 1/2MS. PCR analysis confirmed that rol B gene of TL-DNA of Ri plasmid was integrated into the genome of hairy roots-regenerated *P. cablin* plants. The results presented provide a possibility for breeding of a new cultivar of *P. cablin*.

Key words: *Pogostemon cablin*, *Agrobacterium rhizogenes*, Plant regeneration, C58C1, ATCC15834.

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INTRODUCTION

Pogostemon cablin, of the genus *Pogostemon*, is a perennial herbal plant native to the Philippines. It is a traditional chinese medicinal material commonly used in removing dampness, relieving summer-heat, exterior syndrome, stopping vomiting and stimulating the appetite. Recent studies showed that *P. cablin* has an *in vitro* antivirus effect.[1-3] In China, *P. cablin* is propagated by cutting propagation generally because it doesn't bloom in southern China. This mode of reproduction leads to weaker resistance of progeny and decline in yield and quality.[4] It is very difficult to breed new cultivars using the traditional methods. Consequently, it is not only necessary to develop new technologies to create germplasm resources but also breed new cultivars. Many reports showed that genetic transformation of plants with *Agrobactrium rhizogenes* can be used as an efficient way of breeding new cultivars for improvement and creation of germplasm resources.[5] So far, hairy roots which grow rapidly with stable and comparatively high content in secondary metabolites, induced by genetic transformation of *A. rhizogenes*, were successfully used for the production of essential oils from medicinal or aromatic plants, such as Rose-scented geranium (*Pelargonium sp.*),[6] *Plumbago indica*[7] and *Psoralea drupacea* (Fabaceae)[8] and for improvement and creation of germ- plasm for *Nierembergia scoparia*,[9] *Kalanchoe blossfeldiana*[10] and *Glycine max*.[5] The transgenic plants also produced from hairy roots are usually non-chimeric because the hairy roots originate from single cells and each hairy root consists of uniformly transformed cells. To date, there are only a few reports on breeding transgenic *P. cablin* plants with pest resistance by genetic transformation with *A. tumefaciens*.[11-12] In the present study, we will describe the development of an efficient system for genetic transformation of the medicinal and aromatic plants *P. cablin* with *A. rhizogenes* and its plant regeneration from hairy roots.

MATERIALS AND METHODS

Plant material

P. cablin was cultivated in the medicinal botanical garden of Guangdong Pharmaceutical University. Leaves were sterilized, cut and placed at the medium of MS[13] (Murashige and Skoog 1962) + 0.1 mg l^{-1} NAA + 0.2 mg l^{-1} BA, then cultured at 25^{0}C under a 14h photoperiod.

Bacterial Strains and Culture Conditions

A. rhizogenes strains ATCC15834 and the disarmed *A. tumefaciens* strain C58C1 (which carries *A. rhizogenes* Ri plasmid pRiA4)[14] were used to determine the transformation efficiency. The bacterial strain ATCC15834 and C58C1 were shaken at 28°C in liquid YEB medium in the dark supplemented with 40 mg l^{-1} rifampcine, respectively. These strains were cultured in 25 mL of YEB on a gyratory shaker at 160 rpm for 10 h at 28°C until the OD600 was approximately 1.0. The bacterial suspension was centrifuged at 5,000 rpm for 10 min and the pellet was re-suspended in 50 mL 1/2 MS liquid medium and used for co-cultivation of explants.

Induction and culture of P. cablin hairy roots

Young leaves (second and third leaves from the apex) excised from the seedlings which were micropropagated 25 days after last subculture were cut into 1.0-1.5 cm^2 leaf explants and pre-cultured in the dark at 25^{0}C on solid, growth regulator-free MS medium for 24 h, 48 h and 72 h. The leaf explants were then infected by dipping them into Agrobacterium suspension containing 10 mg l^{-1}, 15 mg l^{-1} and 20 mg l^{-1} acetosyringone for 10 min, 15 min, 20 min, 25 min, and 30 min. Following infection, the agrobacterium suspension was blotted with sterilized filter paper to remove excess agrobacterium. After 1, 2 or 3 days of co-cultivation on solid, hormone free MS medium at 28^{0}C

in the dark, the leaf explants were transferred and placed on the MS medium containing 500 mg l^{-1} cefotaxime (filter-sterile before addition to the medium) and kept at 25^{0}C, under a 14 h photoperiod to induce hairy roots. All leaves were sub cultured every 7 days until there was no plaque's appearance. Control explants were given the same treatment but were dipped in sterile growth regulator-free MS medium. Each treatment consisted of 36 explants and the infection experiments were repeated twice.

PCR analysis of hairy roots

The isolation of genomic DNA from 100 mg of sterile hairy roots and natural (non-transformed) roots was conducted using the established methods of Zhou *et al.*[15] Polymerase chain reaction (PCR) identification of the rooting locus genes rol B was performed using DNAs from the hairy root as template and the non-transformed roots as control. The primers of rol B was P1: 50-GCT CTT GCAGTG CTA GAT TT-30 P2: 50-GAA GGT GCA AGC TAC CTC TC -30.[16] For amplification, the PCR parameters of rol B consisted of a pre-denaturation step of 5 min at 94^{0}C, denaturation of 30s at 94^{0}C and 35 cycles (each consisting of 30s at 58^{0}C and 1 min at 72^{0}C), followed by a final extension at 72^{0}C for 10 min.

The amplified products were detected by ethidum bromide staining after 0.8% agarose gel electrophoresis.

The induced hairy roots were placed in different mediums for screening the best medium.

Plant regeneration from hairy roots

To induce callus, the positive transformed hairy root, maintained on 6,7-V medium, was excised into 2–3 cm-long segments and transferred to the callus induction media, in which MS medium was supplemented with BA (1.0, 2.0 or 3.0 mg l^{-1}) and NAA (0, 0.1 or 0.2 mg l^{-1}) in combination, 3% (w/v) sucrose and 0.7% (w/v) agar Each treatment consisted of 15 segments of hairy roots (3 segments per flask). The percentage of callus induction was calculated 30 days after inoculation. To induce adventitious shoot formation, the yellow-green calli with vigorous growth were selected and then transferred into the medium MS+0.1 mg l^{-1} BA +0.1 mg l^{-1}NAA. Each treatment consisted of 15 calli of hairy roots (3 calli per flask).The flasks were then kept at 25^{0}C under a 14h photoperiod. When adventitious shoots formed from the calli of hairy roots were 3–4 cm long with 3 leaves and were excised and transferred into ½ MS medium at 25^{0}C under a 14 h photoperiod for root induction. 36 shoots were each transferred into the rooting medium. Rooting percentage (the rooted shoots/total shoots × 100%) and the frequency of rooting (mean number per shoot) were recorded after 2 weeks. 30 regenerated plantlets were subjected to acclimation, transplanted to potting soil, then kept in a greenhouse.

PCR analysis of hairy root-regenerated plants

The method of PCR analysis was the same as that of hairy roots, but using the hairy root-regenerated plant as template and the non-transformed plant in flask as control. The amplified products were detected by ethidum bromide staining after 0.8% agarose gel electrophoresis.

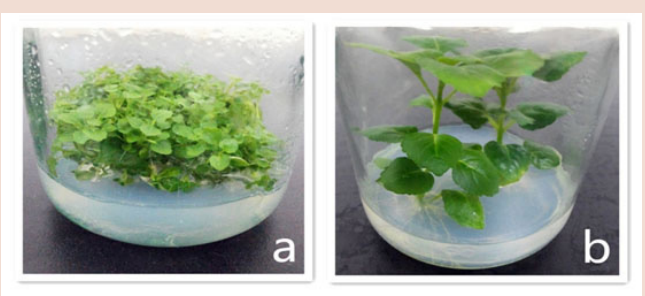

Figure 1: Induction of *P. cablin* aseptic seedling a. Calli and clump buds of *P. cablin* b: Seedlings of *P. cablin*.

RESULTS

Induction and culture of P. cablin seedlings

Leaves of *P. cablin* were cultured about 20 days, calli were induced and buds formed (Figure 1-a), buds elongated and shoots were transferred into 1/2 MS medium for rooting. Well-growth seedlings would be got after 30 days (Figure 1-b). The leaves of these seedlings were materials for hairy root-induction.

Effect of pre-culture duration on transformation frequency

Recent reports suggest that pre-culturing may influence the transformation frequency.[17-18] Prior to infection with *A. rhizogenes*, stem sections were pre-cultured in MS medium for a varying period from 0 to 3 days, after which the standard procedure described in methods was used for the remaining part of the assay.

Transformation frequency differed depending on pre-culture time as shown in Table 1. The results demonstrated that the transformation frequency could be improved after pre-culturing. The highest transformation frequency of two strains (> 80%) were both observed after a 2 days pre-culture. The transformation frequency declined with an extended pre-culture time, with a 3-day pre-culture resulting in a decline of the transformation frequency. Thus, a 2-day pre-culture was used to test the effects of the following parameters on the transformation frequency.

Table 1: Effect of pre culture time on induction rate of *P. cablin* hairy roots

Pre culture time (d)	*A. rhizogenes*	Number of explants	Number of explants with roots	Transformation rate (%)
1	ATCC15834	36	27	75.0
	C58C1	36	24	66.7
2	ATCC15834	36	30	83.3
	C58C1	36	29	80.5
3	ATCC15834	36	24	66.7
	C58C1	36	21	58.3

Effect of acetosyringone (AS) concentration on induction rate of P. cablin hairy roots

The concentration of AS had influence on induction rate of *P. cablin* hairy roots (Table 2). Induction rate of *A. rhizogenes* ATCC15834 and C58C1 both reached the highest with 15 mg l^{-1} AS. *A. rhizogenes* couldn't recognize the host cells with lower concentration of AS, and the toxic effect of higher concentration of AS would block the combination of *A. rhizogenes* and the host cell.

Table 2: Effect of *A. rhizogenes* strain with their ability to induce hairy roots on various concentrations of acetosyringone (AS)

AS (mg/L)	*A. rhizogenes*	Total number of explants	Number of explants with roots	Transformation rate (%)
10	ATCC15834	36	21	58.3
	C58C1	36	9	25.0
15	ATCC15834	36	25	68.1
	C58C1	36	18	50.0
20	ATCC15834	36	5	13.9
	C58C1	36	16	44.4

Table 3 : Effect of infection time on induction rate of *P. cablin* hairy roots

Infection time (min)	*A. rhizogenes*	Number of explants	Number of explants with roots	Transformation rate (%)
10	ATCC15834	36	25	68.1
	C58C1	36	18	50.0
15	ATCC15834	36	21	58.3
	C58C1	36	24	66.7
20	ATCC15834	36	26	72.2
	C58C1	36	28	77.8
25	ATCC15834	36	29	80.5
	C58C1	36	29	80.5
30	ATCC15834	36	24	66.7
	C58C1	36	23	63.9

Table 4 : Effect of co-culture time on induction rate of *P. cablin* hairy roots

Co culture time (d)	*A. rhizogenes*	Number of explants	Number of explants with roots	Transformation rate (%)
1	ATCC15834	36	15	41.7
	C58C1	36	14	38.9
2	ATCC15834	36	30	83.3
	C58C1	36	29	80.5
3	ATCC15834	36	20	55.6
	C58C1	36	21	58.3

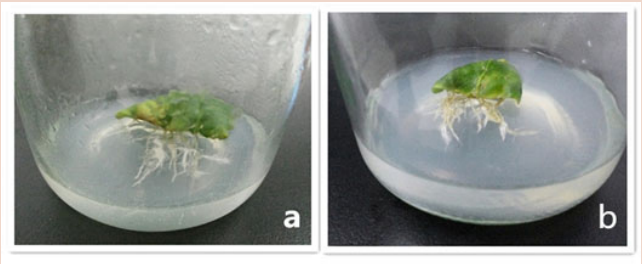

Figure 2: Hairy roots of *P. cablin* induced by *Agrobacterium rhizogenes* ATCC15834 and C58C1 a : Hairy roots of *P. cablin* induced by ATCC15834; b : Hairy roots of *P. cablin* induced by C58C1.

Effect of infection time on induction rate of P. cablin hairy roots

Both *A. rhizogenes* strains reached the highest transformation rate after 25 min infection (Table 3).

Effects of co-culture time on induction rate of P. cablin hairy roots

After infection, the explants were placed on the hormone free MS medium to allow T-DNA transfer from the plasmid into plant cells. Co-cultivation duration was tested in order to assess its impact on transformation frequency, the stem sections with one node were precultured for 2 days, infected with *A. rhizogenes* corresponding to OD600 around 1.0 and then placed on MS medium at 25°C for 1, 2 or 3 days. After this, the explants were placed on MS medium containing 500 mg l^{-1} cefotaxime for hairy root production. As shown in Table 4, both two highest transformation frequency (83.3% and 80.5%) were achieved with a 2-day co-cultivation. The transformation frequency was lower at both shorter and prolonged co-cultivation.

Figure 3: PCR analysis of *P. cablin* hairy roots. M : DL 2000 DNA marker; a1, a2, a3: fragment amplified from *P. cablin* untransformation root; b1, b2, b3: fragment amplified from hairy roots of *P. cablin* induced by ATCC15834; c1,c2,c3: fragment amplified from hairy roots of *P. cablin* induced by C58C1.

Induction of hairy roots

When leaf explants were inoculated with freshly grown *A. rhizogenes* suspensions in MS medium, hairy roots were induced directly from the cut edges of leaf explants (Figure 2). The first adventitious roots were visible 8 days after inoculation. With increasing incubation time, the percentages of rooted leaf explants also increased. The frequency of explants with adventitious root formation 25 days after inoculation with *A. rhizogenes* ATCC15834 and C58C1 were about 83.3% and 80.5% respectively. The hairy roots had characteristics of transformed roots such as high lateral branching and lack of gravitopism. As a control, adventitious roots excised from *P. cablin* sterile plants were cultured on solid, growth regulator-free MS medium, these roots without lateral roots were observed to grow very slowly and died after 18 days.

Hairy roots were placed on different mediums like MS, 1/2MS, 6-7V and B5, it was found that hairy roots grew slowly in these mediums, but slowly browned in 6-7V relatively.

PCR analysis of hairy roots

Rol B is one gene of the TL-DNA (T-DNA left arm) of Ri plasmid in *A. rhizogenes*. In this study, by using DNAs from the hairy roots as template and the non-transformed roots as control, PCR products amplified with rol B primers, could be detected (Figure 3). It was demonstrated that rol B fragment (423bp) was amplified from hairy root cultures and the colony of *A. rhizogenes* but not from untransformed roots. The result indicated that the rol B gene from the Ri plasmid of *A. rhizogenes*

Table 5 : Results of callus induction of hairy root induced by *Agrobacterium rhizogenes* ATCC15834

No.	6-BA/ (mg/L)	NAA/ (mg/L)	Number of hairy roots	Number of calli	Induction rate of calli(%)	Growth state
1	0.0	0	15	0	0.00	No calli
2	1.0	0	15	10	66.67	Light-green, loose
3	2.0	0	15	12	80.00	Light-green, compact
4	3.0	0	15	12	80.00	Light-yellow, loose
5	2.0	0.1	15	15	100.00	Light-yellow, loose
6	2.0	0.2	15	14	93.33	Light-green, compact

Table 6 : Results of callus induction of hairy root induced by *Agrobacterium rhizogenes* C58C1

No	6-BA/ (mg/L)	NAA/ (mg/L)	Number of hairy roots	Number of calli	Induction rate of calli(%)	Growth state
1	0	0	15	0	0.00	No calli
2	1.0	0	15	10	66.67	Light-green,compact
3	2.0	0	15	13	86.67	Light-green,loose
4	3.0	0	15	12	80.00	Light-green,compact
5	2.0	0.1	15	15	100.00	Light-yellow,loose
6	2.0	0.2	15	13	86.67	Light-yellow,compact

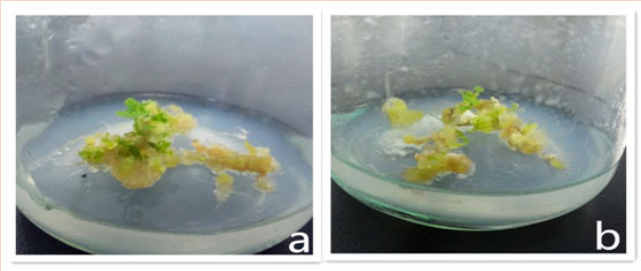

Figure 4: Calli from hairy roots of *P. cablin* a : Calli from hairy root induced by Agrobacterium rhizogenes C58C1 : b : Calli from hairy root induced by Agrobacterium rhizogenes ATCC15834.

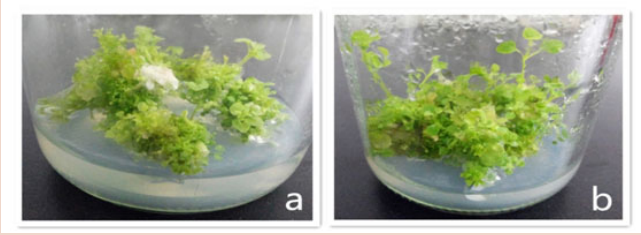

Figure 5: Buds induction from callus of *P. cablin* hairy root a : Buds induction from Callus of hairy root induced by Agrobacterium rhizogenes C58C1; b : Buds induction from Callus of hairy root induced by Agrobacterium rhizogenes ATCC15834.

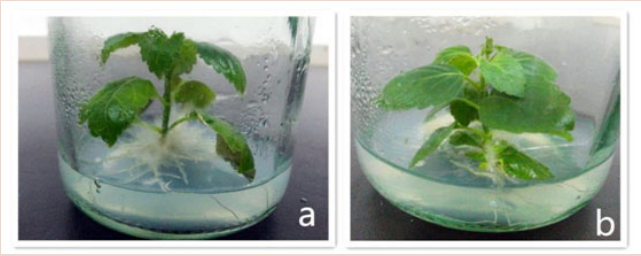

Figure 6: Regeneration plant of hairy root induced by *P. cablin* a : Regeneration plant of hairy root induced by Agrobacterium rhizogenes C58C1; b : Regeneration plant of hairy root induced by Agrobacterium rhizogenes ATCC15834.

ATCC15834 and C58C1 was integrated and expressed into the genome of *P. cablin* hairy roots.

Plant regeneration from hairy roots

When 2-3 cm root segments excised from the transformed hairy root were transferred into the callus induction medium for 7 days, the segments of hairy roots began to swollen and formed small light-green calli from the cut ends. After 30 days, the formation rates of calli were counted (Table 5 and 6).

The highest rate of calli induction was obtained at MS+2.0 mg l^{-1} BA+0.1 mg l^{-1} NAA, where the hairy roots could form light-yellow and loose calli. Some calli began to differentiate adventitious buds (Figure 4).

When transferred into shoot inducing medium MS+0.1 mg l^{-1} BA+0.1 mg l^{-1} NAA for 35 days, they began to form adventitious buds at a frequency of 100%, a lot of buds were generated and grew well (Figure 5).

When adventitious shoots were 3-4 cm height with 3 leaves, strong shoots were chosen and transferred to 1/2MS medium for rooting. Cultures grown in complete darkness had thickened roots. The regenerated shoots were vigorous in appearance with dark-green and wide leave (Figure 6). Wrinkling of leaves in hairy root-transformants is common and has been reported in several species as a symptom developed after insertion of the Ri T DNA of *A. rhizogenes*.[19-20] Mei[21] reported wrinkle-free normal leaves in transformed plants of *Alhagi pseudoalhagi*.

And there were many adventitious roots with high lateral branching and lack of geotropism formed on the contact surface of the regenerated plant and the medium.

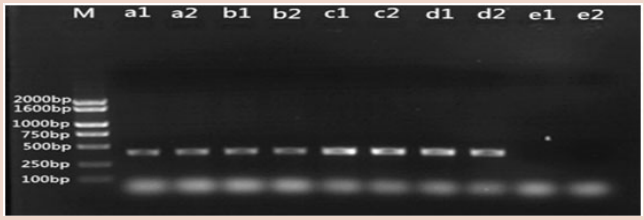

Figure 7: PCR analysis of regeneration plant of hairy root induced by *P. cablin* M: 100 bp DNA marker; a1, a2: fragment amplified from regeneration plant 1 of hairy root induced by *A. rhizogenes* ATCC15834; b1, b2: fragment amplified from regeneration plant 2 of hairy root induced by *A. rhizogenes* ATCC15834; c1, c2: fragment amplified from regeneration plant 1 of hairy root induced by *A. rhizogenes* C58C1; d1, d2: fragment amplified from regeneration plant 2 of hairy root induced by *A. rhizogenes* C58C1; e1, e2: fragment amplified from *P. cablin* aseptic seedling.

PCR analysis of regeneration plant

PCR products amplified with rol B primers could be detected (Figure 7). It was demonstrated that rol B fragment (423bp) was amplified from the regenerated plant of hairy root but not from untransformed plant. The result indicated that the rol B gene from the Ri plasmid of *A. rhizogenes* ATCC15834 and C58C1 was integrated and expressed into the genome of regenerated plant of *P. cablin* hairy roots. The rooted plantlets were transferred to greenhouse conditions where they showed about 95% survival.

DISCUSSION

A. rhizogenes causes hairy root disease in plants. The neoplastic (cancerous) roots produced by *A. rhizogenes* infection are characterized by high growth rate, genetic stability and growth in hormone free media.

These genetically transformed root cultures can produce levels of secondary metabolites comparable to that of intact plants. Hairy root cultures offer promise for high production and productivity of valuable secondary metabolites (used as pharmaceuticals, pigments and flavors) in many plants.[22] But the introduction of hairy roots still can be affected by many factors.

AS is an amino acid derivative and a phenolic compound, which must be constant for biological activity and maintenance of vir gene expression in Muskmelon explants.[23] Many other reports also pointed out that the vir genes are inducible in response to the monocyclic phenolic compound like AS.[24] The results obtained from higher (20 mg l-1) concentration of AS indicated an inhibitory action rather than stimulatory.

A. rhizogenes C58C1, a disarmed *Agrobacterium tumefaciens*, is widely used for the production of important pharmaceutical or aromatic chemicals from many medicinal plants, such as *Anisodus acutangulus,*[25] *Linum Album*[26] and *Camptotheca acuminate.*[27] Wang reported that camptothecin and 10-Hydroxy-camptothecin detected in the hairy roots inducted from C58C1 was the highest and C58C1 was the best one in the study of effects of different strains (including A4, 15834, R1601 and C58C1) on induction of hairy roots from *Camptotheca acuminate.*[27] In our study, *A. rhizogenes* C58C1 could induce the hairy roots of *P.cabin*.

Pre-culture time had some effect on the transformation rate. The cut on the leave would go brown after infection, which could cause low induction rate. But after a period of pre-culture, the cell of cut in explants began to divide and get into competence which could increase the transformation rate. In our study, 2 days was the best processing time, but Lu reported 3 days was the best pre culture time for transformation in hairy roots induction of *Hyoscyamus niger,*[28] and the transformation rate would decrease sharply with the pre culture time over 3 days.

Although hairy roots were induced successfully by ATCC15834 and C58C1, those hairy roots didn't grow fast. PCR analysis indicated that the hairy root gene had been transferred into, so it was speculated that TR-DNA which was responsible for the hormone synthesis was not transformed into hairy roots. It was suspectd that *A. rhizogenes* may have some effects on the second metabolites of the regenerated plants of hairy roots, so the chemical constituents of the regenerated plants would be studied further.

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ABBREVIATION USED

BA: Benzyladenine, **MS:** Murashige and Skoog, **NAA:** Naphthaleneacetic acid, **Ri:** Root inducing, **rol B:** Rooting locus B, **rol C:** Rooting locus C, **YEB:** Yeast Extract Broth, **AS:** Acetosyringone, **PCR:** Polymerase Chain Reaction.

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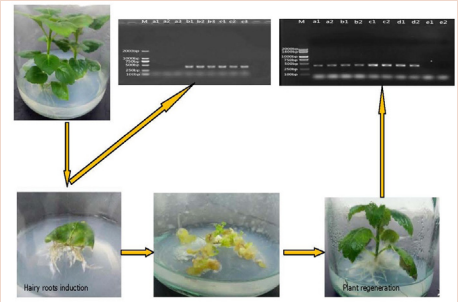
PICTORIAL ABSTRACT



SUMMARY

- Hairy roots formed from the cut edges of leaf explants by using *Agrobacterium rhizogenes* ATCC15834 and C58C1, but the hairy roots grew slowly.
- Plants could be regenerated from the hairy roots induced by *Agrobacterium rhizogenes* ATCC15834 and C58C1.
- The hairy roots and plants regenerated both contained rol B fragment of *Agrobacterium rhizogenes*.

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