Original Research Article

Effect of different *Agrobacterium rhizogenes* strains on *in-vitro* hairy root induction for cucurbitacin E production in *Citrullus colocynthis*

ABSTRACT

The present investigation examined two-fold higher accumulation of cucurbitacin E in hairy roots compared to normal *in vitro* root cultures. Leaf explants were more suitable for production of hairy roots through *A. rhizogenes*. This protocol facilitates mass production of bioactive compounds from medicinal plant roots. Hairy root cultures of *C. colocynthis* were established after infecting cotyledon, leaf, stem and callus segments with *Agrobacterium rhizogenes* strains-A4, Ar532, ARqua1 and R1000 were cultured on Murashige and Skoog phytohormone-free media. Among all the *Agrobacterium*- strains used, R1000 induced hairy roots more effectively with a frequency of transformation of 80.1% in leaf explants. Addition of 150 µM acetosyringone to the co-cultivation medium resulted in a two-fold induction of root hairs from leaf explants infected with strain R1000, followed by 20 min and three days of co-cultivation also demonstrated to increase the percentage of infection. Cefotaxime at a concentration of 300 mg/L was found to be optimum for the hairy root production without any damage to explants and control the growth of bacterial culture.

Keywords

Agrobacterium rhizogenes; Citrullus colocynthis; Cucurbitacin E; Transformation

1 INTRODUCTION

Citrullus colocynthis (L.) Schard is a medicinal plant widely grown outside and in Asian countries such as India and Turkey, a great multipurpose plant for health promotion. The whole plant is widely used in folk and Ayurvedic medicine. The plant contains several bioactive compounds, including cucurbitacin E, which is a very effective anticancer agent [1]. Today's market has a growing demand for natural and renewable commodities that have turned attention to *in vitro* plant materials as potential sources of secondary phytochemical products. This paved the way for new investigations to investigate the expression of *in vitro* secondary product [2].

The extract of *C. colocynthis* inhibits the growth of human breast cancer cells and inhibits the growth of ER-MCF-7 and ER-MDA-231 human breast cancer cell lines in the G2/M phase of the cell cycle [3, 4]. The root extract of *C. colocynthis* also revealed the heavy presence of steroids and flavonoids [5]. Similar or comparable, hairy roots exhibit a diverse range of enhancements in the production of physiologically active chemicals compared to regular plant roots. Hairy root culture promotes the production of plant derivatives, illuminates biosynthetic pathways, therapeutic proteins, and enhances phytoremediation efforts under eco-friendly conditions [6, 7].

The process of *in vitro* transformation of root cultures using soil-based natural bacteria *A. rhizogenes* is now considered as a new biotechnology tool for over production of plant secondary metabolites from a wide variety of plant species [8]. Non fundamental components produced in

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very little amount during plant growth are known to be secondary metabolites [9]. To meet the necessary demand, they are difficult to produce and procure from agriculturally grown plants. Therefore, to overcome this problem, it is necessary to find other ways to meet the demand of these important compounds [10].

The hairy root system shows the combined advantages of rapid growth of cell suspension culture and whole plant development in controlled sterile environments. However, hairy roots grown *in vitro* are genetically and biochemically stable and free from various contaminants such as pathogens and herbicides. Primary improvement of the hairy root system is the ability of the roots to develop in a hormone-free medium; the culture system is also unaffected by their general environment [11]. Hairy roots sometimes exhibit the advantage of accumulating metabolites absent in native plants [12] Georgiev and others also reported the application of the hairy root system in the field of physiological processes and biosynthetic pathways [7].

In the present study, we have examined the responses of different *A. rhizogenes* strains for the hairy root production and evaluated the concentration of a bioactive compound that is Cucurbitacin E from these cultures. Inoculation of *A. rhizogenes* was performed with different explants of *C. colocynthis*.

2 Material and Methods

The seeds of *C. colocynthis were* collected from the surrounding area and germinated plants were maintained in the departmental greenhouse (18.0264138°N, 79.5589066°E). The explants (leaf, stem, node, cotyledons, and shoot tips) were collected from polyhouse grown plants, which were thoroughly washed under the running tap water. Then the explants were surface sterilized by 0.1% fresh aqueous mercuricchloride ($HgCl_2$) solution for 1-2 minutes, subsequently washed thoroughly with sterile distilledwater to remove any traces of chemical sterilant. MS medium was prepared and the pH of the medium was adjusted to 5.7 either by adding 0.1% NaOH (or) 1N HCl before autoclaving. About15-20 ml of the medium were dispensed in each culture tube and sealed with non-absorbent cotton plugs prior to autoclaving at 121°C for 15 min under 15 lbs pressure. The leaves shootapex and nodal explants were inoculated onto MS solid medium supplemented with 2.0 mg/L 2, 4-D and 1.0 mg/L Kn for callus induction.

2.1 Bacterial strains

A. rhizogenes strains R1000 and A4 were obtained from Prof. Andy Ganapathi Bharathidasan University, Ar532 from MTCC Chandigarh and ARqua1 from BRC, University of Szeged, Hungary. These strains were used for transformation of Ri-plasmid into *C. colocynthis* to produce hairy roots.

2.1.1 Development of *Agrobacterium rhizogenes* inoculums

The different cultures of *A. rhizogenes* strains R1000, A4 and Ar532 were taken from glycerol stock and initiated to grow in liquid Luria-Bertani (LB) medium and ARqua1strain in Tryptone-Yeast (TY) medium for 48 hrs at 28ºC with 120 rpm up to mid- log phase (OD at 600 nm = 0.5). The *A. rhizogenes* cells were collected by centrifugation and poised in liquid inoculation MS medium with 3% sucrose. The *A. rhizogenes* cell density was adjusted to 0.5 to 1.0 at 600 A^{o} for inoculation.

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2.1.2 Hairy root induction

An individual colony of the selected type *A. rhizogenes* strains R1000, A4, Ar532 and ARqua1were grown in LB medium and TY medium separately and collected cell pellet was washed thrice with half strength liquid MS medium and the re-suspended bacterial cultures were used for infection. The cotyledon, leaf and stem explants were pierced with a sterile hypodermic needle and immersed into the bacterial culture for 20 minutes and blotted on sterile tissue paper for 10 minutes. Blotted explants were shifted to half-strength MS medium fortified with 100µM acetosyrigone, 3% sucrose and 0.8% agar, maintained at 25± 2ºC in dark condition. These explants were co-cultivated for different time intervals of 10, 15, 20, 25, 30 minutes.

The treated explants were washed with sterilized distilled water, followed by MS liquid medium containing cefotaxime (300 mg/L) after the Co-cultivation phase. Later after 15– 20days of culture on the half strength MS solid media fortified with cefotaxime, the hairy roots were started appearing at the infected sites of the explants. These putative hairy root explants were shifted to half strength MS medium fortified with 3% sucrose, 0.8% agar and 300 mg/L cefotaxime until the left over bacteria have been exclusively killed.

2.1.3 Assay for β-glucuronidase *(GUS)* activity and statistical analysis

Transient *GUS* expression was examined after initiation of hairy roots from different explants as per Jefferson et al., (1987). Histochemical staining was performed using *GUS* assay buffer (1g/L^{-1} X-Gluc - 5-Bromo 4-chloro 3-indolyl β- glucuronide - cyclohexyl ammonium salt, 0.05 M Na_2HPO_4, 10 mM EDTA and 0.1 % (v/v) Triton X-100) by incubating the hairy roots at 37°C for 24–48h. Thus contaminated tissue samples were denatured using glacial acetic acid: alcohol (1:3v/v). After staining, blue foci (spots) were examined under a dissecting microscope and were scored to test the efficiency of transient GUS expression; non transformed roots were used as control. Each treatment consists of at least three replicates and was duplicated thrice. Total DNA from hairy roots and control or non-transformed roots was used for the molecular analysis in order to confirm the integrity of transgenes into the host plant genome.

The genomic DNA was extracted following the CTAB (Cetyl trimethyl ammonium bromide) method from the roots of both transgenic and normal plants of *C. colocynthis*. Isolated DNA was amplified using C1000TM programmable PCR thermal cycler with 5′CCCCAACCCGTGAAATCAAAAAACT3′ as forward primer and 5′CCCTGCTGCGGTTTTTCACCGAAGT3′ as reverse primer. The PCR reaction mixture (50µl) consisted of 100 mg of DNA, 10×PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs, 10 pmol of each specific primers and 1 U of Taq DNA polymerase. The DNA amplification program was designed using the following conditions, 94ºC for 5 mins as initial denaturation, followed by 30cycles at 94°C for 30s, 55°C for 45s for annealing, primer extension at 72°C for 2 mins and a final extension at 72°C for 5 mins. The amplified PCR products were separated using 1% agarose gel and visualized by gel documentation system.

2.2 Extraction of bioactive compound (CucurbitacinE) and HPLC analysis

The *in vitro* roots (control and hairy) were collected, dried and powdered separately. 5gms of each powder was suspended in 50 ml of methanol separately and was incubated for two days in orbital

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shaker, then filtered by Whatman No.1 filter paper. The extracted solvents were dried and re-suspended in DMSO. The samples were filtered using 0.2µ syringe filter and then analyzed with C18 column in Shimazu liquid chromatography. The compound was detected by injecting 20µl of the sample through ultraviolet spectrometric detector and the columns used were maintained at a controlled ambient temperature (18-20°C). Solvents acetonitrile and water used are of HPLC grade. Crude plant extracts usually contain complex mixture of different bioactive constituents, whereas the cucurbitacin E was evaluated quantitatively in hairy roots cultures by comparing with standard curve of cucurbitacin E.

3 Results and Discussions

3.1 Significance of *Agrobacterium* strains on induction of hairy root

Four different *A. rhizogenes* bacterial strains (A4, Ar532, ARqua1 and R1000) were used for co-cultivation with leaf, cotyledon, stem and callus explants (Fig.1). All the four bacterial species showed notable transformation efficiency in *C. colocynthis* plant. All these strains produced hairy roots at the infected sites of explants, whereas the effectiveness of transformation is diverse between different bacterial strains. The study was performed by infecting 180 explants of each variety with different *A. rhizogenes* strain. Among all the *A. rhizogenes* strains used, R1000 exhibited more induction of hairy roots in all type of explants in a short time period. Four strains showed the frequency of transformation in their descending order as R1000, ARqua1, A4 and Ar532 (Fig. 2, 3), of which R1000 showed the best results with a frequency of 80.1%, followed by ARqua1, A4 and Ar532 with 74%, 67.7% and 63.3% respectively (Table 1).

The *Agrobacterium rhizogenes* strains were reported to show varied levels of virulence in different plant species and the transforming frequency was also found to vary among different plant parts [13]. The results of the present study were found to be in accordance with the previous reports. Similarly, the R1000 strain was also found to be efficient for hairy root induction in species like *Withania somnifera*, where high frequency of hairy roots (88%) was obtained with R1000 strain, followed by A4 stain with 79% [13]. Marwani and others also reported that R1000 strain was found to induce high frequency infection (60%) in cotyledonary explants of *Andrographis paniculata* [14]. *A. rhizogenes* strains such as MSU 440 and R1000 are considered as high virulence and ARqua1 and K599 are relatively low virulence [15].

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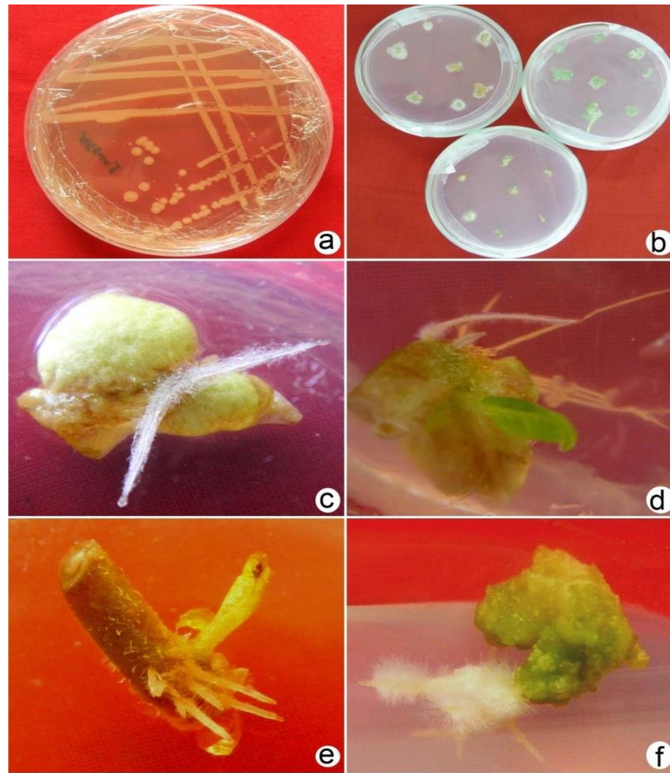

Figure 1. **Genetic transformation through *Agrobacterium rhizogenes*in *C. colocythis* (a) *Agrobacterium rhizogenes* strain, (b) Co-cultivation of different explants, (c) hairy rootinitiation from cotyledon explants, (d) hairy root initiation from leaf explants, (e) hairy root initiation from stem explants, (f) hairy root initiation from callus.**

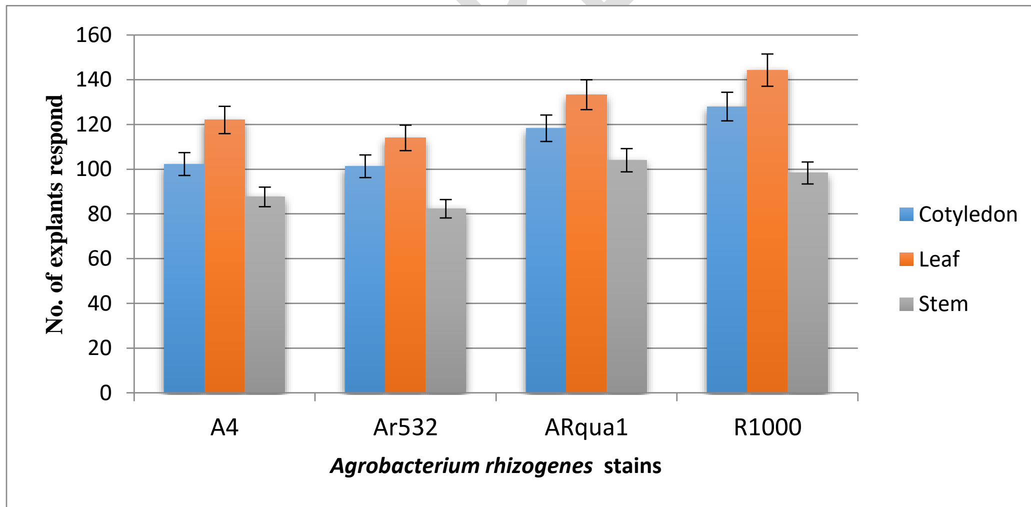

Figure 2. **Effect of different bacterial strains for hairy root initiation on various explants of *C. colocynthis*.**

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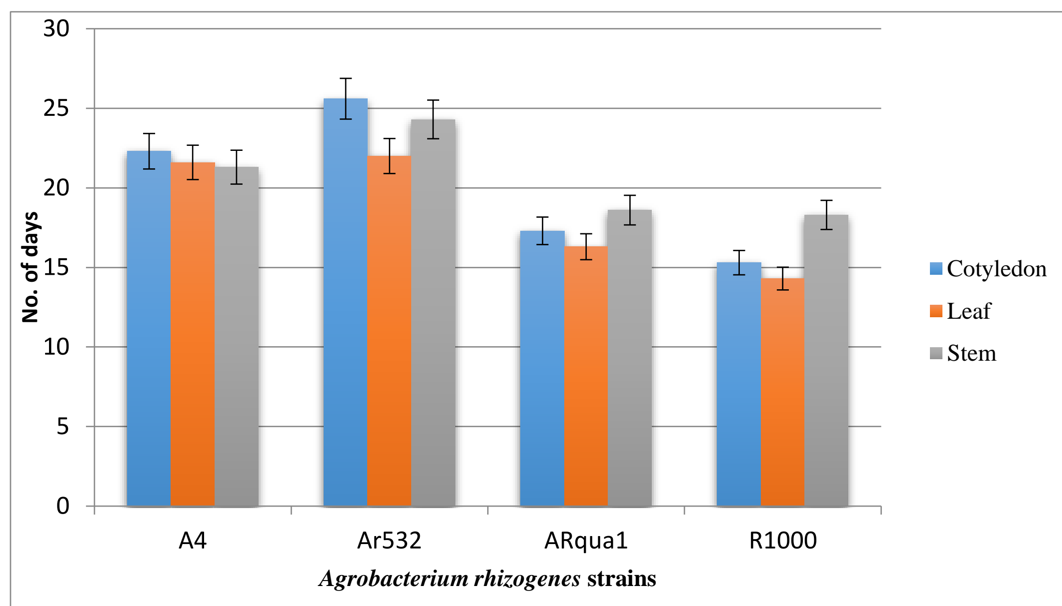

Figure 3. **Effect of time duration for hairy root initiation of various explants by using different *Agrobacterium rhizogenes* strains in *C. colocynthis.***

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Table 1: **Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots in *Citrullus colocynthis.***

Bacterial strains-explants	Total number of explants infected	Number of transformed explants	Frequency of transformation	No. of days for hairy root induction	No. of hairy roots
A4					
Cotyledon	180	102.33±0.88	56.8	22.33±0.33	4.33±0.33
Leaf	180	**122.00±0.57**	**67.7**	**21.67±0.33**	**8.33±0.33**
Stem	180	87.67±0.88	48.7	21.33±0.33	5.00±0.57
Ar532					
Cotyledon	180	101.33±0.88	56.2	25.67±0.33	3.33±0.33
Leaf	180	**114.00±0.33**	63.3	22.00±0.57	**8.00±0.57**
Stem	180	82.33±0.88	45.7	24.33±0.33	3.00±0.57
ARqua1					
Cotyledon	180	118.33±0.88	65.7	17.33±0.33	11.33±0.88
Leaf	180	**133.33±0.88**	**74**	**16.33±0.33**	**14.33±0.33**
Stem	180	104.00±0.57	57.7	18.67±0.33	8.00±0.57
R1000					
Cotyledon	180	128.00±0.57	71.1	15.33±0.33	10.33±0.88
Leaf	180	**144.33±0.66**	**80.1**	**14.33±0.88**	**16.33±0.33**
Stem	180	98.33±0.33	54.6	18.33±0.33	9.00±0.57

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3.2 The Significance of explants on induction of hairy roots

Cotyledon, leaf, stem and callus cultures were used as explants for testing all the four strains. Among all the explants tested, leaf explants were found to be efficient for production of superior number of hairy roots with four strains. The average numbers of leaf explants transformed were 144, 122, 114 and 133 upon infection with strains R1000, A4, Ar532 and Arqua1 respectively (Table 1, Fig. 2). Whereas in the cotyledon explants, maximum transformation frequency of 71.1%

was achieved with R1000 stain, followed by other three strains with 56.8%, 56.2% and 65.7% frequencies respectively.

Stem explants showed moderate results with all the bacterial strains tested and highest frequency (57.7%) was observed with Arqua1 strain, followed by R1000, A4 and Ar532 with 54.6%, 48.7% and 45.7% transformation frequency (Table.1). However, only negligible transformation rate was observed in callus treated with all bacterial strains and only R1000 initiated hairy roots in callus without further growth. The maximum amount of hairy root production was noted with the leaf explants.

The transforming efficiency of different plant parts was also found to differ. Among the explants tested, leaf explants exhibited greater transformation frequency with all the *A. rhizogenes* strains. Sivanandhan et al. reported that among leaf and cotyledon explants, the leaf explants had shown a greater number of roots with high transformation frequency in *Withania somnifera* [16]. Similar result of high frequency hairy root induction was achieved in leaf explants (89%) than cotyledon explants in *Cucumis anguria* [17]. However, in contrast, cotyledon explants showed maximum transformation frequency than leaf explants in *Andrographis paniculata* [14] and *Cucumis sativus* treated with *A. rhizogenes* [18].

3.3 The Significance of acetosyringone on induction of hairy root

Acetosyringone plays a vital function in induction of hairy roots with differences in time of infection. In present study, we observed the effect of acetosyringone on different bacterial strains and explants. Acetosyringone was added to the co-cultivation medium at different concentrations ranging from 0– 300 μ M. To assess the effect of acetosyringone concentration, the number of hairy roots initiated per explants was considered. Initially, co-cultivation carried without acetosyringone achieved negligible results. The addition of acetosyringone to co-cultivation media (50 μM to 150 μM) had led to gradual increase of hairy roots. Highest number of hairy roots was achieved at 150 μM concentration with all bacterial strains in all explants.

The addition of acetosyringone (150μM) to co-cultivation media doubled the hairy root induction in leaf explants infected with R1000 bacterial strain. The leaf, cotyledon and stem explants produced an average of 14.3, 11.3 and 8.0 hairy roots respectively.

Acetosyringone was extensively used as an enhancer for agrobacterium mediated hairy root induction [16]. The co-cultivation medium augmented with acetosyringone was found to induce hairy roots in cultured explants. Addition of acetosyringone to the media showed drastic improvement in explants response to *agrobacterium.* Similar, report was observed in *Berberis aristata*, where the hairy root production was improved in the leaf and callus explants when the co-cultivation media is enriched with acetosyrigone [19].

3.4 The Significance of infection and co-cultivation periods on induction of hairy root

The present study also evaluated the significance of infection and co cultivation periods on induction of hairy roots. The difference in infection and co-cultivation time intervals influenced the efficiency of the transformation. All the three types of explants were immersed in the bacterial culture for different time intervals like 10, 15, 20, 25, 30 minutes. Among these, 20 minutes

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infection time had shown maximum percentage of frequency in all experiments (Fig. 4). Findings of this experiment reflect that 15 minute of infection time also showed values nearer to 20 minutes infection time. Subsequent increase in the infection time has shown not much better results. Co-cultivation phase was checked in the leaf, stem and cotyledon explants with R1000 bacterial strain, where the explants were co-cultivated from the first day to 5th day differently. A co-cultivation for 3 days improved the effectiveness of the hairy root initiation. Co-cultivation period above 3 days resulted in adverse effect of over growth of bacteria on and around the co-cultivated explants (Fig. 5).

Co-cultivation is a period for successful transformation of plant. The period of co-cultivation varies among the species and type of agrobacterium strain used. Brijwal and Tamtaalso reported that the infection time period also plays the major role in the initiation of hairy roots in *Berberis aristata* [19]. Prolonged and shorter co-cultivation periods effect the transformation rate in explants. Co-cultivation of explants for 3 days was found to be successful in *C. colocynthis*. Similarly, in *Withania somnifera* co-cultivation period for 2ndand 3rd day was found to be appropriate for hairy root induction [20]. Whereas in the same plant, a co-cultivation for 5th day increased the efficiency of the hairy root induction in leaf explants [16].

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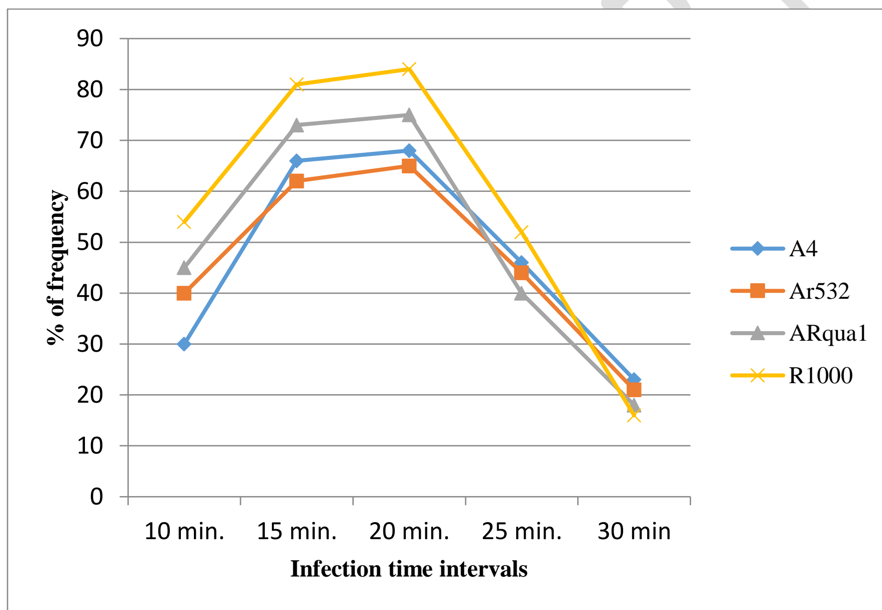

Figure 4. **Effect of different infection time for hairy root initiation in *C. colocynthis.***

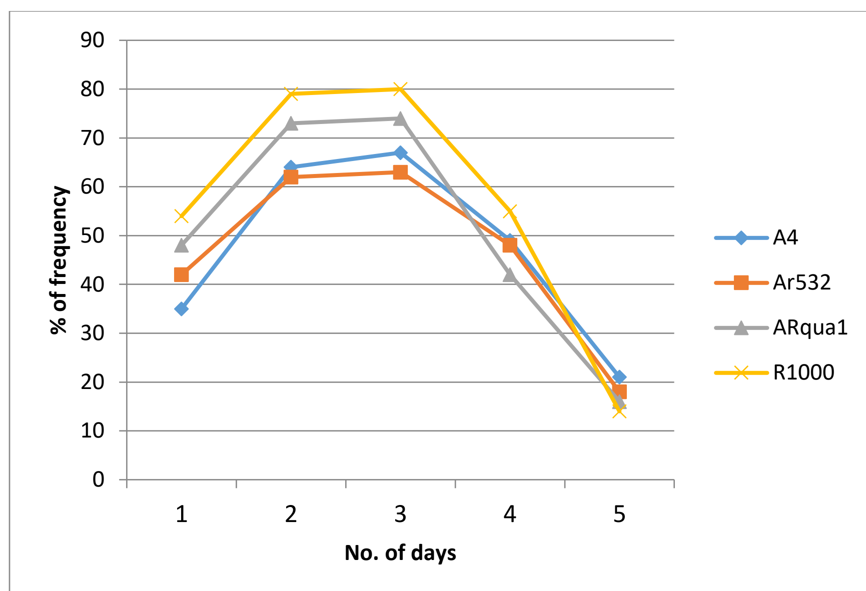

Figure 5. **Effect of Co-cultivation days for hairy root initiation in *C. colocynthis*.**

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3.5 The effect of cefotaxime on explants and bacterial strains

Varying concentrations (100, 200, 300, 400 and 500 mg/L) of cefotaxime antibiotic was added to MS medium after co-cultivation. Among these concentrations, 300 mg/L cefotaxime was found optimum for the hairy root production without damage to explants and no evidence of bacterial growth. Below 300 mg/L concentration, bacterial growth was observed after 3-5 days in every transformation and above 300 mg/L concentration, there is no growth of bacteria, but the explants have color changed initially and after one week all type of explants were totally dried. The time period to dry out also differed in various explants, like the leaf was dried very soon compared with cotyledon and stem explants.

Cefotaxime was the commonly used antibiotic for agrobacterium mediated transformation. But higher concentration of cefotaxime may induce negative effects on cultured explants, so it is necessary to optimize the concentration of cefotaxime for efficient transformation [21]. Similar concentrations of cefotaxime (300 mg/L) were used for bacterial growth in *Artemisia annua* [22]. Our results were in accordance with results obtained in the optimization of cefotaxime in *agrobacterium* mediated transformation in apple [23].

3.6 Histochemical analysis of *GUS* activity

Transgenic hairy root induction by *A. rhizogenes* in *C. colocynthis* was analyzed by histochemical method. Consistent *gus* activity was induced independently by ARQUA1 strain of *A. rhizogenes*. The PCR amplification of DNA reputed transgenic lines using *GUS* based primers has shown positive result with a banding pattern corresponding to 540 bp, whereas the respective band was not observed with DNA from the roots of control plants (Fig. 6).

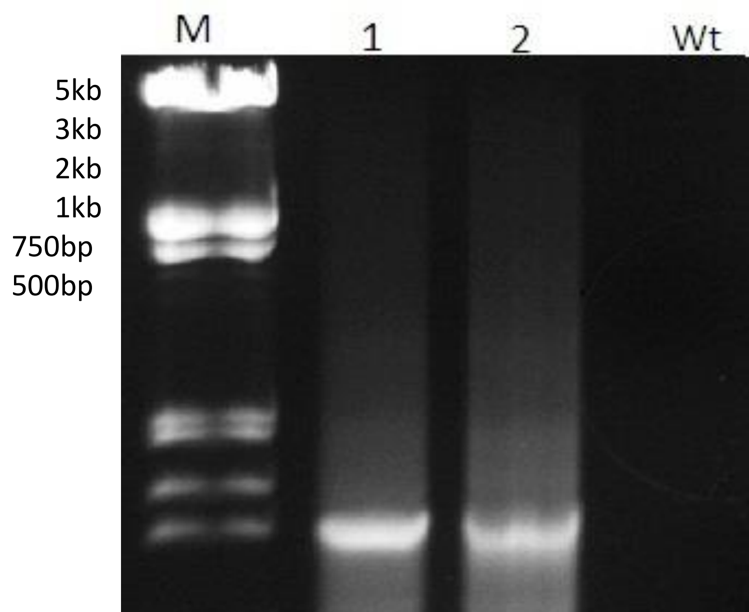

Figure 6. **PCR conformation of *GUS* showing 540bp amplicon.Lane (M): Marker, Lane(1&2):Transformed root, Lane (3): Control.**

3.7 Analysis of cucurbitacin E from Hairy root cultures

Analysis of a crude hairy root extract was performed to estimate the presence of cucurbitacin E, an oxidated steroid consisting of a tetracyclic triterpene. *C. colocynthis* hairy roots were shade dried and ground into powder. Extracted with the methanolic solvent system, the extract was dried and re-dissolved in DMSO. The cucurbitacin E present in the extracted solution was examined by high performance liquid chromatography with a C18 column and acetonitrile : water (60:40) was used as the mobile phase with a flow rate of 1ml/min. At 275 nm UV detection andisocratic elusion was chosen. The results were compared with the standard cucurbitacin E at a concentration of 1mg/ml, 20µl of standard cucurbitacin E injected in to HPLC column, for 20µg of cucurbitacin E standard, retention time is 7.6 to 7.9 min. with the area 1773.76 mAU and percentage of 31.73 inside entire peaks. Control (*in vitro*) roots were checked in parallel with hairy roots based on standard peak area with known concentration of cucurbitacin E. The concentrations of *in vitro* roots and hairy roots were 6.46 µg with peak area 523.65 mAU and 12.04 µg with peak area 1068 mAU, with percentage of peaks were observed 2.08 and 8.02 respectively (Fig. 7, 8, 9).

Cucurbitacin E was found to possess anticancer potential by inducing apoptosis and arresting the cell cycle at G2/M [24].The amount of cucurbitacin E was found to increase in hairy roots and was analyzed by HPLC method. The similar method of determination was employed to extract the cucurbitacin E from *Gratiola officinalis* using HPLC [25].

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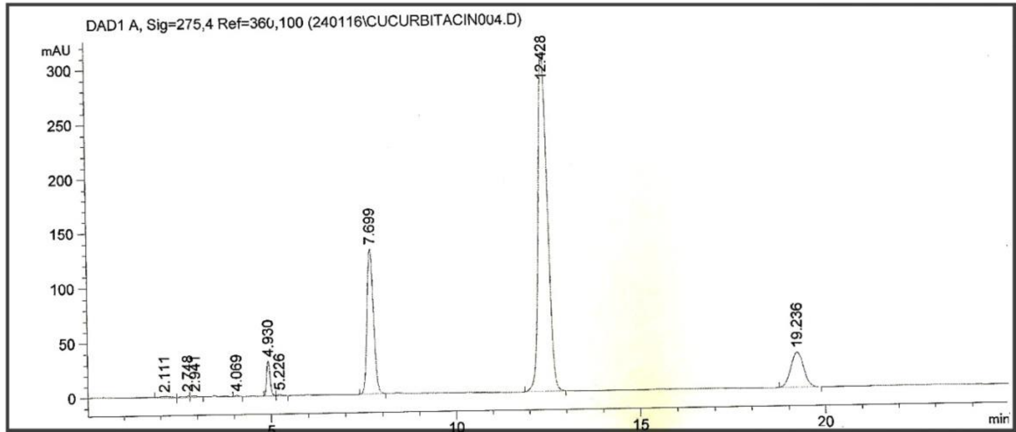

Figure 7. **HPLC chromatogram of standard Cucurbitacin E.**

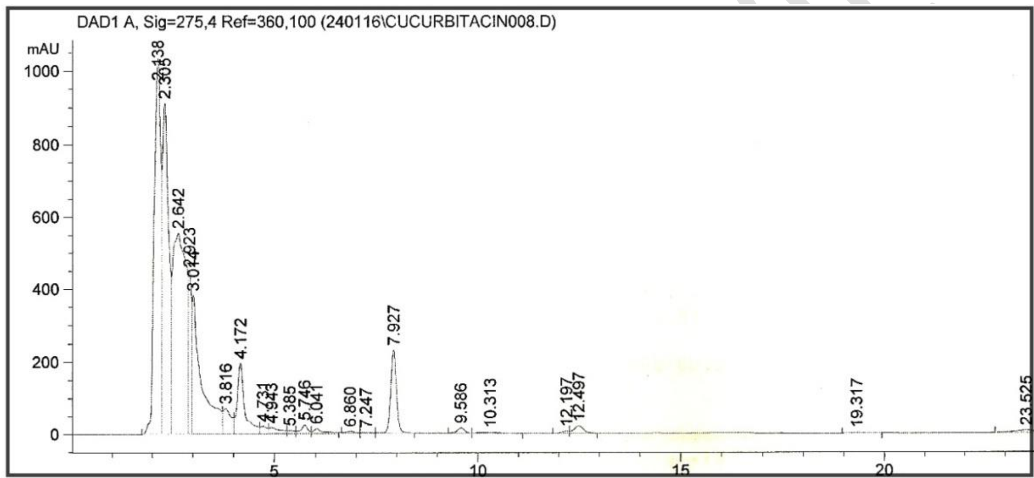

Figure 8. **HPLC chromatogram of *in vitro* roots extract of *C. colocynthis*.**

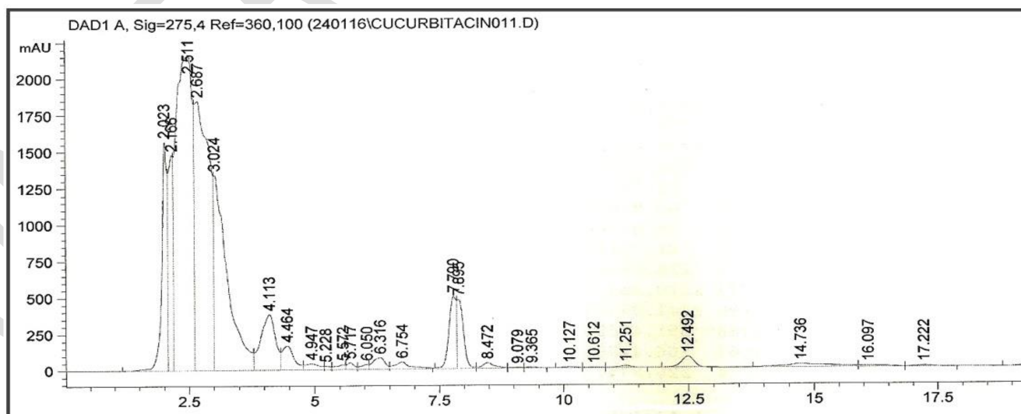

Figure 9. **HPLC chromatogram of hairy roots extracts of *C. colocynthis*.**

4 Conclusion

The present investigation describes the practice of hairy root induction in *C. colocynthis* and the effect of various strains, concentrations of acetosyringone, cefotaxime, infection and co-cultivation time period on explants. Among all the four strains, R1000 was highly virulent and shown maximum hairy root frequency. Leaf explants were more suitable for production of hairy roots through *Agrobacterium rhizogenes*. Infection of explants for 20 minutes gave efficient results in hairy root induction and co-cultivation for 3 days was the optimum time for good results. For control of over growth after infection, 300 mg/L cefotaxime is sufficient for all explants tested. Bioactive compound cucurbitacin E extracted from *in vitro* roots and hairy root cultures exhibited that the hairy roots produced double the concentration than control. Thus, the present study described the induction of hairy root protocol and the presence of cucurbitacin E in hairy roots. This protocol facilitates mass production of bioactive compound (Cucurbitacin E) in medicinal plants.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Abbreviations

A. rhizogenes : *Agrobacterium rhizogenes*

C. colocynthis : *Citrullus colocynthis*;

MS : Murashige and Skoogs medium; Kn- Kinetin;

LB : Luria-Bertani

TY : Tryptone–Yeast

MTCC : Microbial Type Culture Collection

HPLC : High Performance Liquid Chromatography

DMSO : Dimethyl sulfoxide

GUS : β-glucuronidase

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PCR : Polymerase Chain Reaction

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