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Original article

Effect of different *Agrobacterium rhizogenes* strains for *in-vitro* hairy root induction, total phenolic, flavonoids contents, antibacterial and antioxidant activity of (*Cucumis anguria L.*)

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ABSTRACT

The present study aimed to investigate the effect of different *Agrobacterium rhizogenes* on the induction of hairy root of *Cucumis anguria* and determine its total phenolic, flavonoids contents, antibacterial and antioxidant activity. In this investigation *A. rhizogenes* strains such as, 15834, 13333, A4, R1200, R1000, LBA9402, R1301 and R1601 are all investigated, were developing hairy root conception in cotyledon and leaf tissue explants. Polymerase chain response (PCR) and the converse transcription-PCR are transgenic clones of hairy roots has been utilized *rol*A and *rol*B particular primers. In the middle of the different attention of better regulators the extreme transformation frequency was achieved in (IBA + NAA) cotyledon explant. Transgenic hairy roots increase in MS liquid medium added to with IBA + NAA (2.46 + 1.07) displayed the maximum accumulation of biomass 0.68 g/l dry weight (DW) and 6.52 ± 0.49 g/l fresh weight (FW) were obtained at the 21 days of cotyledon explant. The flavonoid and total phenolic contents were estimated using aluminium chloride method and Folin-Ciocalteu method. The amount of phenolic compounds in *Cucumis anguria* L non transformed root (124.46 ± 6.13 mg GA/g) was lower than that in the methanol extracts of *Cucumis anguria* L. hairy roots (160.38 ± 5.0 mg GA/g), being was *Cucumis anguria* L non transformed root lower (42.93 ± 1.58 mg rutin/g) than that in the concentration of total flavonoids in *Cucumis anguria* L. hairy root (16.26 ± 1.84 mg rutin/g). Additionally, transgenic hairy roots professionally produced various phenolic and flavonoid composites. The total antimicrobial activity, phenolics, flavonoids content and antioxidant were more in the hairy roots related to non-transformed roots. In our discovery, the *A. rhizogenes* R1000 is promising candidate for hairy root initiation of *C. anguria* from cotyledone explants were realized large number of hairy roots compared with leaf explants. The antioxidant potential of methanol extracts of flavonoid and phenolic compounds from the hairy roots have great potential to treat various diseases.

1. Introduction

Cucumis anguria L. is otherwise known as, West Indian gherkin, bur gherkin is one of the important vegetable and most common medicinal crops. It is widely used in United States, Cuba, Brazil, India, Zimbabwe, Brazil, Cuba, United States and Africa [Nayar and Singh, 1998]. Boiled, roasted, stewed, pickled, salads and hamburgers were absorbed by the fruits and leaves of the gherkin [Ju et al., 2014]. Also, seeds, roots and fruits of gherkins have been used to prevent kidney stone formation, hemorrhoids, jaundice

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and stomach pain. Due to need for pickled gherkins a growing worldwide, many food businesses are learning the possiblities for creating gherkins. The bioactive compounds such as, cucurbitacin G, cucurbitacin D and Cucurbitacin B were reported from the plant *Cucumis anguria.* Among these bioactive compounds, the role of cucurbitacin B has been studied and has the capacity to prevent the development of cancer. Flavonoids, tannins, alkaloids, saponins and steroids are the useful compounds of the gherkin having antibacterial action.

Nowadays, the need for *C. anguria* species has increased owing to its clinical significance. Their low development rate *in-vivo* condition is very hard to motivate plant production. Therefore, the tissue culture technique plays very critical role in micropropagation. The *in-vitro* plant micropropagation and recreation of *C. anguria* species was already studied utilizing leaf explants.

Agrobacterium rhizogenes is belonging to the family Rhizobiaceae is a Gram-negative bacterium, which can survive in the hair root of plants. Hairy Root stimulating (Ri)-plasmids, *A. rhizogenes* improved T-DNA into plant cells to create while using hairy root diseases [Hamill et al., 1987]. Recently, *A. rhizogenes* were utilized to stimulate hairy roots to various leguminous plants [Guillon et al., 2006). In recent years, there is a significant interest in hairy root cultures for rapid development and capacity to generate more amount of secondary metabolites than genetic and biochemical stability [Srivastava and Srivastava, 2007]. Because of their different pharmacological applications [Swarup et al., 2007], there is a constant need for production of secondary metabolites from various plant foundations. Phenolic and flavanoides has a strong beneficial properties such as antioxidant and anti-inflammatory properties [Rocha et al., 2015]. The contact of elicitor in higher plant is mainly associated with synergistic mechanism, disease and plant pests trigger in variety plant species [Bong et al., 2015] and to increase production of secondary metabolites. Studies on optimization of secondary metabolite production are performed by the analysis of phytohormones and elicitors and also using various bioinformatics tools [Tambunan et al., 2014]. In recent investigation *C. anguria,* Anthraquinones and saponins are utilized for the antibacterial and antifungal function [Abdullahil Baque] et al., 2010. Plant evolution regulators (PGRs) are more important influencesdisturbing for the cell improvement, metabolic processes and differentiation [18]. The types and concentration of PGRs have been presented to change in particular auxins and/or cytokinins in the biosynthesis of phenolic acids to change the shikimate/phenylpropanoid pathways, perhabs by flexible the action of chalcone synthase and phenylalanine ammonia lyase [Moyo et al., 2014]. Many bioactive compounds have been synthesized in *in vitro* plant culture technique. Here we have used various *A. rhizogenes* strains utilizing growth hormones, examined the initiation of hairy roots from *C. anguria*, utilizing growth studies of hairy roots. Additionally, we compare the flavonoids and phenolic content, antioxidant activity and antibacterial of hairy roots were established from *C. anguria* explants.

2. Materials and methods

In this study, *C. anguria* L. (gherkin) seeds were collected from Nunhems Seeds Pvt., Ltd., (Bangalore, India). The collected seeds were sterilized externally additionally by the previous experiment of the Chung and Thiruvengadam et al. (2014a, 2014b), and the seeds were immunized with MS medium without any hormone [Murashige and Skoog, 1962] added with sucrose (3.0%, v/v) and agar (0.8%, w/v). The seedlings were critically improved in white fluorescent light (40 $\mu molm^{-2}$ s^{-1}) with 16/8 h of light/dark photoperiod and at 25 °C. The explants of leaves, cotyledons, hypocotyls and shoot nodes (5 × 5 mm in length) were utilized for revolution in vitro seedlings 10 days ago.

2.1. Agrobacterium rhizogenes and growth conditions

The selected strains from the bacteria *A. rhizogenes* utilized in this investigation like, LBA9402, A4, 15834, 13333, R1200, R1000, R1301 and R1601 were retrieved from glycerol stocks. These were grown in a Luria-Bertani liquid medium, with the overnight culture (O.D 600 = 0.5) overnight maintained at 28 °C at 180 rpm. The cell densities of *A. rhizogenes* were analyzed through using a spectrophotometer.

2.2. Effect of different strains

There was sample for establishing hairy roots Leaves of *C. anguria* L., were cut at 7 × 7 mm. The selected leaves were sliced into pieces and placed in the culture of *A. rhizogenes* strains with 10 min in the liquid inoculation medium. Later these models were dried in the form of sterilized filter paper and further incubated in dark on MS medium at 25 °C. After 2 days of culture, the explants were transferred to a MS medium (agar-8 g/L; sucrose-30 g/L; cefotaxime – 500 mg /L; and vitamins) containing without any hormone. Root hair was formed from the injured site within two weeks and the newly formed hairy roots were detached from explants and further cultured in the same culture medium at 25 °C. After the development of hairy structure, it was transferred to MS liquid culture medium in 100 ml Erlenmeyer flask. Then it was placed in a development chamber, further flux rate (35 $\mu mol\ s^{-1}m^{-2}$) and other factors were maintained in standard condition. The Erlenmeyer flask was incubated for 21 days and the dry weight was of hairy root was calculated. Three flasks were utilized for each culture and trials were repeated three times.

2.3. Hairy root culture (R1000)

Cotyledon, hypocotyls, shoot nodes and leaf (0.5 cm, 20 day old) explants were used from *in vitro* seedling of *C. anguria* L. as explants for outside root culture. They were further cultured in MS medium and various combinations of IBA, NAA and IAA (0.08–1.48 μM for IBA and 0.57–1.71 μM for IAA and 0.54–1.61 μM for NAA). The pH of the medium has been adjusted before autoclaving at 5.8. All the cultures were placed at 25 ± 2 °C under total darkness. After 20 days, approximately 0.2 g FW root tips (20 mm length) were disconnected from three explants and moved to 150 ml Erlenmeyer flask comprising hormonal MS liquid medium. After that, it was it was kept in gyratory shaker at 120 rpm (Orbitek, India) in total darkness and sub-cultured in the respective media (every 15 days) were periodically. The cultures were harvested after 45 days to describing the growth index (GI).

2.4. R1000 inoculation and culture of transformed roots

Depend on the former analysis on adventitious root culture; we used cotyledon explants for *A. rhizogenes* transformation. For analysis, cut was made in explants at various places with hypodermic needle (DISPOVAN) wiped out by bacterial suspension to infectivity. Excised cotyledon explants were cotyledon mid-rib and lamina wounded. LB medium which was not inoculated by bacteria was used. Co-cultivation was maintained for 3 days at 25 ± 2 °C by keeping the infected explants on MS basal medium with 0.2% agar, in 150 ml Erlenmeyer flask with supplemented phytagel (Sigma, USA). All the cultures incubated in dark condition. The explants were further washed several times with sterile distilled water and then washed four to five times in cefotaxime (300 mg/l) (Alkem Laboratories, Mumbai, India) to remove bacteria. In

150 ml Erlenmeyer flask, 300 mg/l cefotaxime was found in dark on 30 ml of solid MS basal medium. All the cultures were incubated at 25 ± 2 °C, and cultured for three successive subcultures (every 15 days). Hairy roots were developed in injured areas of the precious explants were excised and refined below total darkness in 150 ml Erlenmeyer flask including 30 ml MS basal solid medium additional with cefotaxime (300 mg/l) to develop axenic transferred root cultures. Later 20 days, about 0.2 g FW of root tips (20 mm length) was transferred to MS liquid basal medium added with 200 mg/l cefotaxime to create mass culture of hairy roots and kept on a Gyratory shaker at 25 ± 2 °C for 120 rpm. The cultures were harvested 45 days after to determining the evolution index (GI).

2.5. Growth studies

The dry (DW) and Fresh weights of (FW) of both adventitious and hairy roots were inspected as follows. The hairy and adventitious roots were distributed from the centre and root FW once washed with sterile double distilled water before measuring and blotting out on the tissue paper, and root DW was dried up to get constant weight at room temperature.

The growth index was derived using below formula,

$$GI = \frac{\text{Harvested dry weight (g)}}{\text{Inoculated dry weight (g)}}$$

2.6. PCR analysis for the rol B and rol B

The *rol* A and *rol* B genes were extracted from *A. rhizogenes* into the plant genome. A total of 490 bp fragment of *rol* A gene was amplified using forward (5′-ACGGTGAGTGTGGTTGTAGG-3′) and reverse (5′-GCCACGTGCGTATTAATCCC-3′) primer; whereas, a 383 bp fragment of *rol* B gene was amplified using 5′-GCTCTTGC AGTGCTAGATTT-3′ (forward) and 5′-GAAGGTGCAAGCTACCTCTC-3′ (reverse) primer.

2.7. Total phenolic content determination

Total phenolic content of the methanol extract was performed from *C. anguria* L. using the Folin- Ciocalteu method [Kaur and Kapoor, 2002]. In brief, the crude methanol extract from the expand was mixed with double distilled water appropriately. To this sample, Folin-Ciocalteu reagent (0.6 ml) was mixed and incubated for 3 min. Gallic acid was used as the standard. The absorbance of the sample was read at 760 nm using a spectrophotometer.

2.8. Determination of flavonoid content

The aluminum chloride colorimetric technique defined by modified method of Chang et al., 2002] crude methanol is using to quantity overall flavonoid content of the increase in*C. anguria* L.. In short blend the 4 ml of purifiedwater and 0.3 ml of 5% $NaNO_2$ was then added reaction mixtures with 100 ml of crude methanol extract (1 mg/mL) and blends completely. Afterward, 0.3 ml of 10% $AlCl_3$ solution was added. After 5 min of incubation, the combination was allowed to stand in 6 min. Two milliliters of 1 M NaOH solution was then included, and the last volume of the combinationis complete to 10 ml utilizing double-distilled water. The combination was recovered at the room temperature for 15 min, and the absorption was calculated utilizing spectrophotometer in 510 nm. The total flavonoids contented were calculated based on the calibration curve and the result was expressed as mg equivalent to g dry weight.

2.9. Antibacterial screening

2.9.1. Bacterial strains and cultivation

The bacteria such as *Stephylococcus haemolyticus, E. coli* (KF 918342), *E. coli* (ATCC 35150), *Aeromonas hydrophila* (KCTC 12487), *Aeromonas salmonicida* (KACC 15136) and *Cronobacter sakazakii* (ATCC 29544) were used for antibacterial screening. These bacterial strains were obtained from Bharathidasan University. The selected strains were inoculated into 100 ml culture medium in 250 ml Erlenmeyer flask. After 18 h, the growth was monitored (0.1 OD) at 600 nm and diluted appropriately.

2.9.2. Disc diffusion method

The selected bacterial strains were swabbed on LB agar plates. Sample was loaded on the Whatman filter paper disc and placed on LB agar plates. Streptomycin was utilized as a standard.

2.10. Antioxidant activity

2.10.1. DPPH assay

The DPPH scavenging assay of *Cucumis anguria* L. extracts was assayed as suggested by Al-Dhabi et al. [2015]. DPPH was prepared at 0.15% concentration in methanol and different concentration of extracts (20–100 μL) was added with 200 μL of DPPH solution. It was keep at room temperature absorbance was measured at 517 nm after 30 min. To plot a standard curve, Vitamin C was used as a standard. The DPPH radical scavenging activity was calculated using the following equation:

DPPH radical scavenging movement (%) = $[(A0 - A1/A0) * 100]$, where the absorbing the management is *A*0 at 30 min and the absorbing model *A*1 is at 30 min. All models are studied in triplicate.

2.10.2. Superoxide dismutase (SOD)-like activity

Superoxide dismutase-like activity of the sample was assayed based on the method of Marklund and Marklund [1974]. SOD –like activity was calculated based on the following equation.

Superoxide dismutase-like movement (%) = $[(A0\text{-}A1/A0 \times 100]$,

Where the absorbing in the management is A0 and the absorbing model is A1. All the samples were studied in triplicate.

3. Results and discussion

Cucumis anguria L. explants were highly susceptible for disease by the tested strains of *A. rhizogenes*. No significant dissimilarity was obtained in the morphology of hairy roots. Hairy root was developed at various rates were; R1000 (93.1 ± 7.3%), 13,333 (92.2 ± 7.4%), R1200 (91.7 ± 6.4%), LBA9402 (90.6 ± 8.1%), 15,834 (90.5 ± 6.8%), R1301 (89.7 ± 6.8%), R1601 (88.3 ± 7.2%) and A4 (87.6 ± 5.9%) (Fig. 1).

3.1. Adventitious root culture

The adventitious root cultures were studied in MS medium also with IBA either only or in mixture with IAA or NAA. Additional auxins applied only or in mixture did not display positive response. Four kind of explants i.e., cotyledon, hypocotyl, leaf and shoot nodes were tested. Between these only cotyledon and leaf explants responded finitely. Therefore, these two explants are utilized for root culture trial. Following 20 days of culture rooting was attained in MS medium supplemented with IBA, or IBA/IAA or IBA/NAA mixture. The cotyledon explants when cultured in IBA/NAA mixture, observed the maximum number of roots (9.2) and percentage of response (96.7%) (Table 1). The leaf explants reported the percentage of response (88.9%) in the similar mixture and the maximum

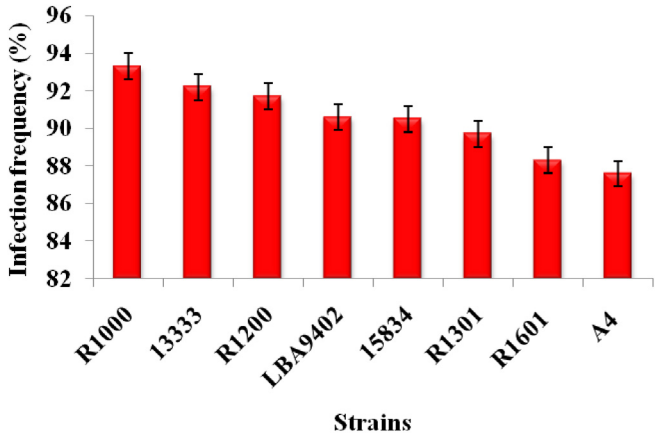

Fig. 1. Effect of strains of *A. rhizogenes* on the infection frequency of *Cucumis anguria* L hairy root cultures (The values represent the mean ± SD of three independent measurements).

number of roots (7.8). Though IAA and IBA mixture also displayed rooting other than it is lesser than the individual IBA therapy.

As good results was observed at 2.46 μM IBA, this absorption was keptsteady for mixture therapy of IAA (1.14 μM) and NAA (1.07 μM). IBA and NAA (2.46 + 1.07 μM) mixture developed largest number of roots (113.42) with 15.24 cm root limit is observed in the cotyledon explants derived cultures. In the similarmethod the largest numbers of roots (106.32) with 13.19 cm shoot length were observed in the leaf explant derived cultures in the similar absorption (Table 2). Though the IBA (2.46 μM) and IAA (1.14 μM) mixture developed roots in the subculture, the number of roots were lower than the medium containing IBA alone. Similarly trend in biomass and growth index experiments were observed. The maximum biomass (fresh weight-6.52 g; dry weight-0.68 g) and growth index 1.88 were recorded in cotyledon explants derived cultures in IBA and NAA combination. The highest biomass (fresh weight-7.62 g; dry weight 0.74 g) with 2.05 growth index was noted in leaf derived roots cultures at IBA with NAA combination. Hence the IBA (2.46 μM) and NAA (1.07 μM) combination may be considered as best hormonal combination and cotyledons are the most suitable explant for root culture studies of *C. anguria* L (see Table 3).

It was previously reported that many growth regulators influenced, biomass production, root elongation and induction [Balestri and Bertini, 2003]. Auxin in the culture medium specifically increased the mass of *C. anguria* L. Supplementation of IBA in the culture medium has been increased biomass production. However at higher concentrations, biomass production has also been decreased in the case of *lobelia* culture. And the most effective concentration was determined as 25 μM IAA and 10 μM NAA [Balvanyos et al., 2001]. Similarly, the cotyledons and leaf explant was developed maximum regularity of root induction in the *Taraxacum platycarpum* [Lee et al., 2004] and *Gymnema sylvestre* [Nagella et al., 2013]. In our investigation, leaf explants developed maximum hairy roots. These results were highly correlated with the results of *Torenia fournieri* and *Artemisia vulgaris* hairy root induction [Sujatha et al., 2013).

3.2. Optimization of Agrobacterium infectivity

The selected bacterial strain R1000 induced root formation in the explants (Fig. 2). Analysis was performed within 20 days in removed cotyledon explants of *C. anguria* L. About 96% of revolutioneffectiveness was experimental in cotyledon explants with 19.8 numbers of roots and 13 cm root length (Table 3). Roots never become obvious on the control cotyledon explants. The beginnings of changed roots were significantly associated with bacterial pressure and explants. These results indicated that *Cucumisanguria* L. is highly susceptible to strain R 1000 (Table 3). It has been reported that that the bacteria from the genus *Agrobacterium* showed varying virulence [34] and the efficacy of transformation varied among bacteria [Hu, 1993].

Afterward, the explants were maintained in MS hormone free culture medium in the presence of cefotaxime. During the first

Table 1
Effect of different concentration and combination of auxins (IBA, IAA and NAA) on adventitious root induction from different explants (cotyledon and leaf) of *C. anguria*L.

PGR concentration and combination (μM)	Cotyledon explants			Leaf explants		
	% of response	Total No. of roots[a]	Root length (cm)[a]	% of response	No. of roots[a]	Root length (cm)[a]
IBA						
0.08	48.2	3.4 ± 0.14	5.0 ± 0.23	41.5	3.2 ± 0.18	3.4 ± 0.62
1.99	84.4	5.6 ± 0.17	7.2 ± 0.42	76.1	4.7 ± 0.17	4.4 ± 0.17
2.46	**94.2**	**6.8 ± 0.11**	9.7 ± 0.28	83.7	**6.1 ± 0.25**	7.2 ± 0.24
4.43	76.6	6.0 ± 0.15	8.8 ± 0.62	69.2	5.4 ± 0.20	4.3 ± 0.77
3.44	60.4	5.2 ± 0.18	7.5 ± 0.73	54.3	4.5 ± 0.27	3.3 ± 0.25
0.49	52.7	4.1 ± 0.19	6.9 ± 0.24	47.7	3.6 ± 0.18	2.4 ± 0.56
1.48	48.2	3.6 ± 0.12	3.1 ± 0.32	40.5	3.0 ± 0.21	1.2 ± 0.38
IBA + IAA						
2.46 + 0.57	68.4	2.0 ± 0.20	2.2 ± 0.32	59.5	2.4 ± 0.25	1.1 ± 0.44
2.46 + 1.14	**70.8**	**4.3 ± 0.17**	4.6 ± 0.47	62.7	**3.5 ± 0.26**	3.4 ± 0.32
2.46 + 2.85	66.7	3.4 ± 0.22	3.4 ± 0.28	56.4	3.0 ± 0.23	2.6 ± 0.22
2.46 + 3.99	64.5	3.1 ± 0.14	2.8 ± 0.62	52.1	2.8 ± 0.22	2.1 ± 0.19
2.46 + 5.14	60.4	2.8 ± 0.13	2.3 ± 0.18	49.5	2.2 ± 0.24	1.4 ± 0.71
2.46 + 0.57	58.7	2.3 ± 0.17	2.1 ± 0.22	46.8	1.3 ± 0.18	1.1 ± 0.57
2.46 + 1.71	50.6	2.0 ± 0.12	1.3 ± 0.37	43.6	1.1 ± 0.22	1.0 ± 0.25
IBA + NAA						
2.46 + 0.54	80.4	6.4 ± 0.28	7.2 ± 0.34	71.8	6.3 ± 0.24	5.6 ± 0.34
2.46 + 1.07	**96.7**	**9.2 ± 0.21**	10.4 ± 0.19	88.9	**7.8 ± 0.16**	9.4 ± 0.22
2.46 + 2.69	86.5	8.1 ± 0.20	9.6 ± 0.52	83.5	6.1 ± 0.18	8.5 ± 0.76
2.46 + 3.78	80.8	7.2 ± 0.19	8.7 ± 0.28	73.1	5.0 ± 0.14	6.6 ± 0.23
2.46 + 4.83	68.6	5.2 ± 0.18	5.1 ± 0.72	59.3	4.2 ± 0.13	3.2 ± 0.67
2.46 + 0.54	60.9	3.1 ± 0.23	3.8 ± 0.67	51.4	2.9 ± 0.14	2.1 ± 0.37
2.46 + 1.61	40.7	3.1 ± 0.20	2.7 ± 0.34	38.0	2.4 ± 0.18	1.4 ± 0.75

[a] Each value represents mean ± S.E of three replicates recorded after 21 days of culture. Each mean represented five replications with 10 explants.

Table 2
Proliferation of adventitious root culture from different explants of *C.anguria* L. on MS medium fortified with IBA (2.46 μM), IBA + IAA (2.46 Mm + 1.14 μM) and IBA + NAA (2.46 μM + 1.07 μM).

PGR concentration and combination (μM)	Cotyledon derived roots			Leaf derived roots		
	% of response	No. of roots[a]	Root length (cm)[a]	% of response	No. of roots[a]	Root length (cm)[a]
Control	–	–	–	–	–	–
IBA (2.46)	97.23	90.38 ± 5.98	13.23 ± 0.23	95.23	81.24 ± 4.21	10.16 ± 0.32
IBA + IAA (2.46 + 1.14)	96.11	30.31 ± 5.82	10.11 ± 0.21	94.21	23.21 ± 3.28	8.14 ± 0.28
IBA + NAA (2.46 + 1.07)	98.18	113.42 ± 1.21	15.24 ± 0.28	96.28	106.32 ± 3.81	13.19 ± 0.38

[a] Each value represents mean ± S.E of three replicates recorded after 45 days of culture. Each mean represented three replications with 50 explants.

Table 3
Effect of co-cultivation on hairy root induction from cotyledon explants of *Cucumis anguria* L. using *A. rhizogenes* strain R1000 on MS basal medium under total darkness.

Experiment no.	No. of transformed explants	Transformation efficiency (%)	Number of roots per explant[a]	Root length (cm)
1	45	90	19.8 ± 1.12	11.8 ± 0.2
2	48	96	18.5 ± 1.22	12.5 ± 0.8
3	47	94	19.0 ± 1.27	13.0 ± 0.6
4	47	94	18.0 ± 1.31	10.0 ± 0.5
5	46	92	17.5 ± 1.22	13.0 ± 0.5

Each experiment was conducted with 50 explants Transformation efficiency (%) = No. of explants infected / No. transformed explants × 100.

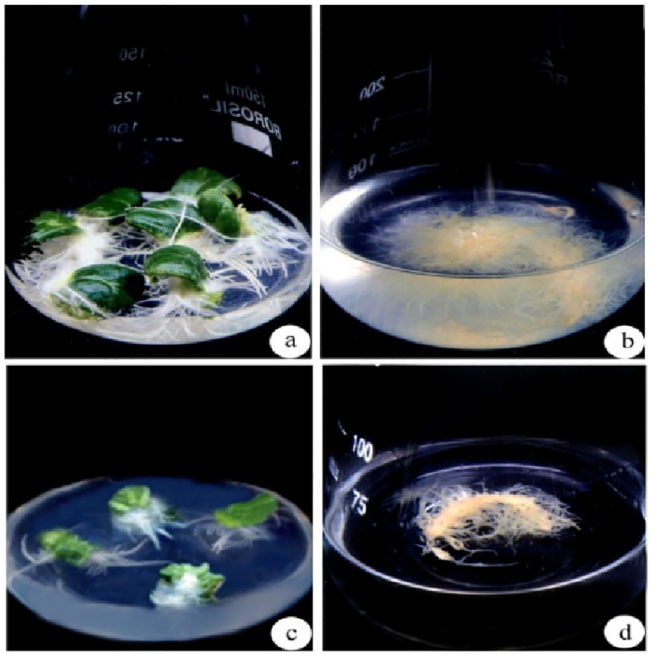

Fig. 2. Adventitious root culture using various explants of *Cucumis anguria* L. a, c. Origination of adventitious roots from cotyledon and leaf explants b, d. Pr oliferation of adventitious roots from cotyledon and leaf explants.

week of culture the changed cotyledon explants formed roots and the non transformed explants have not produced any roots. Putative different hairy roots are encouraged at a high rate at the proximal end of the cotyledon. Within twenty days all the converted cotyledons produced hairy roots. Transformation rates were measured from the explants after 20 days of culture on MS medium containing Cefotaxime (300 mg/l). The number of hairy roots per cotyledon explant changed from 17.5 to 19.8 with root length of 10 to 13 cm.

Hairy root cultures were conventional from primary roots (0.2 g) formed at each wound sites in MS liquid medium without growth regulator. The roots grow hastily and developed lateral roots within 28 days in MS liquid medium with 200 mg/l cefotaxime. Within 45 days, 99% of cultures presented maximum number of hairy roots (132) with 18 cm length from cotyledon consequent hairy roots (Table 4). In general, the transformed roots showed loose morphology and dispersed with very long primary roots with lateral branches without any root hairs (Fig. 2). Non changed roots under same experimental conditions showed poor growth associated with the deficiency of lateral branching. Further, the developed roots were transferred into MS basal culture medium without any specific antibiotics and revealed rapid growth. Similarly root clones showed a higher degree of branching and rapid plagiotropic growth.

The first successful cucumber transformation was described by Trluson et al. [1986] using hypocotyl explants with *A. rhizogenes* containing *nos-npt* II gene. They have restored transgenic cucumber plants from roots of hypocotyl explants diseased with *A. rhizogenes*. Ping et al. (1998) *definition*hairy roots of following 10 days of inoculation of *Cucumis sativus* L. cotyledon explants with the strain of *A. rhizogenes* R1000 and R1601. But our result was contrary to Ping et al. (1998). In this study, after 20 days of treatment with the strain R1000 hairy root of cotyledon explants was observed. So the R1000 strain was found more appropriate to *Cucumis anguria* L. than *Cucumis sativus* L. Matsuda et al. [2000] reported that the 10–14 hairy roots were produced from the single cotyledon explants of *Cucumismelo* L. Our results are comparable and we found cotyledon of this plant classes are suitable explants for the production of hair root.

3.3. Growth studies

After incubation period, biomass development was found to be more than two fold than control (Table 5). The present finding revealed variation in root tip thickness and length. Cotyledon derived hairy roots verified the best FW biomass accumulation,

Table 4
Proliferation of hairy root cultures and biomass production from cotyledon explants of *Cucumis anguria* L. on MS liquid medium with all plant growth regulators under total darkness after 45 days of culture.

Experiment no.	Percentage of response	No. of hairy roots per explant	Length of the hairy root	Biomass Production		
				FW[a]	DW[a]	GI
1.	97	130.27 ± 7.28	18.55 ± 2.86	9.63 ± 0.18	1.00 ± 0.17	2.77
2.	96	127.23 ± 7.34	18.12 ± 3.14	9.40 ± 0.13	0.98 ± 0.12	2.72
3.	**99**	**132.38 ± 8.27**	**18.86 ± 3.16**	**9.78 ± 0.14**	**1.02 ± 0.19**	**2.83**
4.	94	123.21 ± 9.24	17.55 ± 3.19	9.10 ± 0.11	0.94 ± 0.13	2.61
5.	95	125.29 ± 8.35	17.84 ± 2.94	9.26 ± 0.15	0.96 ± 0.18	2.66

Table 5
Fresh weight, dry weight and growth index of *C. anguria* L. root cultures after 45 days of culture in the dark on media supplemented with different concentrations and combinations of IBA, IBA + IAA, IBA + NAA.

PGR (μM)	Cotyledon derived roots			Leaf derived roots		
	FW (g) [a]	DW (g) [a]	GI	FW (g) [a]	DW (g) [a]	GI
IBA 2.46	5.33 ± 0.12	0.56 ± 0.41	1.55	4.42 ± 0.28	0.46 ± 0.26	1.27
IBA + IAA 2.46 + 1.14	1.67 ± 0.60	0.18 ± 0.38	0.51	1.27 ± 0.34	0.13 ± 0.32	0.36
IBA + NAA 2.46 + 1.07	**6.52 ± 0.49**	**0.68 ± 0.57**	**1.88**	5.82 ± 0.27	0.60 ± 0.38	1.67

[a] Each value represents mean ± S.E of three replicates with recoded after 45 days of culture. Each mean represented three replications with 50 explants.

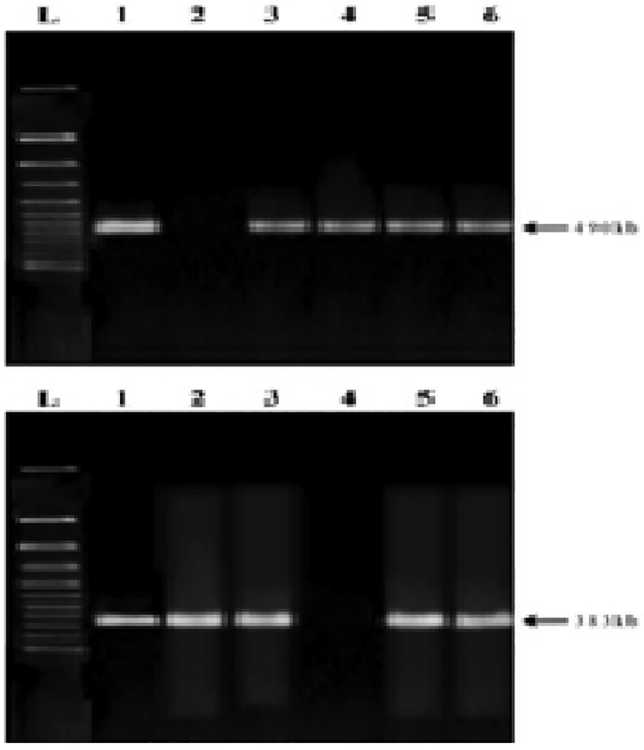

Fig. 3. Confirmation of transformation.

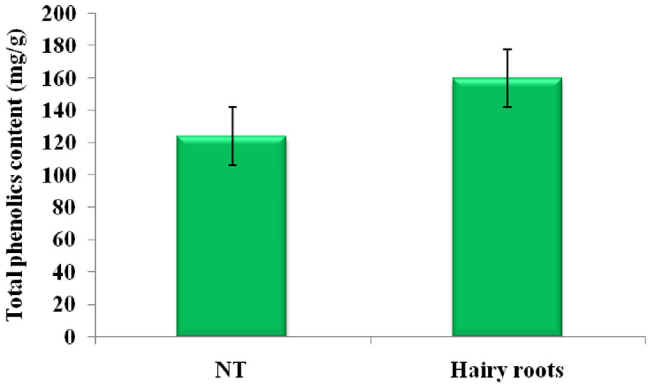

Fig. 4. Total Phenolics content in the methanol extracts of *Cucumis anguria* L. root cultures.

9.78 g (GI of 2.83), which was 2 fold than non transformed roots. Difference in the accumulation of biomass between various explants consequent hairy roots have been reported earlier in *Duboisia leichhardtii* [Mano et al., 1986], *Atropa belladonna* [Aoki et al., 1994] and *Catharanthus reseus* [Batra et al., 2004]. The exact mechanisms included in the formation of lateral roots are not clearly understood, however transformation of roots by *A. rhizogenes* are mainly by the development of laterals and involved in biomass quantity. Also, the physiological mechanism of root enhancing process is not very clear, however high growth was obtained by inhibiting the accumulation of polyamine [Ben-Hayyim et al., 1996).

3.4. Transformation analysis

The transformed root sample showed positive for the genes, *rol* A and *rol* B. The sequence of these gene products were, 490 bp and 383 bp, respectively. The negative control showed negative result for the genes, *rol* A and *rol* B (Fig. 3).

3.5. Phenolic and flavonoid content of the sample

The phenolic content of hairy roots has been described previously. Polyphenol synthesis was affected by various factors,

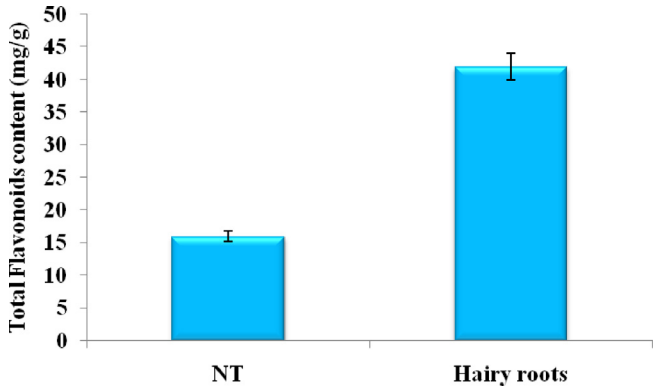

Fig. 5. Total flavonoid content in the methanol extracts of *Cucumis anguria* L. root cultures.

including, post-harvest handling, ontogenetic stage, plant breeding, and other abiotic and abiotic factors [Bruni et al., 2009]. In our study, the total phenolic content was maximum than control (Fig. 4). Generally, flavonoids showed various hydroxyl groups and showed high antioxidant properties [Kim et al., 2013]. These phytochemicals, (phenolic compounds and flavonoids) were highly useful for human health and involved in antioxidant, anticarcinogenic and cardioprotective activities [Grassi et al., 2010]. In our study, flavonoid content of the experimental methanol extract showed 42.93 ± 1.58 mg rutin/g, and total flavonoids from the methanol extract of non control was 16.26 ± 1.84 mg rutin/g (Fig. 5). Generally, phytochemicals such as, flavonoid and polyphenol were involved in antioxidant and antibacterial activity [Daglia, 2011].

3.6. Antimicrobial screening

The crude methanol extract of *C. anguria L.* showed activity against tested pathogenic bacterial strains. Among the solvent tested, methanol extract showed more activity. *C. anguria L.* sample showed against various pathogenic bacteria and the results were tabulated in Table 6. The hairy root of plants showed antibacterial activity against various Gram-positive bacteria than Gram-negative bacteria [Wang et al., 2012). In a study, potent antimicrobial activity was determined from the non -transformed roots [Thiruvengadam et al. (2014a, 2014b)]. In the present study observed higher antibacterial activity from the transformed hairy roots.

Table 6
The results from the disc diffusion method against the bacterial strains.

		Zone of inhibition(mm) from hairy root extracts			
strains	EA	Ethanol	Control(NT)	Methanol	Streptomycine
E. coli (KF 918342)	10.5 ± 0.7	14.6 ± 0.7	13.5 ± 1	21.3 ± 0.6	27.6 ± 0.6
S. Haemolyticus	6.7 ± 0.2	10.8 ± 0.4	10.8 ± 0.6	24 ± 0.7	26.3 ± 0.6
Aeromonas hydrophila	12.6 ± 0.8	13.2 ± 0.7	12.7 ± 0.9	21 ± 1	27 ± 0
E. coli (ATCC 35150)	13.3 ± 0.5	11.4 ± 1	9.5 ± 0.7	20.6 ± 1.1	28.3 ± 0.6
Cronobacter sakazakii	16.5 ± 0.8	13.5 ± 0.5	13.5 ± 1	22.3 ± 0.6	25.6 ± 0.6
Aeromonas salmonicida	11.8 ± 0.9	14.5 ± 0.9	16.5 ± 1.2	19.6 ± 1.5	27 ± 1

NA- No activity, DE- Di ethyl Ether, EA- Ethyl Acetate, NT- Non Transformed.
Each value is the average of three analyses ± standard deviation.

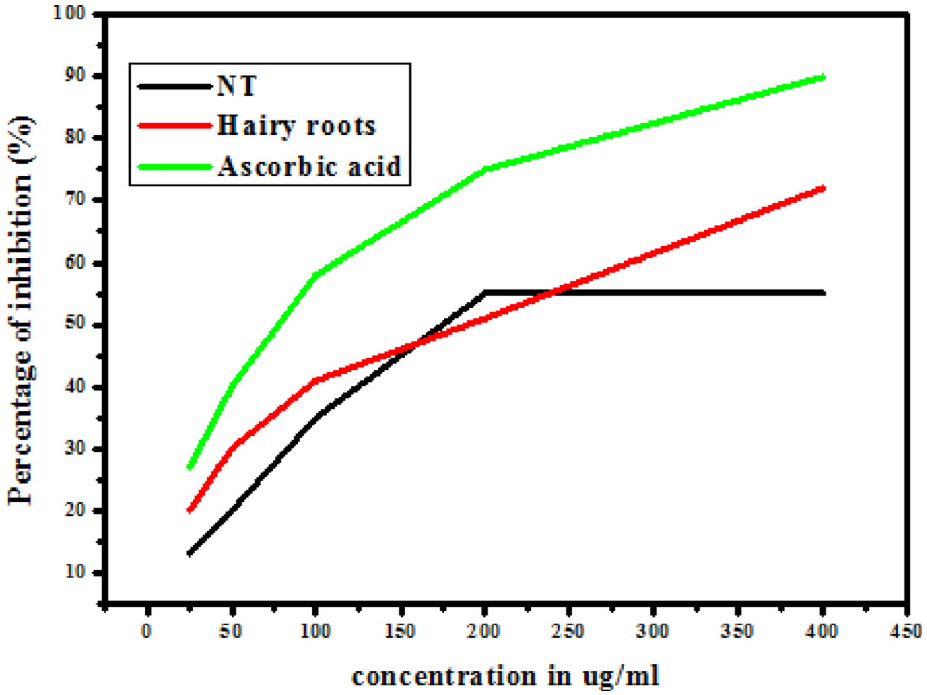

Fig. 6. DPPH assay of the methanol extracts of *Cucumis anguria* L. hairy roots.

3.7. Antioxidant activity

Methanol extract of *C. anguria* L. hairy roots showed antioxidant activities. DPPH antioxidant potential of the extract was described in Fig. 6. The antioxidant power of the sample was based on the concentration of the sample. The methanol extract of the sample showed high activity than non transformed root. The present find revealed that the SOD-like activities did not show any significant results. The present finding revealed maximum antioxidant activity this was highly associated to untransformed root hairs. This king of observation was made earlier in the hair roots of *D. moldavica* (Weremczuk-Jezyna et al., 2013).

4. Conclusion

To conclude, the transformed roots showed potential variations in biomass accumulation and development. Root culture showed rapid growth and accumulation of biomass in liquid media than control. Among all seven strains the successful transformation of *C. anguria* L. by *A. rhizogenes* strain R 1000 showed beneficial effect. Thus, we found that the antioxidant activities of the phenolic and flavonoid contents in the ethanol abstracts of *Cucumisanguria* L. hairy roots depend on the attention of these compounds in the quotations. Our findings clearly revealed that the hairy roots have high antioxidant potential due to the presence of flavonoids and phenolic compounds.

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Declaration of Competing Interest

The authors do not have any conflict in this manuscript.

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