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Efficacy of *Rhizobium rhizogenes*strains on hairy root transformation in *Stevia rebaudiana* L. Bertoni

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Abstract
Two strains of R. rhizogenes, ATCC15834 and MTCC 532 were tested for their virulence on different explants of Stevia rebaudiana. In case of R. rhizogenes strain ATCC 15834, out of different co-culture period, best results were observed after 48h treatment with maximum mean percentage of root induction on shoot tips (91.66%) as well as maximum mean number of hairy roots (20.6). In case of strain MTCC 532, 3h co-culture period was found to be best with maximum mean percentage of root induction (94.33%) and maximum mean number of hairy root (24.33). The overall study revealed that R. rhizogenes strain MTCC 532 was more effective than ATCC 15834 in terms of hairy root induction in S. rebaudiana. However, both the strains of R. rhizogenes (15834 and 532) were able to produce hairy roots in S. rebaudiana, where strain MTCC 532 was found better in terms of transformation frequency (94.33 %) as well as number of roots per explants (24.33).

Keywords: Hairy roots, Rhizobium rhizogenes, Stevia rebaudiana, ATCC15834 and MTCC

Introduction
Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavour and fragrance ingredients, food additives, and pesticides [1]. In the search for alternatives for production of desirable medicinal compounds from plants, biotechnological approaches, specifically, transgenic hairy root through *Rhizobium rhizogenes* infections, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites. These transformed roots have unique genetic and biochemical stability and faster multiplicity, which may provide continuous, reliable source of plant pharmaceuticals across all seasons.
Stevia rebaudiana, widely used natural calorie-free sweetener [2, 3, 4], belongs to family Asteraceae. The leaves of *S. rebaudiana* contain diterpene glycosides namely, stevioside, steviolbioside, rebaudioside A-F and ducloside A which are responsible for the typical sweet taste [5]. Stevioside is regarded as a valuable natural sweetening agent because of its relatively good taste and chemical stability. In addition diterpene, triterpenes, sterols and flavonoids are some of the non-sweet secondary metabolites that have also been identified from the leaves of *Stevia rebaudiana* [6, 7]. Beside sweetness, stevioside may also offer therapeutic benefits, as they have anti-microbial, anti-fungal, anti-hyperglycemic, anti-hypersensitive [8, 9], anti-inflammatory, anti-bacterial, anti-viral, anti-yeast, anti-tumor, diuretic, and immune influencing properties [10].
The present study has been comprehended to check the efficacy of two strains of *R. rhizogenes*, ATCC15834 and MTCC 532 in hairy root transformation of different explants of *S. rebaudiana*.

Materials and methods
Plant material and *R. rhizogenes* strains: The plants were established in the medicinal garden of College of Biotechnology, Birsa agricultural University, Ranchi. The explants were taken from the aseptically grown plantlets multiplied in MS medium supplemented with BAP 2 mg L^{-1}, adenine sulphate 50 mg L^{-1} and citric acid 1 mg L^{-1}.The strains ATCC15834 and MTCC 532 of *R. rhizogenes* were obtained as a kind gift from Professor Kunal Mukhopadhyay, Dept. of Bio-Engineering, BIT Mesra, Ranchi.

Bacterial culture condition and explant: *R. rhizogenes* strain ATCC15834 was cultured on YEB (Yeast Extract Beef extract) broth supplemented with 5 mg L^{-1}rifampicin and Strain MTCC 532 was grown on YEN (Yeast Extract with Sodium Chloride) broth and both were incubated in dark at 28°C for 48h. The cultures were then harvested at optical density 0.6 and pelleted by centrifugation at 5000 rpm for 10minutes at room temperature.

The pellets were acclimatized in 100 ml of MS broth at 28°C in rotary shaker at 100 rpm for 4h before infection.

Transformation and establishment of Hairy root culture
In the laminar air hood, aseptically grown shoot tip explants were excised and wounded using sterile scalpel and needle respectively. These were co- cultured in MS liquid medium for 1h, 2h, 3h, 4h, 5h, 6h, 24h, 48h and 72h. Explants were then washed with 250 mg L^{-1} cefotaxime sodium salt in MS liquid medium for I minute followed by final rinse in sterile distilled water. The explants were then inoculated in solid MS basal medium. The cultures were maintained in the culture room at 25± 2°C with 16 h lights (3000lux) - 8h dark photoperiod regime. Observations were taken at 8days, 16 days and 24 days after infection.

Statistical analysis: The work was performed in a Completely Randomized Design (CRD), 3 plates each with 8 explants per plate in each set and the experiment was replicated 3 times. The results are represented as mean with Standard Deviation (SD).

Results and discussion
Hairy root induction by ATCC 15834: The *S. rebaudiana* explants were wounded and co-cultured with acclimatized bacterial culture in MS brothfor 1,2,3,4,5,6,24,48 and 72 h. Observations were recorded after 8, 16 and 24 days. There was marked difference in hairy root induction at different periods of co-culture. In case of strain ATCC 15834, a gradual increase in mean percentage of root induction and mean number of roots was observed up to co-culture period of 48 h and further declined with an increase in co-culture period. After 8 days of inoculation no root were observed in control (Fig.2A). Hairy root induction was found to increase with increase in co-culture period with a sharp increase at 24h and 48 h co-culture, 63.3% and 68.3% respectively. The percentage root induction declined sharply after 72h co-culture resulting in browning of explants. 48h co-culture showed highest transformation frequency 91.6% after 24 days (Table 1, Fig.1). Similar observations were reported in *P. zeylanica* L., with an increase in co-cultivation period from 1 to 3 days there was a gradual increase in hairy root induction and further increase in co-culture period decreases root induction [11]. Tissue degeneration was observed in *Chlorophytum borivilianum* explants when co-cultivation time exceeded 48 hours due to bacterial overgrowth [12]. Several authors reported that co-culture time has got effect on transformation of the explants [13, 14].

Along with percentage of root induction, number of hairy roots was also observed. Amongst different co-culture periods, number of hairy roots increased with time, with a maximum number observed at 48 h co-culture period (Fig 4 A, 4B and 4C). As depicted in table 2, after 24 days of culture, a gradual increase in number of roots was observed up to 48 h. Co-culture period. The mean number of hairy root per explant was found to be 20.66after 48h. However, after 72h co-culture period, 15.16 roots per explants were observed after 24 days of infection (Table 2, Fig 3).
Among different co- culture periods, 48h co-culture period was found to be best for the root induction with respect to both mean percentage as well as mean number of hairy roots in *S. rebaudiana* after 8, 16 and 24 days (Fig. 4).On shoot tips of *Artemisia annua* L. 80% transformation frequency and 22 hairy roots per explant was achieved after 6 weeks of inoculation with strain ATCC 15834 [15].
Hairy root induction by MTCC 532: As described above, same co-culture periods as in strain ATCC 15834, was maintained for MTCC 532. No growth of hairy roots was observed on control plates (Fig. 2B). In this case also mean induction percentage and mean number of root was observed after 8, 16 and 24 days. In this strain, root induction gradually increased up to 3h of co-culture period and a gradual decline in percentage of root induction was observed after 4h onwards. After 16 days of inoculation maximum percentage of hairy root induction was observed 81.6%which increased maximum up to 94.33% after 24 days at 3h co-culture period. On the other hand, explants co-cultured for 4h, 5h, 6h, 24h and 72h observer lower percentage of root induction (Table 3, Fig. 5).
Similarly, effect of co-culture time on mean number of roots was also observed. Minimum mean number of roots 2.66 was observed after 8 days at the co-culture time of 1h. It was increased to 6.16 and 10.83 at 2h and 3h co-culture respectively. After that mean number of roots decreasedalong with the co-culture period. After 16 days mean number of roots was found to be 5.33, 18.0 and 21.83 at co- culture periods 1h, 2h and 3h respectively.This was increased up to 8.66, 15.16 and 24.33 respectivelyafter 24 days of inoculation. But after that mean number of root decreased gradually with further increase in co-culture periods. It was observed that after 24 days of inoculation 3h co-culture period was found to be best for both mean percentage and mean number of root induction (Table 4, Fig. 6 and 7). In hairy root culture of *P. zeylanica* leaf explants with *R. rhizogenes* MTCC 532, frequency of explant transformation was about 93% [11].

Table 1: Effect of various co-culture periods with *R. rhizogenes* strain ATCC 15834 on mean percentage of root induction in *S. rebaudiana* explants.

Co- culture period in h	Mean percentage of hairy roots per explant		
	8 Days	16 Days	24 Days
1h	8.33± 0.15	15± 0.47	16.6± 0.3
2h	13.3± 0.1	21.6± 0.42	21.6± 0.26
3h	23.3± 0.3	28.3± 0.55	33.3± 0.49
4h	26.6± 0.29	35± 0.4	38.3± 0.46
5h	35± 0.15	43.3± 0.4	46.6± 0.32
6h	38.3± 0.4	51.6± 0.91	58.3± 0.71
24h	63.3± 0.35	76.6± 0.7	78.3± 0.42
48h	68.3± 0.47	85± 0.4	91.6± 0.86
72h	46.6± 0.4	53.3± 0.59	55± 0.85

Table 2: Effect of various co-culture periods with *R. rhizogenes* strain ATCC 15834 on mean number of root induction in *S. rebaudiana* explants.

Co-culture period in h	Mean number of hairy roots per explant		
	8 Days	16 Days	24 Days
1h	2.5± 0.35	3.83± 0.89	5.66± 0.55
2h	3.66± 0.4	8.66± 0.35	8.66± 1.05
3h	3.83± 0.4	9.5± 0.61	10.66± 0.6
4h	5± 0.66	11.16± 0.61	12.16± 0.2
5h	6.16± 0.51	13± 0.42	14.83± 0.6
6h	7.33± 0.38	15.33± 0.5	16.66± 1.01
24h	8.33± 0.4	16.66± 0.38	19.16± 0.43
48h	9.83± 0.37	19.16± 0.35	20.66± 0.5
72h	6.16± 0.35	14.83± 0.37	15.16± 0.72

Table 3: Effect of various co-culture periods with*R. rhizogenes* strain MTCC 532 on mean percentage of root induction in*S. rebaudiana* explants.

Co- culture period in h	Mean percentage of hairy roots per explant		
	8 Days	16 Days	24 Days
1h	13.33± 0.51	23.3± 0.61	28.33± 0.9
2h	21.6± 1.05	33.3± 0.53	43.3± 0.68
3h	73.3± 0.69	81.6± 1.42	94.33± 0.95
4h	71.6± 0.50	78.3± 1.21	83.33± 1.26
5h	68.33± 0.41	75± 1.22	78.33± 1.21
6h	58.3± 0.75	63.33± 0.72	71.6± 0.68
24h	35± 0.72	43.33± 0.85	51.6± 2.27
48h	26.6± 0.50	35± 1	38.3± 0.64
72h	6.66± 0.35	13.3± 0.75	18.33± 0.50

Table 4: Effect of various co-culture periods with*R. rhizogenes* strain MTCC 532on mean number of root inductionin*S. rebaudiana* explants.

Co- culture period in h	Mean number of hairy roots per explant		
	8 Days	16 Days	24 Days
1h	2.66± 0.42	5.33± 0.81	8.66± 0.59
2h	6.16± 0.59	18± 0.49	15.16± 0.8
3h	10.83± 0.26	21.83± 0.87	24.33± 0.72
4h	9.83± 0.4	20± 0.31	23.33± 0.45
5h	8.33± 0.87	17.66± 1.51	22.66± 0.82
6h	7.33± 0.32	15.33± 0.23	19.16± 1.19
24h	5.5± 0.61	13± 1.12	15.83± 0.93
48h	4.5± 1.03	8.66± 0.7	10.66± 1.01
72h	2.16± 0.36	5.5± 0.65	5.66± 0.56

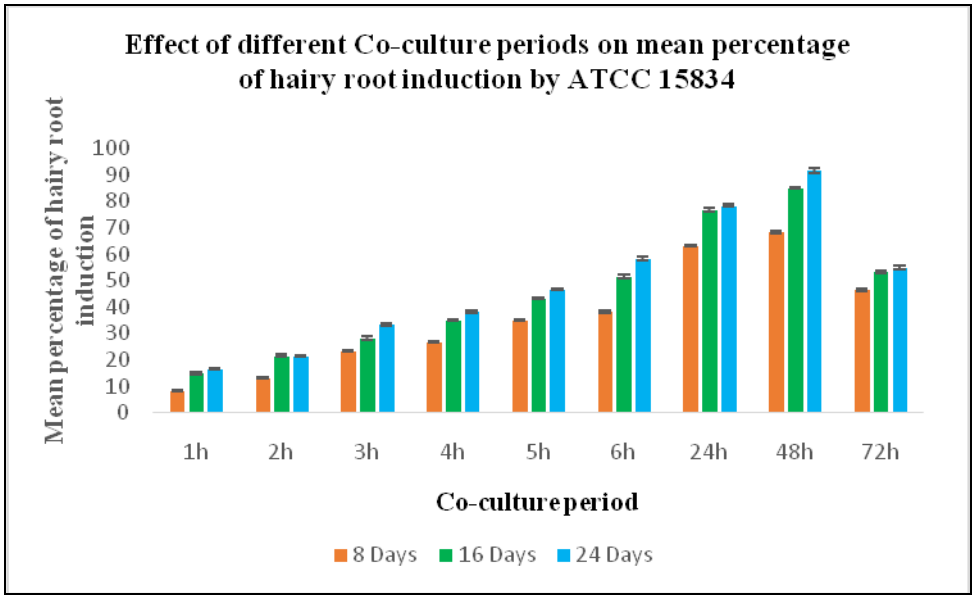

Fig 1: Graphical representation of effect of various co-culture periods with*R. rhizogenes* strain ATCC 15834on mean percentage of root induction in*S. rebaudiana* explants.

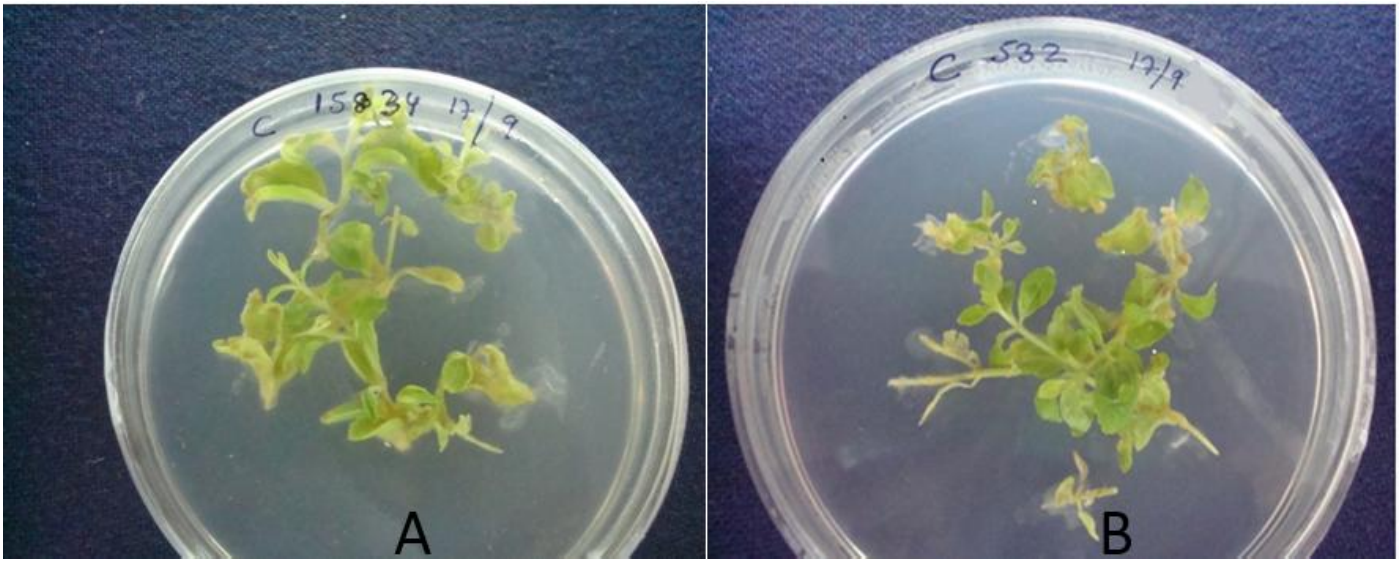

Fig 2: Control plates of *S. rebaudiana* during infection with *R. rhizogenes* strains.
2A: ATCC 15834, 2B MTCC 532.

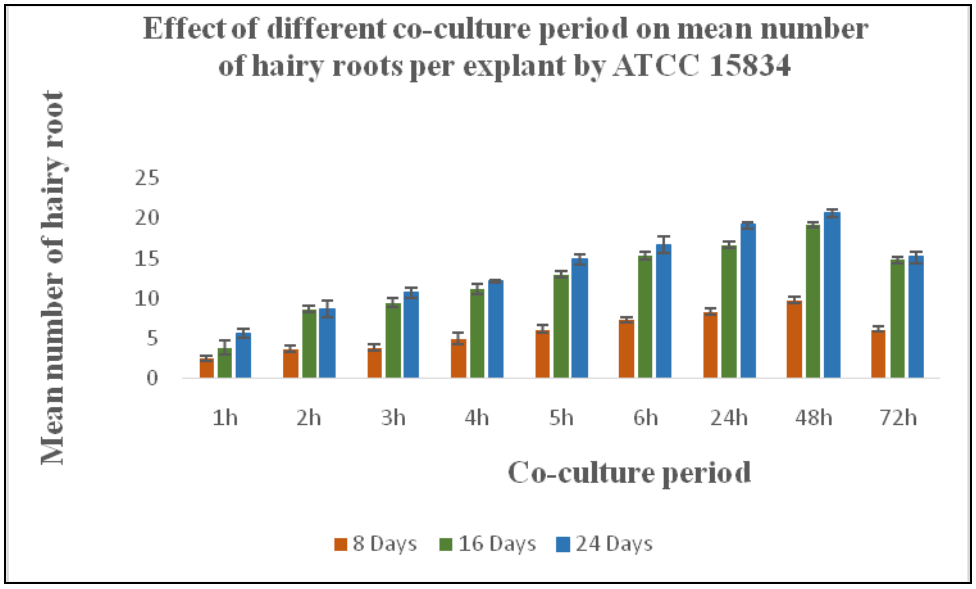

Fig 3: Graphical representation of effect of various co-culture periods with *R. rhizogenes* strain ATCC 15834 on mean number of root induction in*S. rebaudiana* explants.

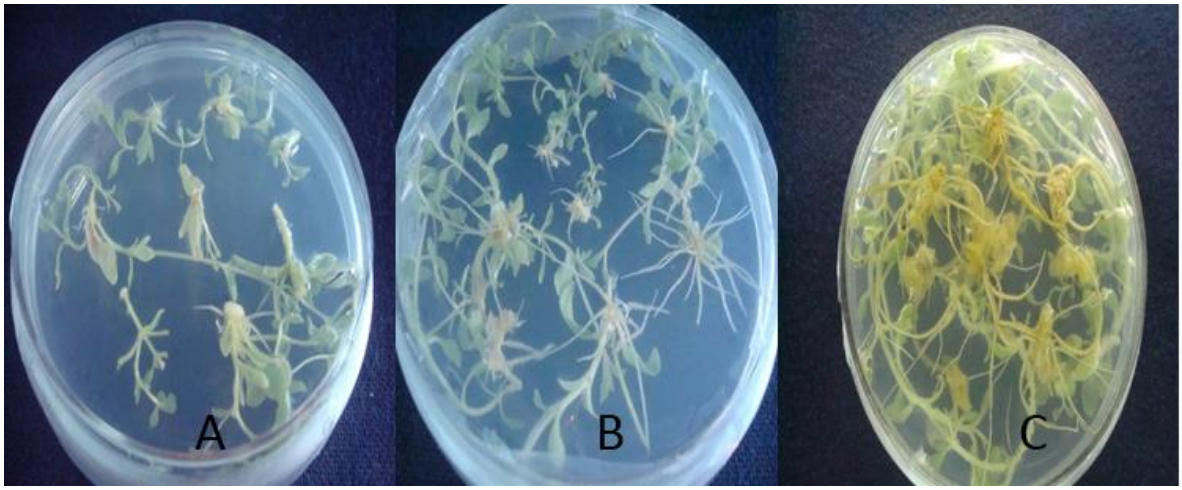

Fig 4: Hairy root induction by *R. rhizogenes* strain ATCC 15834 in *S. rebaudiana*, co-cultured for 48 h.4A: 8 days after infection, 4B: 16 days after infection, 4C: 24 days after infection

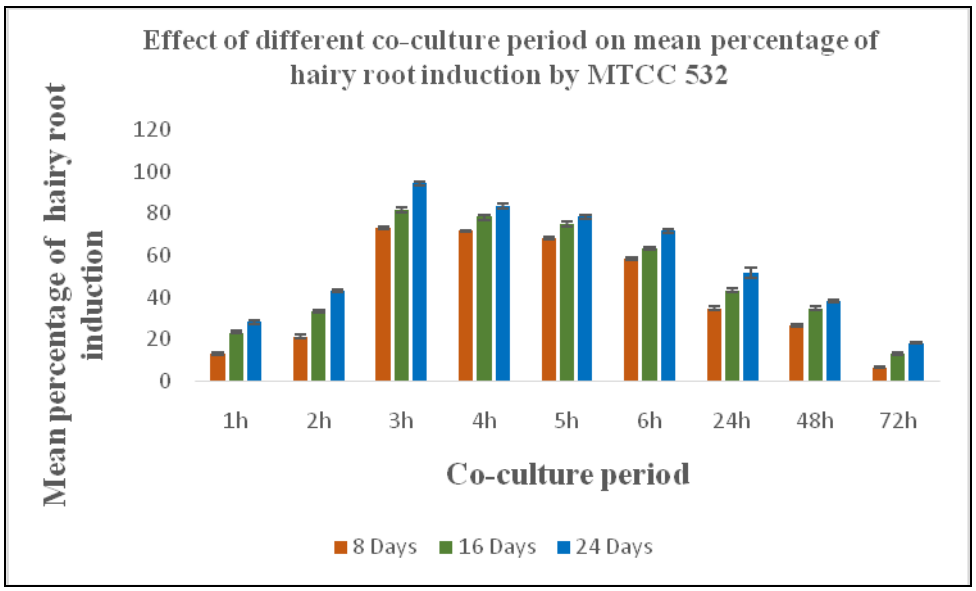

Fig 5: Graphical representation of effect of various co-culture periods with *R. rhizogenes* strain MTCC 532 on mean percentage of root induction in*S. rebaudiana* explants.

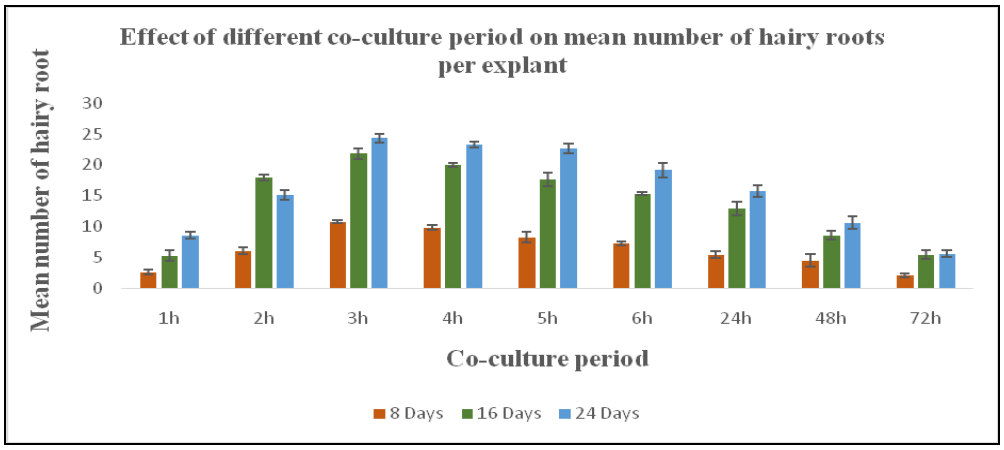

Fig 6: Graphical representation of effect of various co-culture periods with *R. rhizogenes* strain MTCC 532on mean number of root induction in *S. rebaudiana* explants.

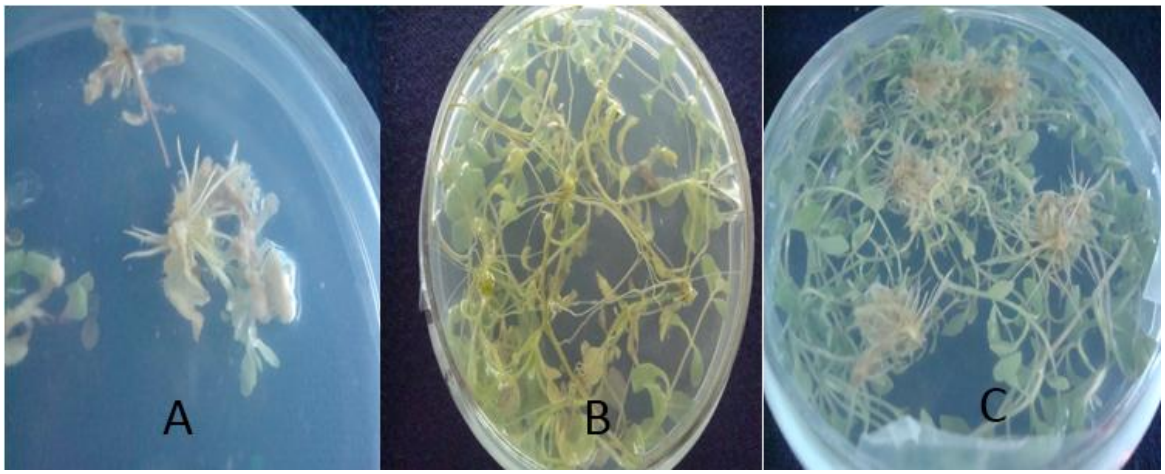

Fig 7: Hairy root induction by *R. rhizogenes* strain MTCC 532 in *S. rebaudiana*, co-cultured for 3 h. 4A: 8 days after infection, 4B: 16 days after infection, 4C: 24 days after infection

Conclusion

Aseptic culture of *Stevia rebaudiana* was used for *Rhizobium rhizogenes* transformation. It was observed that the percentage of hairy root induction and number of hairy roots per explants varied significantly with infection period in both the strains (ATCC 15834 and MTCC 532). Among different co-culture period, 48h was found to be best in case of strain ATCC15834 and 3h in case of strain MTCC 532, for transformation in *Stevia rebaudiana*. However, both the strains of *R. rhizogenes* (15834 and 532) was able to produce hairy roots in *Stevia rebaudiana*, while strain MTCC 532 was found better in terms of transformation frequency (94.33 %) as well as number of roots per explants (24.33). So it may be concluded that strain MTCC 532 was more efficient than

strain ATCC 15834 in inducing hairy roots with the shortest minimum incubation period and showed dominant growth in MS basal medium.

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Conflict of interest: Authors declare no conflict of interest between them.

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