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Shweta Singh
Department of Bio-Engineering, Birla institute of Technology, Mesra, Ranchi, Jharkhand, India

Sunita Murmu
College of Biotechnology, Birsa Agricultural University, Kanke, Ranchi, Jharkhand, India

Anath Bandhu Das
Department of Botany, Utkal University, Vani Vihar, Bhubaneswar, Odisha, India

ZA Haider
College of Biotechnology, Birsa Agricultural University, Kanke, Ranchi, Jharkhand, India

Madhuparna Banerjee
College of Biotechnology, Birsa Agricultural University, Kanke, Ranchi, Jharkhand, India

Correspondence
Madhuparna Banerjee
College of Biotechnology, Birsa Agricultural University, Kanke, Ranchi, Jharkhand, India

Establishment of root to root culture and evaluation of phytochemicals in *Rhizobium rhizogenes* transformed roots of *Stevia rebaudiana*

Shweta Singh, Sunita Murmu, Anath Bandhu Das, ZA Haider and Madhuparna Banerjee

Abstract
Hairy roots provide an efficient way of biomass production due to fast growth and displays high biosynthetic capabilities that are comparable to those of natural roots. For the establishment of root to root culture of *Stevia rebaudiana* L. Bertoni , hairy roots obtained from infection of *S. rebaudiana* with *Rhizobium rhizogenes* strain ATCC 15834 and MTCC 532 were inoculated on MS solid and MS solid medium supplemented with different concentration of Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), α-Napthalenacetic acid (NAA). After 3 weeks of culture, well established roots (20.44) were observed in hormone free MS basal media. Lower concentration of IAA and IBA of 1mg L^{-1} resulted in comparable root growth and multiplication (25.1 and 19.6 respectively). NAA proved detrimental for root multiplication. The developed root culture, when further cultured on growth regulator free MS liquid medium, exhibited fast growth and high lateral branching. Complete dark culture was more effective for the production of roots than light illumination culture. Evaluation of phytochemicals of the transformed hairy roots of *S. rebaudiana* through High- Performance Thin Layer Chromatography (HPTLC) revealed the presence of Stevioside *in vivo* and MTCC 532 infected roots and Stevioside and Rebudioside in ATCC 15834 infected roots.

Keywords: hairy roots, HPTLC, *Rhizobium rhizogenes*, *Stevia rebaudiana*, ATCC 15834, MTCC 532

Introduction
Stevia rebaudiana L. Bertoni is a branched bushy shrub of the Asteraceae family. Presently, *Stevia* is well-known for its high content of sweet diterpene (about 4–20%) in dry-leaf matter [1]. It is the source of a number of sweet ent-kaurene diterpenoid glycosides [2], and the *Stevia* glycosides are the compounds responsible for the sweet taste. Stevioside is described as a glycoside comprising three glucose molecules attached to an aglycone, the steviol moiety that have been analyzed by liquid chromatography coupled with UV, MS and ELS detection [3]. Besides sweetness, stevioside, along with related compounds which include rebaudioside A, steviol and isosteviol, may also offer therapeutic benefits, as they have anti-hyperglycemic, antihypertensive, anti-inflammatory, anti-tumour, anti-diarrhoeal, diuretic, and immunomodulatory effects [4].
Rhizobium rhizogenes is a Gram negative soil bacterium that produces hairy root disease in dicotyledonous plants [5]. *R. rhizogenes* induces the formation of proliferative multi-branched adventitious roots at the site of infection; so called 'hairy roots' [6]. Hairy roots exhibit genetic and biochemical stability, rapid growth and extensive branching in growth regulator-free medium. They have the capability of synthesizing secondary metabolites normally present in roots and organs of the species of origin [7, 8]. For this reason, hairy roots have been induced in several medicinal and aromatic plants and cultured for the production of secondary compounds [9, 10, 11]. Liquid culture of tomato root was successfully achieved [12]. The establishment of root to root culture using different type of medium (solid and liquid) on *S. rebaudiana* is reported for the first time through this study. Several works for analysis of stevioside in leaves of *S. rebaudiana* under different cultural conditions are being performed [13, 14, 15]. Through this study, phytochemical evaluation of *in vivo* and *R. rhizogenes* transformed hairy roots of *S. rebaudiana* has been carried out using HPTLC.

Materials and methods
Plant material and *R. rhizogenes* strains: The *Stevia. rebaudiana*plants were maintained in the medicinal garden of College of Biotechnology, Birsa agricultural University, Ranchi. The hairy roots were taken from three weeks old culture of ATCC 15834 and MTCC 532 infected

S. rebaudiana explants, previously co-cultured for 48 h and 3 h respectively.

Root to Root culture: For root to root culture establishment transformed hairy roots of *stevia rebaudiana* L. Bertoniwere inoculated in different culture media. Full strength MS basal medium alone and half strength MS medium supplemented with different concentrations of auxins namely Indole-3-acetic acid (IAA), α-Naphthalene acetic acid (NAA), Indole-3-butyric acid (IBA) were prepared and 8 gm l^{-1} agar was used as gelling agent. The pH of all media were adjusted between 5.7 - 5.8 and were autoclaved at 121˚C for 15 minutes for sterilization. All cultures were maintained at 25±2˚C temperature with a photoperiod regime of 16 h light (at 3000 lux approx.) and 8 h of dark period and in completely dark conditions.

Preparation of root extract: Air dried roots (5.0 mg) were powdered thoroughly and extracted with HPLC grade methanol (5 ml) for overnight, filtered using 0.45µm nylon filter and used for phytochemical analysis using HPTLC.

Preparation of standard solutions: Same amount of solutions of Stevioside, and Rebaudioside-A (Standard procured from Sigma) was prepared with methanol and used as test solutions for HPTLC analysis.

HPTLC analysis: Pre coated silica gel 60 F254 (10 cm × 10 cm) plate of uniform thickness (0.2 mm) were used for spotting. Spotting was done with Camag Automatic TLC Sampler (Linomat 5) spotter that applied spots through contact transfer (8 µl) as bands using the spray-on technique. The sampler was connected with argon gas and operated with win CATS workstation software. Distance from bottom of plate-10 mm, from side 10 mm, distance between 2 tracks was 5 mm and each trackwas 6.5mm. Chromatogram was developed in twin trough chamber previously saturated with the mobile phase: ethyl acetate: methanol: water (75:15:10 v/v/v). Between the components of the developing solvent and its vapour, equilibrium was established. Mobile phase allowed to run up to adistance of 9 cm from base. Plate was removed, air dried for 15 min. TLC plate was scanned with CAMAG TLC scanner 4 under UV at 254nm with thehelp of Win Cats software. All the data was automatically plotted in 2D and 3D graphical mode as well as tabular form per lane basis. The software collected and evaluated all data and generated analysis reports.

Results and discussion

Establishment of root to root culture: In the present study well established roots were produced from transformed root of *Steviarebaudiana* L. Bertoni on MS solid medium without auxin within 3weeks. Almost all of the adventitious roots showed vigorous growth and abundant lateral branchingon hormone-free media. They also formed a callus in phytohormone-free medium which was appeared after 3-4 weeks in *Stevia rebaudiana*. It is also worth a note that transformed roots grew well in a hormone-free medium, because transformed roots contained genes on the T-DNA caused both root formation and definite growth of the roots on phytohormonefree medium [6]. In this study the best adventitious root growth of *Stevia rebaudiana*was observed in MS media without hormone (Table 1& 2, Fig. 1& 2) (Figure 3 & 4). The mean frequency percentage of root to root culture was found best with 62.51±0.76 percent (Table 1) and mean number of root was 20.40±0.68 (Table 2)in harmone free basal media within 3 weeks (figure 4).

Effect of exogenous auxin on root production: Hairy roots were grown in half strength MS solid medium supplements with various concentrations of different auxins IAA (2.0mg/l and 1mg/l), IBA (2.0mg/l and 1.0mg/l) and NAA (1.0mg/l and 0.5mg/l). Results revealed that the response of root culture production was better in medium supplemented with IAA and IBA. Root culture in MS medium supplemented with IAA (1mg/l) and IBA (1mg/l) showed rapid induction of roots and higher growth rate. Whereas, NAA showed lower growth proliferation (Table 1& 2, Fig. 1& 2). The root initiated in medium containing different auxins, showed low growth and occasional callusing. In general, high level of auxin promoted the production of adventitious roots, although the auxin inhibited the elongation of roots [16]. On contrary, in the present study, IAA and IBA were found effective for the production of roots and NAA strongly inhibited the growth of roots. Maximum growth of roots was noticed at the 3th week of culture and later the growth was slowly declined. Roots turned light brown, flaccid and brittle. For regular maintenance of root culture, it was found that root tips of 3week old culture were the best inoculum.

HPTLC analysis of root extract: Methanol extracts of roots from *in vivo* grown plants, roots induced by *R. rhizogenes* strain 532 and strain 15834 were evaluated for the phytochemicals, against standards of *Stevia*.HPTLC is an inexpensive method for separation, qualitative identification, or semi-quantitative analysis of samples. The use of TLC/HPTLC has expanded considerably due to the development of gradient TLC methods, improved stationary and mobile phase TLC methods, improved stationary and mobile phase selection and as new methods of quantitation methods [17, 18]. One mg/ml concentration of standard solution of Stevioside, Stevioland Rebaudioside-A was applied on HPTLC plates along with methanolic extract of roots of *in vivo* grown plants, roots induced by *R. rhizogenes* strain 532 and 15834 in same concentration. The HPTLC plates were developed in a suitable solvent system and dried in air and scanned densitometrically at 254 nm. The method was validated in terms of precision and accuracy. The peak areas were recorded and relationship between the concentration and peak response was found to be linear within the range of used concentration for standard marker compound.

HPTLC chromatograms for the methanolic extracts of roots of *S. rebaudiana* from *in vivo* grown plants, transformed roots through *R. rhizogenes* strain 532 and 15834 along with standards Stevioside, Steviol and Rebaudioside A were visualized at 254 nm (Fig. 5).The structure of the three standards are illustrated (fig. 6) which displays the structure of steviol as a part of stevioside. LC-MS analysis of extracts from *S. rebaudiana*leaves have shown that stevioside bears a fragment peak which might be a molecule of steviol and rebaudioside also fragmented into steviol [19]. In the present study, the peak areas in HPTLC chromatogram reveals that the root extract from MTCC 532 infected hairy roots bear steviol (10.2%) and stevioside (8.56%) much more then in-vivo root extract (6.32% and 6.09% respectively) and is comparable to those present in the standards (13.64 % and 7.48% respectively). In ATCC 15834 infected hairy root extract much larger amount of stevioside (10.21%) was observed. In this hairy roots, rebaudioside (1.37%) comparable to the standard rebaudioside (1.08%) was present (Table 3).Overall study revealed the presence of all the standards in the root extracts. Hairy roots from *R. rhizogenes*

ATCC 15834 infections were high in stevioside and rebaudioside where as those from *R. rhizogenes* MTCC 532 were high in steviol.

Table 1: Frequency percentages of root to root culture of *S. rebaudiana* on MS solid with and without Auxin.

Percentage of cultures responded			
Medium	**7 Days**	**14 Days**	**21 Days**
MS Basal	11.52±0.22	54.16±1.17	62.51±0.76
MS Basal+ IBA(2mg/l)	5.53±0.30	33.33±0.23	37.50±.1.33
MS Basal+ IBA(1mg/l)	6.80±0.58	37.52±0.76	45.80±0.44
MS Basal+ IAA(2mg/l)	5.54±0.76	45.83±1.59	54.16±1.17
MS Basal+ IAA(1mg/l)	7.94±0.39	50.12±0.57	58.83±0.54
MS Basal+ NAA(1mg/l)	8.33±0.44	15.66±0.44	20.83±0.88
MS Basal +NAA(.5mg/l)	14.10±0.41	19.60±0.53	25.00±0.76

*Each value represents the mean ± standard error (S.E.) of three replicates per each treatment.

Table 2: Mean number of roots (*S. rebaudiana*) per culture on MS solid with and without Auxin.

Mean number of root per culture			
Medium	**7 Days**	**14 Days**	**21 Days**
MS Basal	11.80±0.38	15.40±0.51	20.40±0.68
MS Basal+ IBA(2mg/l)	8.80±0.37	10.81±0.58	12.80±0.73
MS Basal+ IBA(1mg/l)	10.40±0.54	14.40±0.54	19.60±0.55
MS Basal+ IAA(2mg/l)	9.80±0.47	13.20±0.58	14.60±0.42
MS Basal+ IAA(1mg/l)	13.80±0.44	17.50±0.45	25.10±0.68
MS Basal+ NAA(1mg/l)	3.80±0.41	5.20±0.21	7.40±0.68
MS Basal +NAA(.5mg/l)	4.60±0.51	8.30±0.42	12.50±0.55

*Each value represents the mean ± standard error (S.E.) of three replicates per each treatment

Table 3: HPTLC results evaluating Stevioside, Steviol and Rebaudioside-A with Rf values, height and area for standards and different root extracts of at 254 nm.

Track	Peak	Rf	Height	Area	% Area	Assigned Substance
Stevioside (Standard)	1	0.08	222.8	13512.6	86.09	Solvent Peak
	2	0.31	33.7	1008.7	6.43	Steviol
	3	0.38	33	1174	7.48	Stevioside
Steviol (Standard)	1	0.07	236.8	12818	86.35	Solvent Peak
	2	0.29	26.3	762.2	5.13	Steviol
	3	0.38	28.6	872.4	5.88	Steviol
	4	0.46	16	391.1	2.63	Steviol
Rebaudioside-A (Standard)	1	0.08	264.3	14462.7	89.1	Solvent Peak
	2	0.3	26.5	755.2	4.65	Steviol
	3	0.34	26.7	838.6	5.17	Steviol
	4	0.75	15.6	176	1.08	Rebaudioside A
Root extract (*in vivo)*	1	0.08	326	17388.3	87.59	Solvent Peak
	2	0.29	28.7	1255.5	6.32	Steviol
	3	0.38	29.7	1209	6.09	Stevioside
Root extract (532)	1	0.08	248.9	14277.5	81.09	Solvent Peak
	2	0.23	29.8	878	4.99	Steviol
	3	0.3	28.9	943.5	5.36	Steviol
	4	0.38	32.6	1507.3	8.56	Stevioside
Root extract (15834)	1	0.07	216.6	12418.9	88.42	Solvent Peak
	2	0.4	31.9	1434	10.21	Stevioside
	3	0.79	17.7	192.1	1.37	Rebaudioside-A

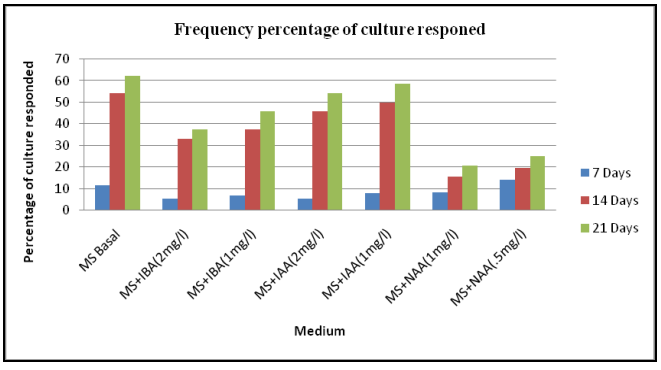

Fig 1: Graphical representation of effect of different culture media on frequency percentage of root to root culture of *S. rebaudiana.*

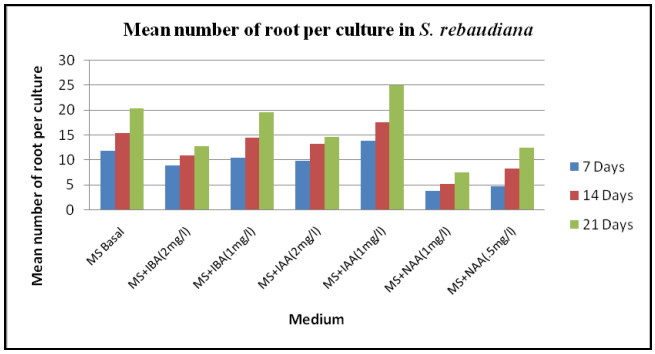

Fig 2: Graphical representation of the effect of different media on number of roots per culture in *S. rebaudiana* hairy roots.

Fig 3: Initiation and establishment of root to root culture from transformed root of *Stevia rebaudiana* in MS solid medium within 3 weeks. **3A:** *R. rhizogenes*ATCC 15834 induced hairy roots, **3B:** *R. rhizogenes*MTCC 532 induced hairy roots in *S. rebaudiana*

Fig 4: Initiation and establishment of root to root culture from transformed root of *Stevia rebaudiana* in MS liquid medium within 3 weeks.

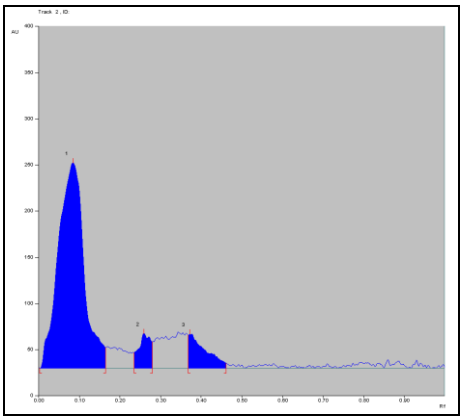

Fig 5A: Chromatogram of standard Stevioside 1mg/ml, application volume- 8 µl

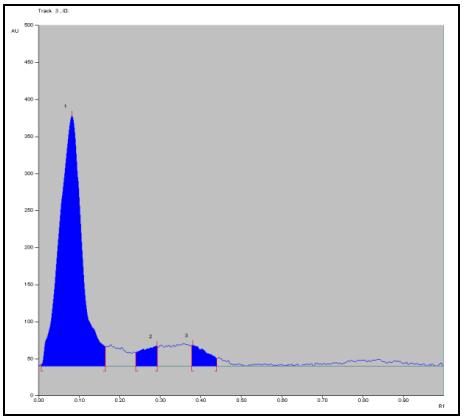

Fig 5B: Chromatogram of *in vivo* root extract 1mg/ml, application volume -8 µl

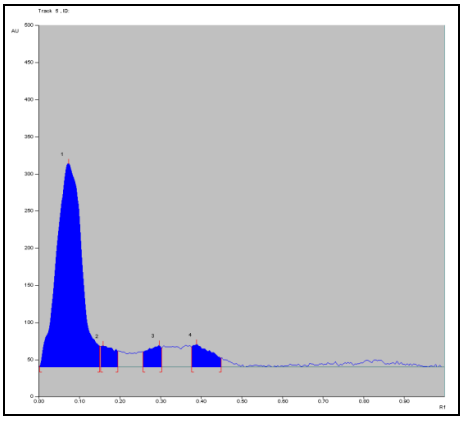

Fig 5C: Chromatogram of transformed root (532) 1mg/ml, application volume -8 µl

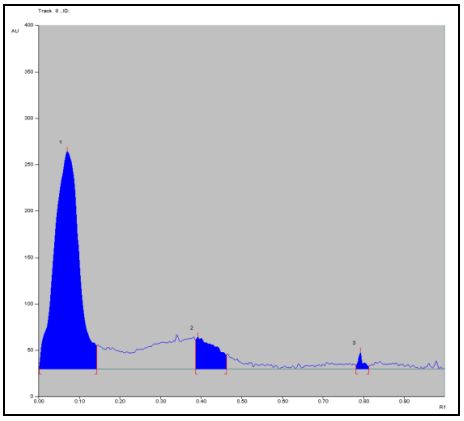

Fig 5D: Chromatogram of transformed extract (15834) extract 1mg/ml, application volume-8µl

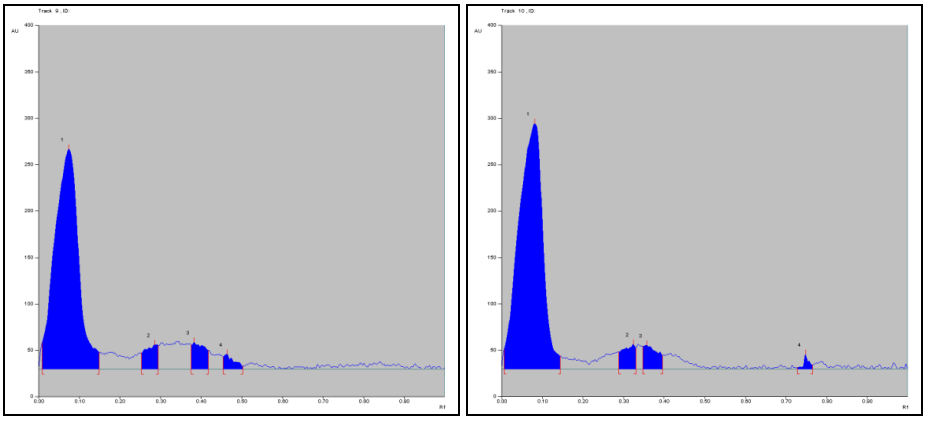

Fig 5E: Chromatogram of standard Steviol **Fig 5F:** Chromatogram of standard 1mg/ml, application volume - 8µlRebaudioside A 1mg/ml, application volume 8 µl
Fig 5: HPTLC chromatogram of Standards, the untransformed and transformed roots

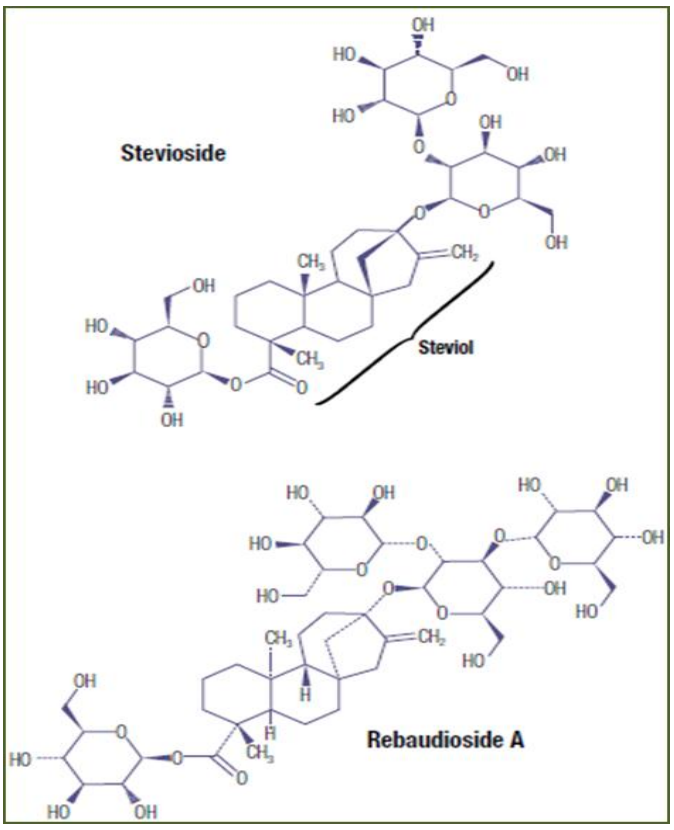

Fig 6: Structural formula of the standards of *S. rebaudiana*

Conclusion

Root to root culture from transformed root of *Steviarebaudiana* L. Bertoni (with hairy roots induction by*Rhizobium rhizogenes*ATCC 15834 and MTCC 532) were tested on different media. The mean frequency percentage of root to root culture was found best on 62.51±0.76 and mean number of root was 20.40±0.68 in MS basal media within 3 weeks. In our result, it was also observed that developed root culture exhibited fast growth and high lateral branching on growth regulator free MS liquid medium. In the present study, thephytochemical compound present in the root extractsof *S. rebaudiana* induced by *R. rhizogenes* strain 532 and 15834were evaluated through HPTLC for the presence of Stevioside, Steviol and Rebaudioside A where ATCC 15834 induced hairy roots were rich in Stevioside and Rebaudioside A while those induced with MTCC 532 were rich in steviol.

Conflict of Interest: There is no conflict of interest between the authors.

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