Studies on the induction of hairy roots in *Stevia rebaudiana* Bertoni

A

Dissertation Report

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in

Biotechnology

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Under the supervision of

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TIET, Patiala

(2018)

DECLARATION

I declare that this thesis entitled **"Studies on the induction of hairy roots in *Stevia rebaudiana* Bertoni"** is an authentic record of my own work, carried out during a period of six months. The work has been completed under the supervision and guidance of Dr. Anil Kumar, Associate Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology (Patiala).

I also declare that matter presented in this thesis has not been submitted by me in any other University / Institute for the award degree.

Lovepreet Kaur 27/08/18

Place: Patiala

Date: Aug. 27, 18

CERTIFICATE

This is to cerify that the thesis entitled "Studies on the Induction of hairy roots in *Stevia rebaudiana* Bertoni" submitted by Lovepreet kaur (Roll no. 301601027) to the Thapar Institute Of Engineering And Technology, Patiala towards partial fulfillment of the requirements for the award of the degree of Master of Science in Biotechnology. This work has been carried out under my supervision.

It is also certify that this thesis or any other part of this thesis has never been submitted, neither in part nor in full to this institute or any other university/ institute for the award of any degree.

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Lovepreet kaur

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LIST OF ABBREVIATIONS

S.No.	Abbreviations	Full form
1.	G	Gram (s)
2.	mg/l	Mili gram(s) per litre
3.	mM	Mili molar
4.	µM	Micro molar
5.	ºC	Degree celsius
6.	Fig.	Figure
7.	w/v	Weight by volume
8.	PGRs	Plant Growth Regulators
9.	MS	Murashige and Skoog medium
10.	EDTA	Ethylenediaminetetraacetic acid
11.	Rpm	Rotation per minute
12.	YMB	Yeast Mannitol Broth
13.	T – DNA	Transfer DNA
14.	*Vir*	Virulence
15.	Ri	Root-inducing
16.	OD	Optical Density
17.	KOH	Pottasium hydroxide
18.	HCl	Hydrochloric acid

ABSTRACT

Hairy roots are the source of secondary metabolites that are present in many medicinal plants from several years because of their characteristic properties such as to grow indefinitely on basal medium and to have stable productivity. The present study was focused on establishment of hairy roots from the leaf explants of *Stevia rebaudiana* Bertoni using various strains of *A. rhizogenes*. Amongst various strains tested, strain R1000 and R1022 were effective in inducing hairy roots. Various factors such as bacterial density of the suspension, infection duration and different period of co-culture were optimized for hairy root induction in *S. rebaudiana*. Among different factors studied, bacterial density of 0.6 with an infection duration of 15 min and 48 h co-cultivation period was found to be most effective. The results indicated that maximum hairy root induction (51.3 %) was observed in case of R1022 after 15 days of infection on explants. However, only 38.7% explants induced hairy roots in R1000 strain after 25- 30 days on medium containing cefotaxime (200 mg/l). The various growth parameters such as secondary root density, root elongation and root biomass production were also studied. Maximum secondary root density (3.8 roots per cm) was observed in roots transformed with R1022 strain while roots transformed with R1000 showed lower secondary root density i.e. 2.1 roots per cm. About 7.5 fold increase in root biomass accumulation was achieved in transformed roots as compared to normal roots. Thus, present study can be expanded for the possible isolation of various secondary metabolites of *Stevia rebaudiana.*

1. INTRODUCTION

Stevia rebaudiana (family *Asteraceae*) is a perennial herbaceous plant (Brandle et al. 1998; Das et al. 2007; Yadav et al. 2011). Plant is also named as madhu patra (sweet leaf) and sweet weed (Chalapathi et al. 1997; Madan et al. 2010; Singh et al. 2015). *S. rebaudiana* is a non-calorie sugar substitute which is 200-300 times sweeter than table sugar (Gupta et al. 2016; Prakash et al. 2008). The height of the mature plant is 65 cm to as tall as 180 cm after its cultivation in fertile soil (Momtazi-Borojeni et al. 2017) and leaves are 2-3 cm long (Singh and Rao 2005). *Stevia* had been used of more than 1500 years by Gurani peoples of South America to sweeten the taste of medicines and tea (Jain et al. 2017; Vanek et al. 2001). Rebaudi was the first chemist who studied the chemical characteristics of *Stevia* extracts in 1952 (Handro et al. 1989; Sapna et al. 2008). At present, the plant is cultivated all over the world in 32,000 hectares due to its sweetening property and major countries producing *Stevia* are Thailand, Korea, Japan, China, Brazil, Malaysia and Paraguay (Chen & Chang 1978; Kim et al. 2002). In India, the plant is successfully cultivated in many states mainly Maharashtra, Orissa, Rajasthan, Tamil Nadu, and Punjab (Kamalakannan et al. 2007; Patil 2010).

Being used as a natural substitute to table sugar (sucrose) for the management of diabetes, demand for *Stevia* is increasing rapidly. Among 230 species, only *S. rebaudiana* tastes sweet (Goyel et al. 2010; Soejarto 2003). The leaf extracts of *Stevia* consists of glycosides – stevioside, rebaudiosides A, B, C, D, and E, dulcoside A, and steviolbioside (Singh et al. 2015; Yadav et al. 2011). Two out of eight glycosides namely stevioside and rebaudioside-A imparts the sweet taste of *Stevia* (Madan et al. 2010). It is also a source of terpenes, flavonoids and phytochemicals such as, riboflavin and β-carotene etc. (Jayaraman et al. 2008). The leaves extract of *Stevia* have also therapeutic properties such as antihypertensive, antimicrobial, anti-fungal, anti-inflammatory, antibacterial, cardiotonic (Gupta et al. 2013; Misra et al. 2015).

Stevia is propagated through seeds. However, there is a limitation of poor seed germination and heterogeneous plant population (Singh et al. 2017; Turgut et al. 2015). Moreover, *Stevia* crop requires more irrigation (Lavini et al. 2008). Alongwith, various factors such as climate changes and water management strategies have to be kept in mind

for cultivation of *Stevia* in the field (Ngaka 2012). The increased amount of secondary metabolite accumulation was observed in hairy roots as compared to cell suspension cultures (Bansal et al. 2014). Thus hairy roots can be valuable source for the isolation of secondary metabolites from *Stevia*.

Plant-based molecules are worldwide accepted because these are source of proteins, carbohydrates and fats (Giddings et al. 2000; Kozlowski 1992; Simopoulos 2004). Plants secrete a variety of components and these are primarily accumulated in the plant roots (Fu et al. 2006; Kumar et al. 1995). Therefore, hairy root lines have been produced in several plants for the isolation of secondary compounds (Guillon et al. 2006; Georgiev et al. 2007; Khatodia et al. 2013; Zhou et al. 2007). Genetic transformation with *A. rhizogenes* was found to be very useful for various applications such as production of phytochemicals (Bhadra et al. 1993), secondary metabolites (Chandra and Chandra 2011; Bourgaud et al. 2001) and phytoremediation (Nedelkoska and Doran 2000). Hairy roots proliferate at the infection site due to transfer of T-DNA of plasmid to nuclear genome of host plant (Chilton et al. 1980; Tzfira et al. 2006). Different bacterial strains results in different morphologies of hairy roots (Kim et al. 2008; Klee et al. 1987). Hairy roots exhibits high stability and potential to produce secondary metabolites (Kim et al. 2002; Ono & Tian 2011). These roots show rapid and plagiotropic growth on basal medium, lack of geotropism and formation of lateral roots (Korde et al. 2016; Shanks and Morgan 1999). The advantage of these properties are higher growth, less requirement of aeration in liquid medium and thus has potential to up-scale in bioreactors (Kim et al. 2008). Under light conditions, hairy roots of all the *Asteraceae* genera are able to form chloroplasts and such green roots are probably adapted to photoautotrophy (Flores et al. 1993; Pandey et al. 2016). Transgenic hairy roots are capable of selection without antibiotic resistant marker genes (Benjamin et al. 1993). A number of factors affect the transformation efficiency and need to be studied. These include degree and mode of injury of plant tissue, genotype, attachment of bacteria, bacterial density and host specificity (Godwin et al. 1992; Karami 2008). Moreover, nature and type of carbohydrate & nitrogen sources in medium, various physical factors such as light and temperature can affect root induction and growth, biomass yield and secondary metabolites (Akula et al. 2011; Yu et al. 2005).

2. REVIEW OF LITERATURE

Many attempts have been made in the past for the induction of hairy roots in medicinal and aromatic plants. Some relevant works in this field are discussed below:

Shunan et al. (1997) reported the hairy root induction from the leaves of *Ginkgo biloba L*. The leaves were surface disinfected with ethanol (70%) for 30 seconds and $HgCl_2$ (0.1%) solution for 10 minutes. These leaves were infected with 15834, A4, R1000 strains of *A. rhizogene*, and then cultured on medium (1/2 MS+0.5 g/l ampicillin) and maintained at 26±1 °C in scattering light. Hairy roots were induced after 25-60 days of incubation. Strain 15834 had highest transformation frequency while strain A4 was failed for hairy root induction. The detection of opines was done by thin layer chromatography.

Ercan et al. (1999) transformed the cotyledons (explants) of *Rubia tinctorum* by 15834, 2628, R1000 and 9365 strains of *A. rhizogenes*. The strains R1000, 15834, 9365 induced hairy roots while strain 2628 resulted only in callus formation. The results indicate that strain 15834 had highest transformation frequency (75%) of hairy root induction as compared to other strains.

Pawar and Maheswari (2004) reported the transformation of stem segments, hypocotyls and leaves of *Withania somnifera and Solanum surattensen* using MTCC532 and MTCC2364 strains of *A. rhizogene*s. Results indicated that hairy roots were induced within 15 days of infection in case of leaf explants of both the plants. The growth of the roots induced was studied on basal MS medium independent of exogeneous hormones. The growth rate of transformed roots was increased by 10 folds as compared to control.

Tenea et al. (2008) induced hairy roots using two strains LBR56 and LBA9402 of *A. rhizogene* in *Glycyrrhiza glabra*. Results showed that LBR56 strain had highest transformation efficiency (80%) but LBA9402 strain was failed to induce hairy roots. The root biomass increased significantly from aseptically grown roots and internodal segments by infection with LBR56 strain of *A. rhizogenes.* The hairy roots thus induced were analyzed at molecular level for the integration and expression of T-DNA genes.

Kim et al. (2008) compared the transformation efficiency of five different strains of *A. rhizogenes* on leaf explants of *Arachis hypogaea L*. The bacterial cultures were overnight grown at 28°C on a rotary shaker at 120 rpm in LB medium till OD_{600} reaches to 0.5 and used timentin to control the bacterial overgrowth. Hairy roots were induced after 3 to 4 weeks of infection using R1000, A4, 15834, R1601 and R1200 strains. Highest transformation frequency of 75.8% was achieved with R1601 strain.

Hashem et al. (2009) studied the induction of hairy roots in *Solanum dulcamara* using 8196 and A4T strains of *A. rhizogenes*. The results indicate that rapid growth and intensive branching was achieved when grown on hormone free MS media. Endogenous phytohormone auxins and cytokinins were determined in an ethyl acetate fractionated extracts using standardized biochemical methods such as hordium coleoptile test for auxins and Cucurbita cotyledonary leaf test for cytokinins.

John et al. (2009) carried out the transformation of tea leaves with *A. rhizogene* strain MTCC532. Among, the various concentrations of acetosyringone tested, transformation frequency was enhanced upto 70% on 300 µM/L of acetosyringone. These transformed roots were established on MS medium containing 5mg/l IAA and maltose as carbon source.

Ahlawat et al. (2012) reported the Artemisinin production in *Artemisia annua L*. using LBA9402 strain of *A. rhizogene*. The optimum infection time and co-cultivation period was found to be 7 min and 48 hrs respectively. However, 100% transformation frequency was achieved using leaves as explants. Hairy roots induced within 5 to 6 days after infection of LBA9402 while A4 strain induced hairy roots within 9 to 11 days. Integration of T - DNA was confirmed by PCR. Various physical factors such as pH, temperature and cultivation media were also optimized for the hairy root induction.

Sarvankumar et al. (2012) compared the efficiency of different *A. rhizogene* strains (ATCC15834, R1000, K599) on leaf, petiole and internodal explants in *Withania somnifera*. However, transformation was achieved in ATCC15834 and R1000 strains. The highest transformaton frequency was achieved in R1000 strain i.e. 64 %. The transformation frequency of R1000 strain was enhanced upto 93.2% by the addition of

acetosyringone. The biomass (withaferin – A) accumulation i.e. 72.3 mg/g dw was also analysed using HPLC.

Khatodia et al. (2013) reported the hairy root induction in *Solanum xanthocarpum* through MTCC532 strain of *A. rhizogene* with 75% transformation frequency. These hairy roots were grown on ½ strength MS liquid medium with doubling time of 7 days. Conformation of transformation was done through PCR to analyze the amplification of rol C gene.

Pandey et al. (2014) reported the transformation of *Coleus forskohlii* through MTCC2364 strain of *A. rhizogene* on nodal segments and mature leaves. The hairy roots were observed after 18 days and transformation frequency was achieved upto 30%. The highest amount of forskolin was recorded in nodal stem parts by HPLC analysis.

Michalec – Warzecha et al. (2016) reported the transformation of leaves and internodes of *S. rebaudiana* with ATCC15384 and LBA9402. Strain LBA9402 had higher transformation efficiency (40%) than ATCC15384. Growth of hairy roots was found to be affected by the light and 24 h light conditions reduced growth.

Panda et al. (2017) induced hairy roots on leaf explants in *Semecarpus anacardium* L using A4, ATCC15834 and LBA9402 strains of *A. rhizogenes*. The hairy roots were induced after 30 days of inocculation. The maximum transformation frequency (61%) was achieved with ATCC15834 strain. These roots were then transferred to ½ strength liquid medium. Hairy roots started differentiating after 3- 4 times subculturing in half strength liquid media.

Table 1: Effect of *A. rhizogene* strains on production of active principle from induced hairy roots

Plant species	***A. rhizogenes* strains**	**Secondary metabolites**	**References**
Aconitum heterophyllum	LBA9402, LBA 9360, A4	Aconites	Giri et al. (1997)
Arachis hypogaea	15834, A4, R1000, R1200, R1601	Resveratrol	Kim et al. (2008)
Cantella asiatica	R1000	Asiaticoside	Kim et al. (2007)
Duboisia myoporoides	HRI	Scopolamine	Deno et al. (1987)
Papaver somniferum	LBA9402, 15834	Alkaloids	Bonhomme et al. (2004)
Brugmansia candida	LBA9402	Scopolamine Hyoscyamine	Giulietti et al. (1993)
Camptotheca acuminate	ATCC15834, R1000	Camptothecin,10-hydroxycamptothecin	Lorence et al. (2004)
Gentiana macrophylla	A4 GUS, R1000, LBA9402, ATCC11325	Secoiridoid glucoside gentiopicroside	Tiwari et al. (2007)
Pueraria phaseoloides	ATCC15834	Puerarin	Shi and Kintzios (2003)
Rhodiola sachalinensis	A4	Salidroside	Zhou et al. (2007)
Salvia miltiorrhiza	LBA9402, ATCC15834, TR 105, R1601, A41027	Diterpenoides	Zhi and Alfermann (1993)
Solanum khasianum	A4, LBA9402	Solasodine	Jacob and Malpathak (2005)
Scopolia japonica	15834, A4, 1855, 2659	Alkaloids	Mano et al. 1986
Valeriana wallichii	A4, LBA9402	Valepotriates	Banerjee et al. 1998

3. OBJECTIVES

The present work was focused on hairy root induction in *Stevia rebaudiana* that can reliably produce and accumulate steviol glycosides with the following objectives:

- To induce hairy roots from leaf explants of *Stevia rebaudiana*
- To study the factors influencing hairy root induction
- To select and optimize the growth of the hairy root lines

4. MATERIALS AND METHODS

The experiments on hairy root induction in *Stevia rebaudiana* were conducted in plant tissue culture laboratory of TIFAC – CORE of this Institute. The materials used and methodology applied during experimentation are as follows;

3.1. Chemicals and glassware

All chemicals (AR grade) were procured from HiMedia laboratories (Mumbai). Experiments were performed in 300 ml culture bottles (Kasablanka Corporation, Mumbai). Glassware like conical flasks, measuring cylinders etc. were purchased from Borosil Glass Works Ltd., Mumbai, India.

3.2. Preparation of culture medium

Murashige and Skoog medium (MS medium) was prepared from the concentrated stock solutions containing different constituents (macronutrients, micronutrients, vitamins). The stock solutions were added in required amount to make the medium and 3.0% (w/v) sucrose was added to it and the final volume of the medium was make up with the help of distilled water. The pH of the medium was titrated to 5.8 with 0.1N KOH or 0.1N HCl. Before autoclaving agar 0.7% (w/v) was added, 50 ml of medium was poured per culture bottle. Medium was autoclaved at 121°C and 15 psi for 20 minutes. Stock solutions of antibiotics were prepared in desired concentrations and filter sterilized using millipore filters (pore size 0.22 µm) and stored at 4 ºC for use. Antibiotic in required concentrations were added to the autoclaved medium after cooling down to a temperature to 45- 50 ºC followed by pouring in autoclaved culture bottles.

3.3. Aseptic culture & Culture conditions

The aseptic cultures of *Stevia* were multiplied on MS1 medium (MS containing 10 µM silver nitrate; Kaur et al. 2017). The cultures were then maintained at 25±2 °C under 16/8 h photoperiod with light intensity of 42 µmol m^{-2} s^{-1}.

3.4. Induction of Hairy roots

Materials

The fully expanded young leaves excised from 21 day old microshoots were source of explants for induction of hairy roots.

Bacterial strains

Two *Agrobacterium rhizogene* strains (R1000, R1022) were tested for hairy root induction from leaf explants taken from microshoots. Both the strains were inoculated in yeast mannitol broth (YMB) and grown at 28±2 ºC on rotary shaker (130 rpm) for 16-18 hrs. Bacterial suspensions were centrifuged (3000 g, 5 min) and pellets were re-suspended in yeast mannitol broth to attain the desired OD_{600}.

Infection

The leaf segments obtained from the microshoots were pricked with sterilized blade and infected with suspension of YMB containing *A. rhizogene* strains (OD_{600} = 0.6) for 10 – 15 minutes.

Co – cultivation:

Following infection, the leaves were blotted on to autoclaved filter paper to remove excess bacterial load and were cultured on MS medium for 48 hrs for co-cultivation under dark conditions.

Washing and inoculation

After co-cultivation infected leaves were washed in autoclaved distilled water (4-5 times). The washed explants were soaked on autoclaved filter paper and inoculated into MS medium containing cefotaxime (200 mg/l). These transformed explants were then incubated in dark.

3.5 Optimization of culture conditions for hairy root induction using *Agrobacterium rhizogenes* R1022

3.5.1. Effect of bacterial concentration

Leaf segments obtained from the microshoots were infected with bacterial suspension with various OD_{600} i.e., 0.4, 0.6, 0.8 or 1.0 for 10 minutes. Following infection, leaves were co-cultivated for two days and then washed with sterile water. These were transferred on to medium with cefotaxime (200 mg/l).

3.5.2. Effect of infection time

Leaf segments were infected with *A. rhizogenes* suspension (OD_{600}= 0.6) for various durations (0-20 min) in petri plates. Following infection, leaves were co-cultivated for two days and were then washed with autoclaved distilled water. These were cultured on MS medium supplemented with cefotaxime (200 mg/l).

3.5.3. Effect of co-cutivation period

Co-cultivation period was optimized when leaf segments were infected with *A. rhizogenes* suspension (OD_{600}= 0.6) for a period of 10 minutes. The excess bacteria were blotted off using sterile blotting paper. These were co-cultivated on MS medium for varied time periods (12, 24, 36, 48 h) in complete dark.

3.6. Growth and morphology of hairy roots

Morphology and growth rate of roots induced with different bacterial strains were assessed vis-à-vis normal roots used as control. Approximately 1 cm long actively growing root tips were cultured to antibiotic medium and incubated for 25-30 days in dark. The various parameters such as root elongation, number of secondary roots per unit length and root growth were recorded.

3.7. Determination of root biomass

The growth kinetics of actively growing hairy roots was examined in 250 ml Erlenmeyer flask containing liquid medium (20 ml). Roots (known quantity and actively

growing) were inoculated in these flasks and incubated in dark at 60 rpm for 3 weeks. These roots were then harvested from culture vessels, followed by washing with distilled water and excess water was blotted off. After recording fresh weight, the dry weight was recorded following drying at 60 ºC till constant weight. The growth was calculated as the ratio of the dry weight of harvested biomass to the dry weight of the inoculum.

3.8. Statistical Analysis

All experiments were performed with three replications and the experiments were repeated thrice. Data recorded was represented in the form of Mean±SE. The graph was plotted in *OriginPro* 8 Software.

4. RESULTS & DISCUSSION

1. Establishment of Hairy roots

Hairy roots from *S. rebaudiana* were induced after infection of leaf explants using *A. rhizogenes* strains R1000 and R1022. The root induction was higher (51.3%) when infected with R1022 strain whereas lower root induction (38.7%) was achieved with R1000 (Figure 1). The explants were inoculated with R1000 strain (OD_{600} = 0.6) for 15 minutes and co-cultivated for 48 hrs. Hairy roots were induced after 25-30 days only on the infected leaves with R1000. On the other hand, hairy roots were formed within 12-15 days after inoculation of leaves with R1022 strain for 15 minutes at OD_{600}= 0.6 and co-cultivated for 48 hours.

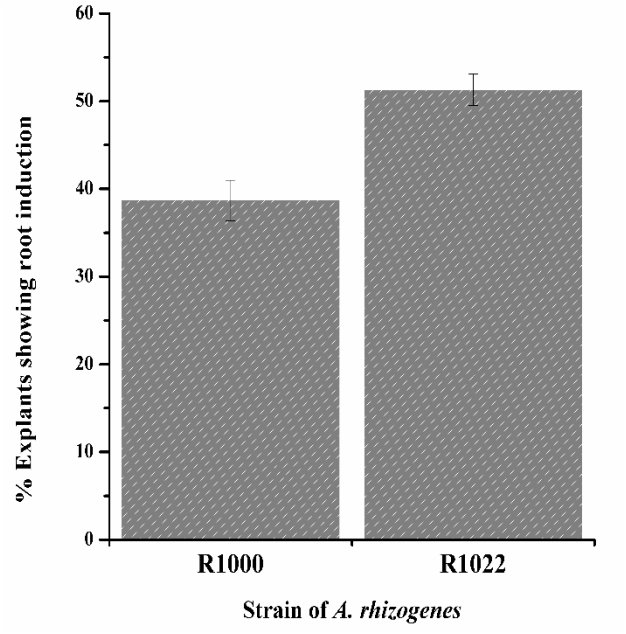

Figure 1: Effect of two strains of *A. rhizognenes* (R1000, R1022) on frequency of explants showing root induction

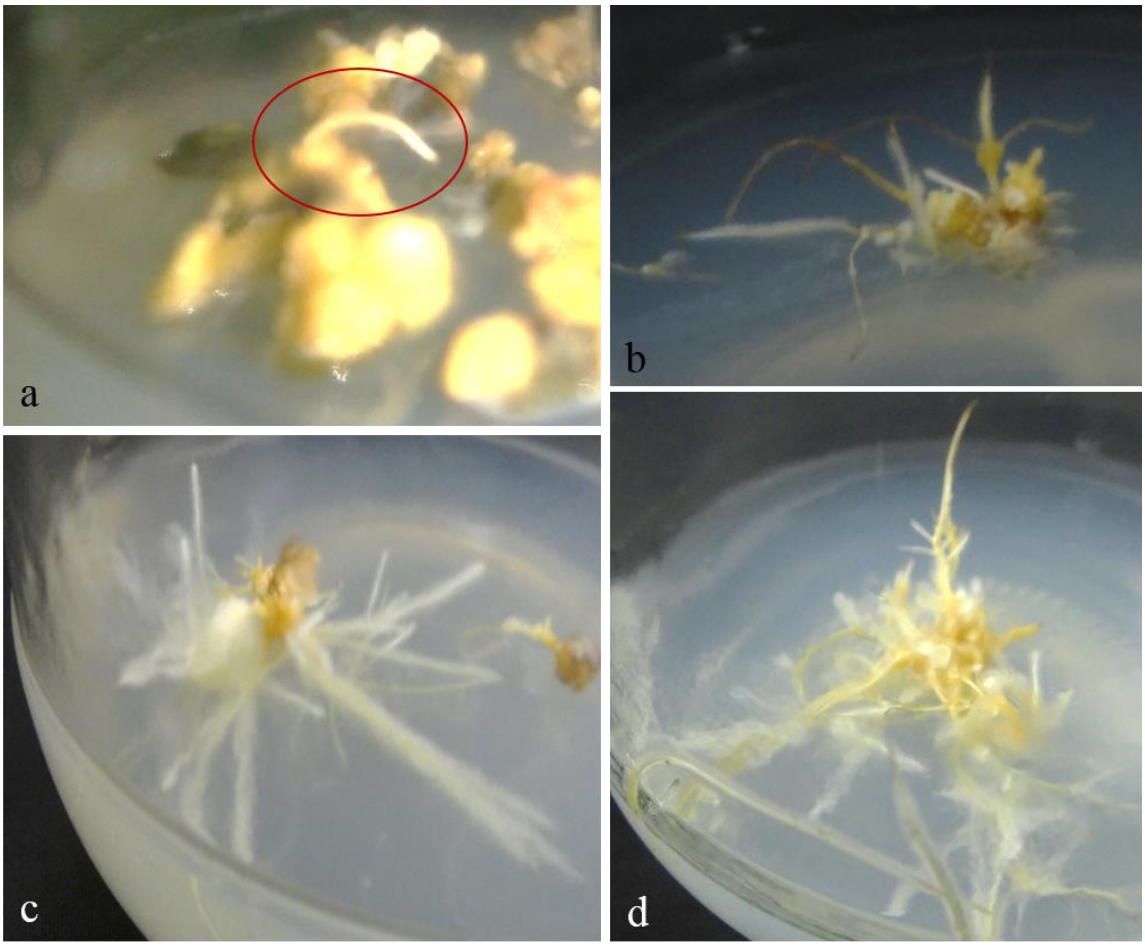

Figure 2: Hairy root induction in *Stevia rebaudiana* using *Agrobacterium rhizogenes* R1022; (a) Emergence of roots from intervening phase within 15 days; (b) rapid growth of roots; (c) root elongation on solid MS medium; (d) formation of Lateral root after repeated culturing on MS medium.

Hairy root induction had been attempted in many plants for the isolation of secondary metabolites (Hamil et al. 1987; Sevon and Oksman 2002; Srivastava and Srivastava 2007). In this context, present work focused on the establishment of hairy roots in *Stevia rebaudiana.* The transformation efficiency was influenced by the bacterial strain and the time of infection (Park and Facchini 2000). The degree of infection varies among different strains of *A. rhizogenes* which had been reported earlier in many plants (Hu and Du 2006). It usually depends on the strain virulence and/or specificity of the host

(Akramian et al. 2008). Michalec-warzecha et al. in 2016 reported that LBA 9402 strain resulted in higher transformation efficiency (40%) than ATCC15384 which resulted in only 2% transformation efficiency in *S. rebaudiana*. Similarly, Ara and Choudhary (2014) concluded that strain ATCC15834 showed maximum 92% transformation frequency while 86% transformation frequency was achieved with MTCC532 from shoot tip explants of *Withania somnifera*. Moreover, Sivanandhan et al. (2014) concluded that R1000 was the most effective strain than A4 as study resulted in 88% and 79% transformation for R1000 & A4 strains respectively from leaf explants. Ahlawat et al. (2012) compared the transformation efficiency of five different strains (LBA920, MTCC532, LBA301, LBA9402, NRRLB193 and A4). The study revealed that NRRLB193 strain exhibits the high transformation efficiency (40%) for leaves, while LBA9402 strain (80%) exhibits maximum transformation efficiency (80%) for stem portions. Moreover, 100% transformation frequency was acheived with LBA9402 strain. However, the other strains were failed for hairy roots induction.

In both the strains, bacteria were eliminated from cultures through MS medium supplemented with cefotaxime. Michalec-warzecha et al. (2016) used cefotaxime (100 mg/l) to eliminate the excess bacteria in transformed roots of *Stevia*. Similarly, Pandey et al. (2014) observed that MS medium containing cefotaxime (500 mg/l) helped to eliminate the bacteria from transformed explants. Although, carbenicillin and cefotaxime are two widely used antibiotics, the effect of claforan or penicillin derivates and ampicillin were also studied to remove bacteria from infected explants (Madhusudanan et al. 2008; Rahman et al. 2004). However, ticarcillin/potassium clavulanate is economical because of the potential to be widely used for carbenicillin and cefotaxime sensitive plants (Ling et al. 1998). Boisson-Dernier et al. (2001) reported the bacterial elimination by the incorporation of augmentin at successive concentrations (400, 200 and 0 mg/l). Moreover, Shackelford and Chlan (1996) conducted a study to identify which antibiotic is effective against *Agrobacterium* strains. Thus, cefotaxime and moxalactam were effective against LBA4404 and EHA101 strains respectively.

2. *Optimization of conditions for hairy root induction using Agrobacterium rhizogenes R1022*

Various factors such as density of bacterial culture, infection duration and cocultivation period influencing hairy root induction were also studied.

2.1 Effect of bacterial density

It had been reported that the bacterial density of suspension influenced hairy root induction in various plant species (Cao et al. 2009). In our present study, the different optical densities at 600 nm such as 0.4, 0.6, 0.8 and 1.0 were tested. The maximum frequency of hairy root induction was observed (53.33%) when a bacterial density was 0.6.

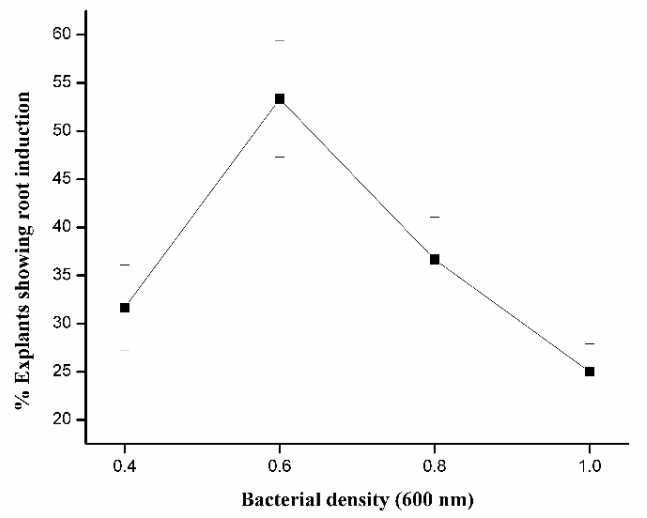

Figure 3: Effect of bacterial density of *A. rhizogenes* (R1022) on the induction of hairy roots

2.2 Effect of infection time

The inoculation time with *A. rhizogenes* also affected the frequency of root induction (Tao and Li 2006). The different infection periods (0, 5, 10, 15 and 20 minutes) were tested. Thus in our study, an infection time of 15 minutes with *A. rhizogenes* was found to be more suitable for the induction of roots.

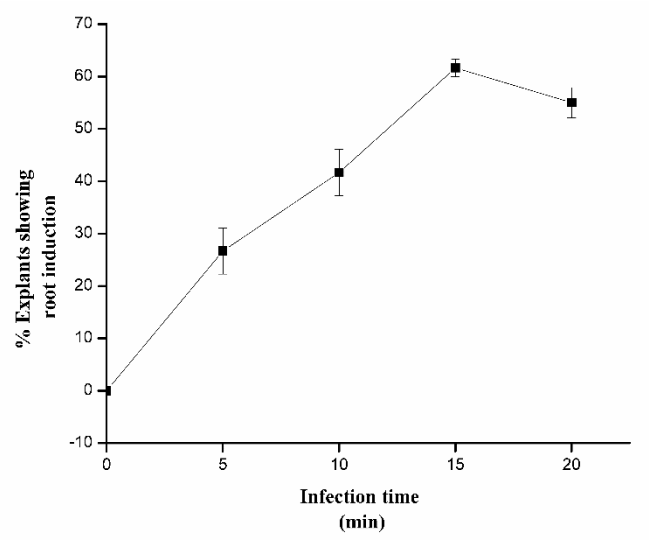

Figure 4: Effect of infection duration on the induction of hairy roots using R1022 strain

2.3 Effect of co-cultivation period

The co-cultivation period following infection also influenced the hairy root induction. The present study revealed that maximum root induction (65±2.89%) was achieved when co-cultivation period of 48 hrs was attempted. However, decline in hairy root production (55±5.78%) was observed when co-cultivation period was extended to 72 hrs.

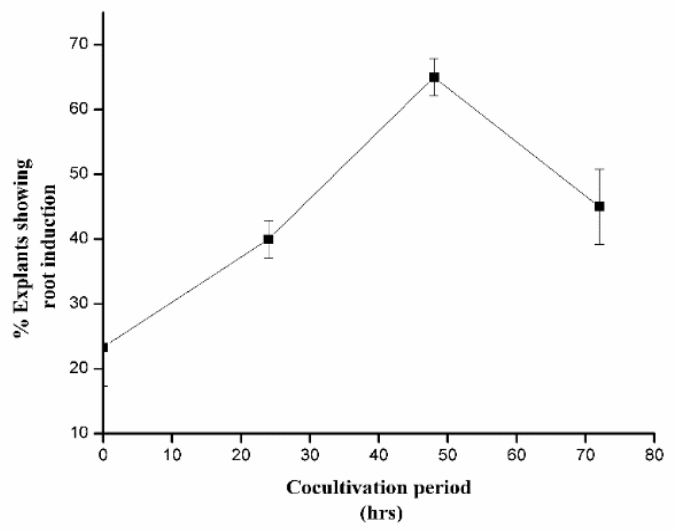

Figure 5: Effect of different co-cultivation periods on the induction of hairy roots using R1022 strain

The efficiency of hairy root induction is closely related to bacterial density and optimum infection time (Bonhomme et al. 2000; Georgiev et al. 2007). Despite high density results in the death of explants, whereas low density alongwith extended infection favours efficient T-DNA delivery resulting in more transformation efficiency (Kabirnataj et al. 2016). The present study revealed that 15 minutes of infection duration resulted in increased hairy root production. This had been reported earlier in many plants (Kim et al. 2008; Fu et al. 2015).

The transformation frequency varied when the explants were co-cultivated for different time durations (Panda et al. 2017). However, a co-cultivation period of 2 days resulted in maximum transformation frequency (Kumar et al. 2006; Kim et al. 2012). Further, on increasing co-culture duration from 48h resulted over bacterial growth on leaves or browning of explants. Thus increase in co-culture periods reduced the mean percentage as well as number of root induction (James et al. 1993; Sivanesan and Jeong 2009).

3. Growth and morphology of roots

In MS medium solidified with agar, the roots showed typical transformed root morphology and rapid growth within 3–5 weeks after infection. After few passages, hairy roots (without bacteria) were transferred to liquid medium. Moreover, morphological variations among different hairy roots from different strains were also recorded after six weeks.

Different lines of hairy roots induced from two different strains were found to vary in growth pattern and morphology such as lateral root density, length and thickness of the roots. Roots induced by R1022 strain were found to be thick whereas thin roots were observed in R1000 strain. However, strain R1022 developed roots through callus phase.

The significant difference was observed in lateral root density among various hairy root lines (Figure 2) and the maximum lateral root density was recorded in roots induced from R1022 strain (3.8 roots per cm). Minimum density of lateral roots was achieved with R1000 strain (2.1 roots per cm). Though, root elongation vary significantly

among strains of *A. rhizogenes*, elongation of hairy roots was maximum with R1022 strain with a length of 4.6 cm.

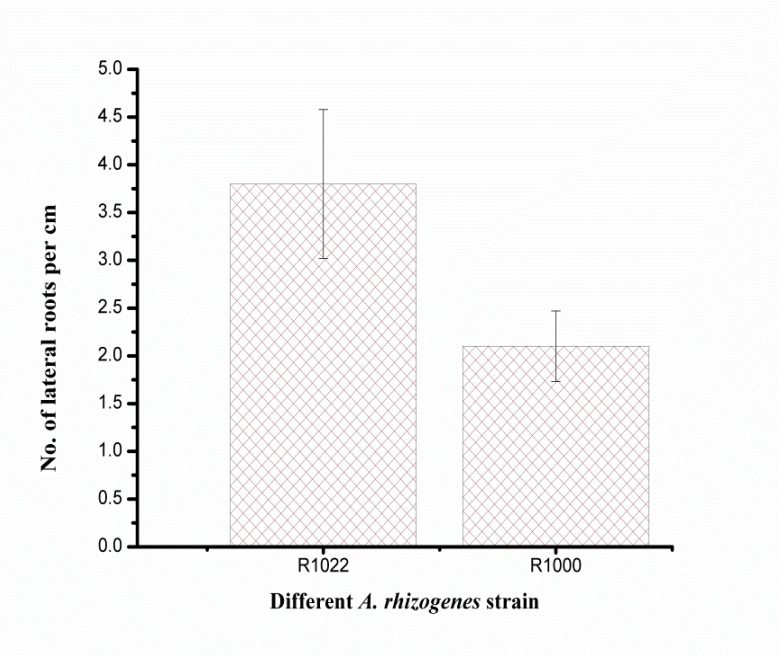

Figure 6: Number of lateral roots (per cm) obtained from two different *A. rhizogenes strains* (R1000 & R1022)

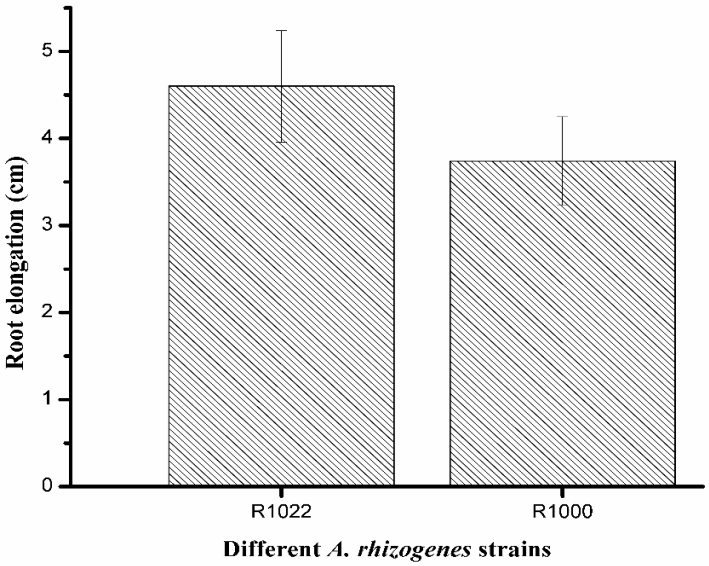

Figure 7: Variation in Root length (cm) using R1000 and R1022 strains of *A. rhizogenes*.

It had been observed that different morphologies of hairy roots obtained with different strains of *A. rhizogenes* in various plant species (Giri et al. 2001) because of the expression of genes present on T-DNA region of Ri plasmid and the location of T-DNA in the host plant genome (Zambryski et al. 1989; Chandra 2012). Saravanakumar et al. 2012 transformed different explants such as leaf, petiole and internodal segments of *Withania somnifera* using three different *A. rhizogenes* strains. The transformation frequency ranged from 0-64% depending upon the type of strain and explants used. The various morphological variations for hairy roots induced from callus and direct hairy roots were observed with R1000 strain from petiole explants. Park and Facchini (2000) studied the efficiency of five different strains of *A. rhizogenes* i.e. 13333, C58C1, R1000, and R1200rolD. The fast growth of hairy roots were produced by inoculation with R1000 than 13333 and R1200rolD strains. Phenotypic alterations on transformed roots such as formation of lateral roots and thin adventitious roots were observed as compared to untransformed roots.

4. Root Biomass determination

The hairy roots thus produced accumulated more biomass (g/l) over transformed roots used as control. Maximum biomass accumulation (8.78 g/l) was achieved in hairy roots using R1022 strain, which showed approximate 7.5 folds increase in root dry weight (Table 3).

Table 2: Biomass accumulation after 4 weeks of inoculation with R1000 and R1022 strains of *A. rhizogenes.*

***A.* Rhizogenes Strain**	**Fresh weight (g/l)**	**Dry weight (g/l)**	**Growth ratio (Fold growth)**
R1022	8.78±0.08	0.468±0.04	7.43
R1000	1.76±0.06	0.207±0.07	3.23
Untransformed	1.28±0.03	0.093±0.02	1.47

The initial dry weight of roots was 0.063 g/l.

Hairy roots showed an increase in biomass accumulation on MS medium solidified with agar and the growth ratio was about 7.5 fold greater than un-transformed roots used as control. Moreover, the hairy roots were more branched than control roots. Thus, in present study, growth pattern and biomass accumulation varied depending on the strain used. In previous reports, Chung et al. (2016) reported 9.2 fold increase in biomass on MS medium containing sucrose (4%) within 3-4 weeks. Moreover, 97.25 g/l fresh weight and 10.11 g/l dry weight accumulation was observed on the same medium from hairy root cultures of *Brassica rapa* sp. *rapa*. More biomass accumulation was achieved when cultures were grown under complete dark conditions. Thus, in order to increase biomass accumulation in transformed explants, various physical factors such as medium to flask volume, pH and temperature, chemical factors such as carbon and nitrogen sources, and aeration and agitation needs to be optimized (Murthy et al. 2014).

5. CONCLUSION

The outcomes of the present study are discussed below:

- Hairy root cultures were established in *Stevia rebaudiana* using R1000 and R1022 strains of *Agrobacterium rhizogenes*.
- Maximum root induction was achieved in strain R1022 as compared to R1000.
- Various factors such as bacterial density, infection duration, and co-cultivation period were optimized for hairy root induction.
- The various physiological factors such as number of lateral roots, root elongation and biomass accumulation were analyzed among two different strains of *Agrobacterium rhizogenes.*

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Annexure

Composition and concentration of various constituents present in Murashige and Skoog (1962) medium.

Constituents	Concentration (mg/l)
Macronutrients	
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Micronutrients	
$MnSO_4.H_2O$	16.9
$ZnSO_4$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Nicotonic acid	0.5
Myoinositol	100
Pyrodoxine HCl	0.5
Thiamine HCl	0.1
Amino acid	
Glycine	2.0
Ferric EDTA	30
Sucrose	3% (w/v)
Agar	0.7%(w/v)