Elite hairy roots of *Raphanus sativus* (L.) as a source of antioxidants and flavonoids

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Abstract

An efficient protocol for hairy root induction in radish was established by optimizing several parameters that affect the efficiency of *Agrobacterium rhizogenes*-mediated transformations. Explants wounded using sterile hypodermic needle, infected with *Agrobacterium* suspension (0.6 OD₆₀₀) for 10 min and co-cultivated in 1/2 MS medium containing acetosyringone (100 µM) for 2 days displayed maximum percentage of hairy root induction using MTCC 2364 (77.6%) and MTCC 532 (67.6%). On further experiments with MTCC 2364 initiated hairy roots, maximum biomass accumulation (fresh weight = 9.50 g; dry weight = 1.48 g) was achieved in liquid 1/2 MS medium supplemented with 87.6 mM sucrose after 40 days of culture. Transgenic state of hairy roots of MTCC 2364 was confirmed by polymerase chain reaction using *rolB*- and *rolC*-specific primers. The MTCC 2364-induced hairy roots produced higher amount of phenolic (33.0 mg g⁻¹), flavonoid (48.0 mg g⁻¹), and quercetin (114.8 mg g⁻¹) content compared to auxin-induced roots of non-transformed radish. Furthermore, the results of ferric reducing antioxidant power and 1,1-diphenyl-2-picrylhydrazyl assay confirmed that the antioxidant activity of MTCC 2364 root extracts was improved when compared to auxin-induced roots of non-transformed radish. The present study offers a new insight in radish for production of phenolics and flavonoids (quercetin) using *A. rhizogenes*-mediated hairy root induction.

Keywords *Agrobacterium rhizogenes* · Acetosyringone · Quercetin · Antioxidant activity · Radish

Introduction

Radish (*Raphanus sativus* L.) belongs to the cruciferae family, which includes white and red cabbage, broccoli, brussels sprouts, and cauliflower (Lugasi et al. 1998). Radish has received much attention in recent years because of its nutritional and health protective value (Patil et al. 2009). In addition, the crop possesses a number of pharmacological and therapeutic properties and consumed as a common vegetable around the world (Ogra et al. 2007). In India, radish is used in the aboriginal system of medicine for the treatment of different human illness. Radish contains essential bioactive components like flavonoids, alkaloids, tannins and phenolic compounds (Wang et al. 2010). The main phytochemicals present in radish include 4-(methylthio)-3-butenyl

isothiocyanate, allyl isothiocyanate, benzyl isothiocyanate, and phenethyl isothiocyanate (Wang et al. 2010). In addition, radish also contains significant amount of flavonoids such as kaempferol, quercetin, myricetin, apigenin, luteolin (Lugasi et al. 2000) and glycosides, peroxidases, and antioxidants. Therefore, radish has been used to treat various gastrointestinal, biliary, hepatic, urinary, respiratory disorders (Ghayur and Gilani 2006), cardiovascular diseases such as hypertension and different types of cancer (Chung et al. 2012).

In recent years, *Agrobacterium rhizogenes*-mediated hairy root culture system has been chosen as a viable alternative for the production of pharmaceutical compound from medicinal plants. Hairy root culture offers opportunities for stable production of a wide range of plant secondary metabolites compared to undifferentiated plant cell/callus cultures (Georgiev et al. 2007; Hu and Du 2006). Hairy roots also offer advantages such as faster growth rate independent of hormones, genetic and biochemical stability, efficient biosynthetic capacity compared to native plant roots, long-term preservation, and sizable biomass production (Kim

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et al. 2013; Ludwig-Müller et al. 2014). Recent progress in the scaling-up of hairy root culture turns this system as an attractive tool for industrial processes (Guillon et al. 2006).

However, to date, there is no report available on optimization of factors affecting hairy root production for the improvement of quercetin content in radish. Thus, optimizing an efficient *A. rhizogenes*-mediated hairy root culture system could be a promising approach to supply flavonoids (quercetin) from radish biomass. Quercetin has gained a lot of attention as powerful antioxidant and antifibrotic compound with cytoprotective effects (Cincin et al. 2014). In medical science, it has been demonstrated that quercetin, in a dose of 150 mg day⁻¹, downregulates from the systolic pressure (Egert et al. 2009). Quercetin displayed anticancer and apoptosis-inducing effects in several tumor cell lines such as colon carcinoma CT-26 cells, prostate adenocarcinoma LNCaP cells, human prostate PC3 cells, pheochromocytoma PC12 cells, estrogen receptor-positive breast cancer MCF-7 cells, acute lymphoblastic leukemia MOLT-4 T cells, human myeloma U266B1 cells, human lymphoid Raji cells and ovarian cancer CHO cells (Hashemzaei et al. 2017). In addition, quercetin was also shown to possess potential antiviral (Wu et al. 2007) and anti-inflammatory activity (Kim et al. 2011).

Thus, the present study is aimed to (1) optimize (wounding, *Agrobacterium* strain, bacterial culture density, infection time, co-cultivation days, acetosyringone concentration, media type, media strength, and sucrose concentration) a hairy root culture system from leaf explants of radish, (2) quantify the total phenol and flavonoid (quercetin) content in transformed hairy roots, and (3) assess the antioxidant activity in methanolic extracts of transformed hairy roots.

Materials and methods

Plant material, in vitro seed germination, and explants preparation

The seeds of white radish cv. co 1 were procured from the Tamil Nadu Agricultural University, Coimbatore, India. The seeds were disinfected with 70% (v/v) ethanol for 2 min, followed by rinsing with 4% (v/v) sodium hypochlorite solution (NaClO) for 15–20 min, and treated with Tween-20 for 15 min. The seeds were then finally rinsed several times with sterile double-distilled water. The surface-sterilized seeds were wiped within sterile Whatman filter paper no 1 and transferred into a sterile culture tube containing half-strength Murashige and Skoog, MS (1962) medium (pH 5.7) without hormones. The seeds were incubated at 25 ± 2 °C under total darkness for 5 days to facilitate seed germination. The tubes with seedlings were then incubated under a 16-h photoperiod with light supplied by cool white

fluorescent lamps (Philips, New Delhi, India) at an intensity of 50 μmol m⁻² s⁻¹. The leaf from 20-day-old in vitro-grown plantlets was cut along the midrib into small segments to obtain explants of 0.5 cm × 0.5 cm.

Bacterial strains and culture preparation

The *A. rhizogenes* strains (wild-type) such as MTCC 2364 and MTCC 532 were used in the present study. A single colony from two *A. rhizogenes* strains was inoculated into 30 ml LB broth containing rifampicin (25 mg l⁻¹) and incubated on an orbital shaker at 28 °C for 14 h at 180 rpm. The bacterial cells were harvested by centrifugation at 3500 rpm for 20 min and suspended in 30 ml liquid half-strength MS medium (pH 5.8).

Optimization of conditions to improve hairy root induction in leaf explants

The leaf explants (with or without wounding) were transferred to *Agrobacterium* (MTCC 2364 and MTCC 532) suspension (0.0–1.0 OD₆₀₀) for 0–30 min (infection time) and co-cultivated (1–5 days) in 1/2 MS medium containing acetosyringone (0.0–125 μM). The co-cultivated explants were washed with sterile double-distilled water and then with sterile liquid shoot induction medium containing 300 mg l⁻¹ cefotaxime and placed on 1/2 MS basal solid medium supplemented with 300 mg l⁻¹ cefotaxime and maintained for 25 days without sub-culture. After co-cultivation (total dark), all the cultures were incubated at 25 ± 2 °C under a 16-h photoperiod (50 μmol m⁻² s⁻¹) provided by cool white fluorescent lamps.

Optimization of media strength and sucrose to improve hairy root biomass

After 25 days of culture, 0.2 g (fresh weight) hairy roots of MTCC 2364 was transferred to 250 ml Erlenmeyer flasks containing 40 ml of liquid medium (1/2 MS, B5 Gamborg et al. 1968, or 1/2 B5). In a separate experiment, 0.2 g (fresh weight) hairy roots of MTCC 2364 were transferred to 40 ml of liquid 1/2 MS medium containing different concentration of sucrose (29.2–146.0 mM). All the cultures were incubated on a rotary shaker (80 rpm) at 25 ± 2 °C under total darkness. The root cultures were replaced with respective fresh media of same composition (at 10-day interval) and maintained for 40 days. The hairy root biomass accumulation was assessed in terms of fresh weight and dry weight. In total, the hairy roots were maintained for 65 days (solid medium = 25 days; liquid medium = 40 days) from the date of culture establishment.

Development of auxin-induced root culture from leaf explants of radish to serve as control

Leaf explants (0.5 cm × 0.5 cm) prepared as mentioned above were cultured on MS solid medium supplemented with 7.38 µM indole-3-butyric acid (IBA) and 87.6 mM sucrose. The cultures were incubated under a 16-h photoperiod with light supplied by cool white fluorescent lamps at an intensity of 50 µmol m⁻² s⁻¹ for 25 days without sub-culture. Well-established adventitious roots (0.2 g) developed from leaf explants were then transferred to 40 ml of liquid 1/2 MS medium containing 7.38 IBA and 87.6 mM sucrose. The adventitious roots were maintained for 40 days as described earlier in materials and methods. These auxin-induced adventitious roots from leaf explants of non-transformed radish were used as control for subsequent experiments.

PCR analysis to screen the presence of *rolB* and *rolC* gene

Two randomly selected hairy roots of MTCC 2364 were analyzed for the *rolB* and *rolC* integration by polymerase chain reaction (PCR). Genomic DNA was isolated from the hairy roots of MTCC 2364- and auxin-induced roots of non-transformed radish using DNeasy® plant mini kit (Qiagen, Germany). The primers, *rolB* FP: 5-GGATCCCAAATTGCT ATCCCC-3 and *rolB* RP: 5-AGGCTTCTTTCATTCGGT TTAC-3 were used to amplify a 437 bp fragment of the *rolB* gene. The primers, *rolC* FP: 5-TGGCTGAAGACGACC TGTGT-3 and *rolC* RP: 5-TAGCCGATTGCAAACCTGC A-3, were used to amplify a 1037 bp fragment of the *rolC* gene. The PCR reaction consisted of 50 ng of genomic DNA, 0.2 mM of dNTPs, 1.0 U of *Taq* DNA polymerase, 1.0 µM of each primer, and 2.5 µl of 10 × *Taq* DNA polymerase buffer in a total of 25 µl reaction. The PCR was initiated in a PTC-100™ thermal cycler (MJ Research, Inc., Waltham, USA) programmed with a hot start at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 57 °C for 30 s, 72 °C for 45 s, and by final extension at 72 °C for 10 min. The reaction products were analyzed by electrophoresis on a 1.0% (w/v) agarose gel and visualized by staining with ethidium bromide.

Extraction for estimation of total phenolics, flavonoids, quercetin and antioxidant content

The hairy roots of MTCC 2364- and auxin-induced radish roots (control) were separated from the liquid medium, rinsed with sterile water, and air dried. The roots were then ground using mortar and pestle into fine powder in liquid nitrogen. About 1 g (DW) of fine root powder was transferred to glass tubes and extracted with 10 ml of methanol (99.9%). The samples were then macerated on an orbital

shaker at 150 rpm for 3 days (22 °C). The extracts were dried using a rotary vacuum evaporator (Cyber Lab). About 1 mg of dried methanolic root extract powder was dissolved in 1 ml of HPLC grade methanol (99.9%) and filtered using 0.45 µm polyvinylidene fluoride syringe filter. The filter-sterilized extracts were used to analyze total phenolic content, flavonoids content, and assess antioxidant potential using FRAP and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay.

Quantification of total phenolic, flavonoid, and quercetin content

About 40 µl of filter-sterilized root extracts (1 mg ml⁻¹) was taken to assess the total phenolic and flavonoid content. Total phenolic compounds were determined according to the method of Singleton et al. (1999) using Folin–Ciocalteu reagent. The gallic acid at 1 mg ml⁻¹ was used as standard. Total flavonoids content was determined by the method described by Crozier et al. (1997) using quercetin (1 mg ml⁻¹) as standard. Quantification of quercetin was performed using Waters 2998 liquid chromatography (Waters, Milford, MA) equipped with the photodiode array detector (PDA). The data were processed with Empower 2 software. Twenty microliters of filter-sterilized root extracts (1 mg ml⁻¹) and standard (1 mg ml⁻¹ quercetin) were injected into the Symmetry® C18 column (4.6 mm × 250 mm, 5 µm) and eluted isocratically with HPLC grade methanol:water (with 0.2% orthophosphoric acid) in a ratio of 65:35 v/v at a flow rate of 1.0 ml min⁻¹. The quercetin was detected with a PDA detector at a wavelength of 375 nm. The amount of the quercetin present in each root extract was calculated by comparing the standard area with sample area. The standard and sample solution were injected in triplicate. The quantification was calculated by the following formula.

$$\text{Quercetin (mg g}^{-1}\text{)} = \frac{\text{Peak area of the sample}}{\text{Peak area of the standard}} \times 1000.$$

Ferric reducing antioxidant power (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The FRAP, a method for measuring total reducing power of electron donating substances, was assessed according to Benzie and Strain (1996). About 20, 40, 60, 80, or 100 µl of filter-sterilized root extracts (1 mg ml⁻¹) were mixed with freshly prepared 6 ml of FRAP reagent (0.1 M acetate buffer:0.02 M FeCl₃:0.01 M TPTZ = 10:1:1). The OD₅₉₂ (Thermo Evolution 201 UV-Spectrometry) was recorded after 30 min incubation at 37 °C.

The ability of the extracts to scavenge the DPPH radical was assessed spectrophotometrically (Gyamfi et al.

1999). About 20, 40, 60, 80, or 100 μl of filter-sterilized root extracts (1 mg ml^{-1}) were mixed with 450 μl of Tris-HCl buffer (50 mmol l^{-1} ; pH 7.4) and 1.0 ml of DPPH (0.1 mmol l^{-1} in methanol). The resultant absorbance was recorded at 517 nm after 30 min of incubation at 37°C . The percentage of inhibition was calculated as percentage of inhibition = $[(A_0 - A_1)/A_0] \times 100$ where A_0 was the absorbance of the control (blank without extract) and A_1 was the absorbance in the presence of the extract.

Statistical analysis

For experiments like optimization of conditions to improve hairy root induction (wounding, *Agrobacterium* strain, bacterial culture density, infection time, co-cultivation days and acetosyringone concentration), each parameter/treatment contained three replicates with at least 30 explants per replicate. For optimization of media type, media strength, and sucrose concentration on root biomass accumulation, each treatment contained three replicates with at least 30 root inoculum mass (0.2 g fresh weight) per replicate. Five randomly selected root biomass (1 g hairy roots of MTCC 2364- or auxin-induced roots) from each replicate (three replicates) were used to quantify the total phenol, flavonoid, quercetin content and assess the antioxidant activity by FRAP and DPPH assay. Data were statistically analyzed using analysis of variance using SPSS version 11.09 (IBM Corporation). Data are presented as mean $\pm$ standard error. The mean separations were carried out using Duncan's multiple range test (DMRT), and significance was determined at 5% level.

Results

Optimization of conditions to improve hairy root induction in radish

The factors affecting transformation efficiency such as, explant wounding, *Agrobacterium* strain, bacterial culture density, infection time, co-cultivation days, and acetosyringone concentration were optimized to obtain maximum hairy root induction from in vitro-grown leaf explants of radish.

Effect of wounding on hairy root induction

The leaf explants wounded using sterile hypodermic needle (27G1/1) (Dispovan, New Delhi, India) and infected using MTCC 2364 and MTCC 532, registered higher percentage (Fig. 1a) of hairy root induction (27.6 and 22.3%, respectively). In the case of explants without wounding, MTCC

2364 and MTCC 532 resulted only with 20.3 and 16.6% of hairy root induction, respectively (Fig. 1a).

Effect of bacterial density (OD_{600}) on hairy root induction

The densities of bacterial suspension used for infection of leaf explants influenced hairy root induction (Fig. 1b). Among different bacterial densities (OD_{600} 0.2–1.0) tested, 0.6 followed by 0.8 showed best results in terms of hairy root induction for both the strains compared to counterparts (0.2, 0.4, and 1.0 OD_{600}). Under optimum condition (0.6 OD_{600}), MTCC 2364 resulted with the highest percentage of hairy root induction (45.3%) whereas, MTCC 532 recorded 37.0%.

Effect of infection time (OD_{600}) on hairy root induction

The duration of 5–30 min was tested to optimize infection time for *A. rhizogenes* MTCC 2364 and MTCC 532 to improve hairy root induction in leaf explants (Fig. 1c). Infecting leaf explants with MTCC 2364 and MTCC 532 for an optimal period of 10 min showed the maximum percentage (57.3 and 48.6%, respectively) of hairy root induction. Infecting the leaf explants beyond or less than 10 min showed a gradual decline in hairy root induction for two strains. In addition, extending the infection time over 30 min resulted with reduced survival of explants (data not shown).

Effect of co-cultivation duration on hairy root induction

The leaf explants co-cultivated with MTCC 2364 and MTCC 532 for different time duration (1–5 days) showed a significant variation in percentage of hairy root induction (Fig. 1d). The hairy root induction (%) increased as co-cultivation time increased, reaching maximum at 2 day followed by decrease thereafter. At this optimal co-cultivation period (2 days), leaves infected with MTCC 2364 (68.6%) and MTCC 532 (58.6%) displayed highest percentage of hairy root induction.

Effect of acetosyringone on hairy root induction

The explants co-cultivated without acetosyringone (Tables 1, 2) displayed reduced percentage of hairy root induction (MTCC 2364 = 34.3%; MTCC 532 = 27.6%), less no. of positive geotropic roots (MTCC 2364 = 1.0/explant; MTCC 532 = 0.6/explant), and decreased no. of lateral roots (MTCC 2364 = 1.6/root; MTCC 532 = 1.3/root). However, inclusion of acetosyringone in co-cultivation medium was found to be more effective in improving the hairy root induction in leaf explants infected with MTCC 2364 and MTCC 532 (Tables 1, 2). Among the five different concentrations

Fig. 1 Optimization of factors influencing genetic transformation and hairy root induction in leaf explants of radish cv. co 1 transformed using *Agrobacterium rhizogenes* (MTCC 2364 and MTCC 532). **a** Influence of wounding (±) on hairy root induction in radish. The leaf explants were wounded (±), infected in *Agrobacterium* suspension (0.8 OD₆₀₀) for 20 min and co-cultivated in 1/2 MS medium for 3 days. **b** Effect of bacterial density on hairy root induction in radish. The leaf explants were wounded, infected in *Agrobacterium* suspension (0.2–1.0 OD₆₀₀) for 20 min and co-cultivated in 1/2 MS medium containing acetosyringone (75 μM) for 3 days. **c** Effect of infection time on hairy root induction in radish. The leaf explants

were wounded, infected in *Agrobacterium* suspension (0.6 OD₆₀₀) for 5–30 min and co-cultivated in 1/2 MS medium containing acetosyringone (75 μM) for 3 days. **d** Influence of co-cultivation time on hairy root induction in radish. The leaf explants were wounded, infected in *Agrobacterium* suspension (0.6 OD₆₀₀) for 10 min and co-cultivated in 1/2 MS medium containing acetosyringone (75 μM) for 1–5 days. The values were recorded after 15 days of culture in respective medium. Each parameter/treatment contained three replicates with at least 30 explants per replicate. Bars represent mean (±) standard error of three replicates

Table 1 Effect of acetosyringone on hairy root induction from leaf explants of radish infected and co-cultivated with *A. rhizogenes* MTCC 2364

Acetosyringone (μM)	No. of explants infected	No. of explants responded*	Percentage of response (%)*	No. of roots with positive geotropism/explant*	Number of lateral roots/root*
0	30	10.3 ± 0.38 ^f	34.3 ± 0.46 ^f	1.00 ± 0.07 ^d	1.60 ± 0.34 ^f
25	30	14.3 ± 0.57 ^e	47.6 ± 0.28 ^e	1.37 ± 0.01 ^d	3.01 ± 0.12 ^e
50	30	17.6 ± 0.78 ^d	58.6 ± 0.59 ^d	2.64 ± 0.03 ^c	5.30 ± 0.23 ^c
75	30	20.6 ± 0.81 ^c	68.6 ± 0.50 ^c	3.07 ± 0.09 ^b	7.64 ± 0.29 ^b
100	30	23.3 ± 1.04 ^a	77.6 ± 0.41 ^a	5.32 ± 0.19 ^a	9.65 ± 0.34 ^a
125	30	21.0 ± 0.91 ^b	70.0 ± 0.32 ^b	3.61 ± 0.08 ^b	5.37 ± 0.15 ^d

The leaf explants were wounded, infected in *Agrobacterium* suspension (0.6 OD₆₀₀) for 10 min and co-cultivated in 1/2 MS medium containing acetosyringone (0.0–125 μM) for 2 days. Each treatment contained three replicates with at least 30 explants per replicate. Data represent mean (±) standard error of three replicates. Values with the different letters within columns are significantly different according to Duncan's multiple range test (DMRT) at a 5% level

*The data were tabulated after 15 days of culture in 1/2 MS medium

Table 2 Effect of acetosyringone on hairy root induction from leaf explants of radish infected and co-cultivated with *A. rhizogenes* MTCC 532

Acetosyringone (μM)	No. of explants infected	No. of explants responded*	Percentage of response (%)*	No. of roots with positive geotropism/explant*	Number of lateral roots/root*
0.0	30	08.3 ± 0.46^e	27.6 ± 0.59^e	0.6 ± 0.24^f	1.3 ± 0.34^f
25	30	09.3 ± 0.47^d	31.0 ± 1.67^d	1.31 ± 0.23^e	3.03 ± 1.16^e
50	30	14.3 ± 0.63^c	47.6 ± 0.68^c	2.63 ± 0.07^d	5.34 ± 0.17^d
75	30	17.6 ± 0.80^b	58.6 ± 0.59^b	3.06 ± 0.37^b	6.64 ± 0.42^b
100	30	20.3 ± 0.90^a	67.6 ± 0.83^a	4.05 ± 0.41^a	7.67 ± 0.46^a
125	30	16.6 ± 0.75^b	55.3 ± 0.59^b	2.61 ± 0.31^c	4.35 ± 0.35^c

The leaf explants were wounded, infected in *Agrobacterium* suspension (0.6 OD_{600}) for 10 min and co-cultivated in 1/2 MS medium containing acetosyringone (0.0 – $125 \mu\text{M}$) for 2 days. Each treatment contained three replicates with at least 30 explants per replicate. Data represent mean ($\pm$) standard error of three replicates. Values with the different letters within columns are significantly different according to Duncan's multiple range test (DMRT) at a 5% level

*The data were tabulated after 15 days of culture in 1/2 MS medium

of acetosyringone (25 – $125 \mu\text{M}$) tested, $100 \mu\text{M}$ was found to be most effective in enhancing hairy root induction with both strains as compared to the control. The maximum of 77.6% hairy root induction, 5.3 positive geotropic roots/explant, and 9.6 lateral roots/root were obtained from leaf explants infected with MTCC 2364 at $100 \mu\text{M}$ acetosyringone (Table 1). Similarly, at same condition ($100 \mu\text{M}$), the explants infected with MTCC 532 resulted with 67.6% of hairy root induction, 4.0 positive geotropic roots/explant, and 4.3 lateral roots/root (Table 2).

Overall, the radish leaf explants wounded using sterile hypodermic needle, infected by *Agrobacterium* suspension (0.6 OD_{600}) for 10 min and co-cultivated in 1/2 MS medium containing acetosyringone ($100 \mu\text{M}$) for 2 days was found to be the optimal regime to obtain maximum percentage of hairy root induction using MTCC 2364 (Fig. 2a, c, e) and MTCC 532 (Fig. 2b, d, f). MTCC 2364 showed better results for all parameters tested compared to MTCC 532. Therefore, further experiments were carried out using the *A. rhizogenes* strain MTCC 2364.

Optimization of media strength and sucrose to improve hairy root biomass

Biomass accumulation in hairy roots of MTCC 2364 significantly varied depending on the nature of media (Table 3; Fig. 2g, h). Biomass accumulation was higher (9.5 g FW , 1.4 g DW), when hairy roots were cultured in 1/2 MS medium compared to counterparts (MS, B5, 1/2 B5). The sucrose concentration also influenced the biomass accumulation in hairy roots of MTCC 2364. Among the different concentrations of sucrose tested, 87.6 mM was optimal to achieve maximum biomass (9.5 g FW , 1.4 g DW) beyond which a steady decline in biomass accumulation was noticed (Table 3; Fig. 2g, h).

PCR analysis

The presence of *rolB* and *rolC* gene in hairy roots of radish transformed with *A. rhizogenes* strain MTCC 2364 were confirmed by PCR. The expected 437 bp (*rolB*; Fig. 3a, lanes 2, 4) and 1037 bp (*rolC*; Fig. 3b, lanes 2, 4) amplified product were observed in the genomic DNA of MTCC 2364 hairy roots. It indicates that the *rolB* and *rolC* gene were integrated into the radish hairy roots genome. Genomic DNA from auxin-induced roots of non-transformed radish did not show any amplified product with *rolB* (Fig. 3a, lane 3) and *rolC* (Fig. 3b, lane 3) gene-specific primers.

Total phenolic, flavonoid, and quercetin content

Total phenolic (33.07 mg g^{-1}) and flavonoid content (48.0 mg g^{-1}) was high in hairy roots of MTCC 2364 compared to control roots (phenolic content = 27.7 mg g^{-1} ; flavonoid content = 39.3 mg g^{-1}) from non-transformed plants (Table 4). The results of HPLC analysis confirmed that the quercetin content was improved in hairy roots (114.8 mg g^{-1}) of MTCC 2364 compared to control roots (24.1 mg g^{-1}) from non-transformed radish (Fig. 4). On an average, 4.7-fold increase in quercetin was achieved in hairy roots of MTCC 2364 compared to control.

FRAP reducing ability and DPPH radical scavenging assay

The reducing ability of the MTCC 2364 hairy root extract was determined using the FRAP assay. The formation of blue colored TPTZ– Fe^{2+} complex from colorless oxidized TPTZ– Fe^{3+} by the action of the electron donating antioxidants was recorded at absorbance 593 nm. The antioxidant activity of MTCC 2364 hairy roots improved as the volume of extracts used for assay was increased from

Fig. 2 *Agrobacterium rhizogenes* (MTCC 2364 and MTCC 532) mediated genetic transformation and hairy root induction from leaf explants of radish cv. co 1. **a** Leaf explants transformed with *Agrobacterium rhizogenes* MTCC 2364, **b** leaf explants transformed with *Agrobacterium rhizogenes* MTCC 532, **c, e** induction (after 15 days of culture) and proliferation (after 25 days of culture) of hairy roots from leaf explants transformed with MTCC 2364 in 1/2 MS medium, **d, f** induction (after 15 days of culture) and proliferation (after 25 days of culture) of hairy roots from leaf explants transformed with MTCC 532 in 1/2 MS medium, **g, h** secondary hairy roots proliferated from 0.2 g inoculum mass of hairy roots regenerated from leaves transformed with MTCC 2364 (after 30 and 40 days of culture on liquid 1/2 MS medium containing 87.6 mM sucrose, respectively)

20 to 100 μ l (Fig. 5). In addition, the antioxidant potential of MTCC 2364 hairy roots was significantly higher when compared to control.

In DPPH assay, MTCC 2364 hairy root extracts (20, 40, 60, 80 and 100 μ l) showed higher potency of inhibition when compared to control root extracts of non-transformed radish (Fig. 5). Furthermore, the extracts of MTCC 2364 hairy roots were able to scavenge DPPH radicals in a concentration dependent manner.

Discussion

Plant secondary metabolites have attracted notice because of their pharmaceutical properties and therapeutic applications in the medical field (Ludwig-Müller et al. 2014; Kalinyan and Agastian 2015). Radish is considered as a staple healthy food worldwide because of the presence of phytochemicals, vitamins, nutrients, and bioactive components

Table 3 Optimization of media and sucrose concentration on biomass accumulation from hairy root culture of radish infected and co-cultivated with *A. rhizogenes* MTCC 2364

Parameters	Fresh weight (g)*	Dry weight (g)*
Medium		
MS	7.22 ± 0.68 ^b	1.03 ± 0.21 ^b
1/2 MS	9.50 ± 0.60 ^a	1.48 ± 0.15 ^a
B5	5.64 ± 0.73 ^d	0.86 ± 0.24 ^d
1/2 B5	6.74 ± 0.70 ^c	0.82 ± 0.19 ^c
1/2 MS + sucrose (mM)		
29.2	2.56 ± 0.32 ^d	0.58 ± 0.34 ^d
58.4	7.65 ± 1.2 ^b	1.08 ± 0.5 ^b
87.6	9.50 ± 0.60 ^a	1.48 ± 0.15 ^a
116.8	8.11 ± 0.22 ^b	1.19 ± 0.16 ^b
146.0	6.34 ± 0.45 ^c	0.81 ± 0.71 ^c

The cultures were initiated with 0.2 g of inoculum mass (FW) in 40 ml medium. Each treatment contained three replicates with at least 30 inoculum mass per replicate. Data represent mean (±) standard error of three replicates. Values with the different letters within columns are significantly different according to Duncan's multiple range test (DMRT) at a 5% level

*The data were tabulated after 40 days of culture in respective medium

such as flavonoids, alkaloids, tannins and phenolic compounds (Ghayur and Gilani 2006). Quercetin, a flavonoid, accumulates to the highest amount in radish leaf and cannot be found in other parts of plants. Quercetin has a wide range of pharmacological properties, such as anti-allergic, antioxidant, antimicrobial, antihistaminic, anti-inflammatory effects, antidiabetic, and anticancer properties (Gao

et al. 2009). Hairy root culture is an effective, simple, and low cost method in biotechnology to produce bioactive compounds. In last few years, transformed hairy roots have become a valuable tool for the production of secondary metabolites (Guillon et al. 2006; Hu and Du 2006). Therefore, an optimized *A. rhizogenes*-mediated transformation is a prerequisite to increase the production of flavonoid such as quercetin in radish. In the present study, factors affecting transformation efficiency were optimized for two *A. rhizogenes* strains (MTCC 2364 and MTCC 532) to improve the percentage of hairy root induction from leaf explants.

In general, wounding of explants releases plant metabolites that transcriptionally activate virulence (*vir*) genes in *Agrobacterium* plasmid. Injuries in plant tissues also facilitate the entry of *Agrobacterium* into the host plant and furthermore, wounded site is required for plant cell competence for *Agrobacterium*-mediated gene delivery (Escudero and Hohn 1997). In the present study, leaf explants wounded using sterile hypodermic needle displayed higher percentage of hairy root induction compared to explants without wounding. Tinland and Hohn (1995) reported that, *Agrobacterium* reacts to wound-excreted signals and could use enzymes (secreted due to wounding) need for DNA replication for the integration of the T-DNA into host cells. In a recent study in *Mucana puriens*, manual wounding method was shown to be essential to develop hairy roots from MTCC 2364-infected leaf explants (Vishwakarma et al. 2017).

In the present investigation, density of bacterial suspension plays an important role in the production of transgenic hairy roots. The results of the present study confirmed that bacterial optical density (OD₆₀₀) of 0.6 favored maximum

Fig. 3 PCR analysis of genomic DNA from hairy roots of MTCC 2364 and auxin-induced roots of non-transformed radish to screen the presence of *rolB* and *rolC* gene. **a** Lane 1 100 bp plus DNA ladder, lanes 2, 4 genomic DNA from hairy roots of MTCC 2364 showing the presence of *rolB* gene (437 bp), lane 3 auxin-induced roots of

non-transformed radish. **b** Lane 1 100 bp plus DNA ladder, lanes 2, 4 genomic DNA from hairy roots of MTCC 2364 showing the presence of *rolC* gene (1037 bp), lane 3 auxin-induced roots of non-transformed radish

Table 4 Total phenolic and flavonoids content in extract from hairy root culture of radish infected and co-cultivated with *A. rhizogenes* MTCC 2364

	Phenolic content (mg g ⁻¹) ^a	Flavonoid content (mg g ⁻¹) ^b
Control	27.73 ± 2.3 ^b	39.36 ± 1.2 ^b
MTCC 2364	33.07 ± 2.8 ^a	48.03 ± 1.9 ^a

Ten ml of methanol (99.9%) was added to 1 g (DW) of fine root powder and placed on an orbital shaker at 150 rpm for 3 days (22 °C). The extracts were dried, and about 1 mg root extract powder was dissolved in 1 ml of HPLC grade methanol (99.9%). About 40 µl of root extracts (1 mg ml⁻¹) was taken for analysis. Data represent mean value of three replicates (five root biomass per replicate) with standard errors. Values with the different letters within columns are significantly different according to Duncan's multiple range test (DMRT) at a 5% level

Control auxin-induced adventitious roots from leaves of non-transformed radish

^amg Gallic acid equivalent g⁻¹ DW

^bmg quercetin equivalent g⁻¹ DW of extract

percentage of hairy root induction in leaves transformed with MTCC 2364 and MTCC 532. In a similar study, OD₆₀₀ between 0.6 and 0.8 was preferred to infect the leaf explants of *Coleus forskohlii* with *A. rhizogenes* strain MTCC 2364 to achieve efficient hairy root induction (Pandey et al. 2014a, b). These results also corroborated with the earlier report on *Bacopa monnieri* where OD₆₀₀ of 0.6 was used to achieve highest hairy root induction in leaf explants infected with *A. rhizogenes* (Bansal et al. 2014). In the present study, the leaf explants infected with MTCC 2364 and MTCC 532 at higher bacterial densities (0.8 and 1.0 OD₆₀₀) turned necrotic, which may be due to increased production of toxic compounds or competitive inhibition due to bacterial overgrowth (Kumar et al. 1991; Sonia Saini et al. 2007). In the present investigation, less induction of hairy roots at suboptimal OD₆₀₀ (0.2 and 0.4) could be attributed to the low availability of bacteria to transform competent cells as suggested by Kumar et al. (1991).

The infection time significantly varies according to choice of the explant and plant species. Therefore, identifying the optimal infection time could be critical to improve transformation efficiency of that particular crop. The relocation and incorporation of T-DNA depend on absorbance (OD) of *Agrobacterium* culture and this would facilitate the bacterial cell to attach with explants (An et al. 2014). In this study, the optimal infection time of 10 min displayed the highest percentage of hairy root induction in leaf explants infected by MTCC 2364 and MTCC 532. Our results are in agreement with earlier report on *B. monnieri* where an infection time of 10 min was used to attain maximum hairy root induction from leaf explants (Bansal et al. 2014). The infection time

adapted to transform the leaf explants above 10 min showed decreased percentage of hairy root induction. Our results suggest that longer infection time (above 10 min) reduced the viability of leaf explants and lowered the root induction potential.

Co-cultivation is very important for gene delivered in plants since bacterial attachment, T-DNA delivery, and integration occurs during this period (Aggarwal et al. 2013; Chen et al. 2008). In our optimization on co-cultivation duration (1–5 days), a period of 2 days displayed highest percentage of hairy root induction from leaf explants infected with both the strains compared to counterparts. Similar to the present study, co-cultivation period of 2 day favored maximum hairy root induction from leaf explants of *Berberis aristata* infected with MTCC 2364 and MTCC 532 (Brijwa and Tamta 2015). The co-cultivation period of 1–2 days displayed the maximum transformation efficiency of MTCC 2364 in leaf and shoot explants of *C. forskohlii* (Pandey et al. 2014a, b). Our findings also suggest that, shorter co-cultivation period (< 2 days) might resulted with incomplete transformation process (insufficient proliferation and reduced virulence of *A. rhizogenes*), while longer co-cultivation period (> 2 days) could have negatively affect radish transformation due to explant cell damage as observed in *Arachis hypogaea* (Liu et al. 2016).

Acetosyringone, a monocyclic phenolic compound, plays an important role on *vir* gene activation and T-DNA transfer (Ridgway et al. 2004). Mohiuddin et al. (2011) reported that acetosyringone, an amino acid derivative, is essential for biological activity and maintenance of *vir* gene expression in host cells. Recently, Srinivasan et al. (2014) reported that carrot transformation efficiency of MTCC 2364 and MTCC 532 was highly dependent on the acetosyringone concentration. In the present investigation, addition of acetosyringone (100 µM) to co-cultivation medium enhanced the percentage of hairy root induction, improved the no. of positive geotropic roots/explant, and increased the no. of lateral roots/root for both the strains when compared to control. In *B. aristata*, co-cultivation medium containing 100 µM acetosyringone was found to be most effective for induction of hairy roots from leaf explant infected with MTCC 532 (Brijwa and Tamta 2015). However, in carrot, supplementation of 150 µM acetosyringone to co-cultivation medium enhanced the transformation efficiency of explants infected with MTCC 2364 and MTCC 532 compared to control (Srinivasan et al. 2014). These differential results recorded in the present study and previous report (Srinivasan et al. 2014) suggests that the optimal requirement of acetosyringone (required to activate *vir* genes) could vary among different crops.

In general, the hairy root induction potential of *A. rhizogenes* differs between the choice of strain and crop. This could be due to the (a) difference in virulence of *A.*

Fig. 4 HPLC chromatograms of quercetin in the hairy roots of MTCC 2364- and auxin-induced roots of non-transformed radish. **a** Standard of quercetin, **b** methanolic extract of auxin-induced adventitious roots from leaves of non-transformed radish and **c** methanolic extract of MTCC 2364 hairy roots. Ten ml of methanol (99.9%) was added to 1 g (DW) of fine root powder and placed on an orbital shaker at 150 rpm for 3 days (22 °C). The extracts were dried, and about 1 mg root extract powder was dissolved in 1 ml of HPLC grade methanol (99.9%). About 20 μ l of root extracts (1 mg ml⁻¹) and standard (1 mg ml⁻¹ quercetin) was taken as injection volume

rhizogenes, (b) differential expression of T-DNA genes between strains, and/or (c) location and integration of T-DNA into the host genome (Cho et al. 1998). In this present study, *A. rhizogenes* strain MTCC 2364 showed better root induction potential in all parameters tested [wounding ($\pm$), OD₆₀₀, infection time, co-cultivation days and acetosyringone concentration] compared to MTCC 532. In previous reports on *Plumbago rosea*, *Rubia tinctorum*, *A. hypogaea* and *Withania somnifera*, the strain MTCC 2364 displayed better results for hairy root induction compared to R1000,

SA79, and MTCC 532 (Yogananth and Basu 2009; Ercan and Taskin 1999; Karthikeyan et al. 2007; Pandey et al. 2010; Vishwakarma et al. 2017).

Optimization of media strength and sucrose concentration is central for the successful scale-up of biomass accumulation. In general, the FW and DW ratio of biomass accumulation differs between various cultures and its value is related to medium osmolarity (Park and Kim 1993). In this present study, an attempt was made to determine the influence of different liquid media (MS, 1/2 MS, B5 and 1/2 B5 media)

Fig. 5 Assessment of anti-oxidant activity in methanolic extracts of MTCC 2364 hairy roots and auxin-induced roots of non-transformed radish using FRAP and DPPH assay. Control: methanolic extract of auxin-induced adventitious roots from leaves of non-transformed radish. Ten ml of methanol (99.9%) was added to 1 g (DW) of fine root powder and placed on an orbital shaker at 150 rpm for 3 days (22 °C). The extracts were dried, and about 1 mg root extract powder was dissolved in 1 ml of HPLC grade methanol (99.9%). About 20, 40, 60, 80, or 100 μ l of root extracts (1 mg ml⁻¹) were taken for analysis. Bars represent the mean values of three replicates (five root biomass per replicate) with standard errors

on biomass accumulation of hairy roots regenerated from leaf explants infected with MTCC 2364. The growth rate was calculated on FW and DW basis after 40 days of culture in respective liquid media. Highest biomass accumulation in terms of FW and DW was achieved with roots cultured in 1/2 MS medium compared to MS, B5 or 1/2 B5. In this study, a reduction in 50% of components in MS medium increased the biomass production of radish roots as observed in *Dracocephalum moldavica* (Jeżyna et al. 2013). Among the different culture media tested (MS, 1/2 MS, and B5), the maximum growth rate (1.61 g FW 50 ml⁻¹) of hairy roots was obtained in 1/2 MS medium using leaf explants of *Agastache foeniculum* (Nourozi et al. 2016). Similar results were also reported in hairy root cultures of *Fagopyrum tataricum*

where 1/2 MS media favored biomass accumulation (fresh weight/dry weight) within 20 days of culture (Bae et al. 2013).

Sucrose is considered to be one of the major carbon sources, and is very essential for the growth of hairy root. Murthy et al. (2014) reported that sucrose has a dual role as (a) carbon source and (b) osmotic agent in plant cell/organ culture media. However, no report currently describes the optimal requirement of sucrose level in the process of obtaining improved biomass accumulation in hairy root cultures of radish. In this present investigation, sucrose at a concentration of 87.6 mM favored maximum biomass accumulation (both FW and DW) of hairy roots after 40 days of culture. These results are in line with earlier report on hairy

root culture of *F. tataricum* where 87.6 mM sucrose showed maximum biomass accumulation (Huang et al. 2016). In this study, increasing the sucrose concentration above 87.6 mM resulted with reduced accumulation of root biomass. Increasing the sucrose concentration above the optimal requirement (87.6 mM) could have raised the osmolality of root culture media and ended with loss of cell viability due to dehydration as presumed by Ma et al. (2015).

Agrobacterium rhizogenes normally transfer one or two T-DNA fragments from root-inducing (Ri) plasmid to the host plant genome. This gene transfer leads to development of hairy roots in infected plants. In general, the hairy root formation from infected plant cells is mostly regulated by *rolA*, *rolB* or *rolC* gene (Pistelli et al. 2010). To date, several studies have reported the stimulatory effect of different *rol* genes in hairy root development and its intervention in improving secondary metabolism, among which *rolB* and *rolC* have gained the highest attention (Bulgakov 2008; Pandey et al. 2014a, b). In the present study, PCR amplifications of *rolB* and *rolC* gene carried out using genomic DNA of MTCC 2364 hairy roots yielded the expected amplicon size confirming their expression in radish root genome.

Plant polyphenols are significant group of compounds which act as primary antioxidants of free radical scavenging. These compounds can chelate with metal ions that could catalyze the formation of ROS which promotes lipid peroxidation (Iqbal et al. 2015). Among polyphenols, flavonoids are of great importance because they help human body to fight against diseases. Molecular structures, position of the hydroxyl group and other features in its chemical structure determines the ability of flavonoids to act as potent antioxidants (Iqbal et al. 2015). In flavonol, quercetin is most abundant with good antioxidant property and possesses structural features for free radical scavenging activity (Kalita et al. 2013). The *A. rhizogenes* strain KCTC 2703-induced hairy roots displayed enhanced production of phenolic compounds than non-transformed roots in *Brassica rapa* (Chung et al. 2016). In *Lactuca serriola*, transgenic hairy roots of *A. rhizogenes* strain AR15834 exhibited a increase in the total phenolic content and total flavonoid content compared to non-transgenic roots (El-Esawi et al. 2017). In a recent study on *Abutilon indicum*, MTCC 2364 and 532 strains were reported for hairy root induction and quercetin production (Sajjalaguddam and Paladugu 2016). Therefore, it is significant to determine the total phenol, flavonoid, and quercetin content in hairy root extracts. In the present study, total phenol, flavonoid, and quercetin content were significantly higher in methanolic extracts of hairy roots when compared to control roots from non-transformed radish.

The FRAP and DPPH are the most common assays to estimate the antioxidant potential of samples. The ferric ion reducing antioxidant potential of samples was estimated from their ability to reduce colorless oxidized TPTZ-Fe³⁺

to TPTZ-Fe²⁺ (blue colored complex). The DPPH molecule that contains stable-free radical has been extensively used to estimate the radical scavenging ability of antioxidants. The DPPH scavenging ability is attributed to the hydrogen donating ability of compounds in plant extracts (Shimada et al. 1992; Tanaka et al. 1988; Ebrahimzadeh et al. 2008). There was an increase in total reducing power and antioxidant potential of *L. serriola* hairy roots infected with strain AR15834 compared to non-transgenic roots (El-Esawi et al. 2017). The antioxidant activity exhibited by KCTC 2703-induced hairy roots was higher than that shown by non-transformed roots of *B. rapa* (Chung et al. 2016). In this present study, the results of FRAP and DPPH assay confirmed that the antioxidant activity of root extracts from MTCC 2364 was significantly higher when compared to control root extracts of non-transformed radish. In addition, in both FRAP and DPPH assay, the antioxidant potential of extracts increased as the volume used to assay was increased from 20 to 100 µl. Our results indicated that the radical scavenging ability of hairy root extracts (20–100 µl) might be mostly related to their concentration of phenolics and flavonoids. Rao et al. (2010) reported that the ability of the extracts to reduce Fe³⁺ could be attributed to the number of hydroxyl groups in the phenolic and flavonoid compounds. Chiang et al. (2017) reported that FRAP and DPPH radical scavenging ability could be attributed to the flavonoids present in plant extract of *Zingiberaceae* family.

Polyphenols are the most abundant antioxidants in human diets and the largest and best studied class of polyphenols is flavonoids (Lima et al. 2014). Flavonoids act as excellent antioxidants in human system and prevent the incidence of cardiovascular disease, diabetes and cancer (Yao et al. 2004). The flavonoids are also involved in several biological activities and possess numerous pharmacological properties with respect to human health. The flavonoid quercetin has received considerable attention because of its potential benefits to overall human health and disease resistance (Aguirre et al. 2011). In the present study, we were able to enhance the production of phenolics, flavonoids, and quercetin using an optimized radish root culture system. The results of our study could be useful to scale up the process for industrial production of several bioactive compounds using radish as a primary source.

Conclusion

The present study was an attempt to establish stable hairy root lines of radish to increase the production of antioxidant compounds. Our optimization on several factors affecting transformation efficiency improved the hairy root induction potential of leaf explants and strain MTCC 2364 showed better responses compared to MTCC 532. The optimization

of media and sucrose concentration enhanced the biomass accumulation of MTCC 2364 hairy roots in vitro. In the present investigation, the genetic and biochemical stability of MTCC 2364-induced hairy roots was confirmed by PCR (*rolB* and *rolC*), total phenol, flavonoid, and quercetin analysis after a period of 65 days from the date of culture establishment. The total phenol, flavonoid, and quercetin content was significantly higher in extracts of hairy roots compared to control. To our knowledge, this is the first report in radish on improving total phenol, flavonoid, and quercetin content using optimized root culture system. In addition, hairy root extracts displayed improved antioxidant activity which was confirmed by FRAP and DPPH assay. Thus, we believe that the current protocol could be useful for the industrial production of antioxidants and bioactive compounds implicated with significant health benefits using radish root culture system.

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Author contribution statement Girija designed research, supervised experiments at all the stages and performed critical revision of the manuscript. Balasubramanian performed all the experiments and analysed data. Anbumegala and Surendran assisted in conducting the experiments and helped in data interpretation. Arun and Balasubramanian wrote the manuscript and prepared figures. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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