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Original article

Influence of cotyledon, hypocotyl extracts and authentic andrographolide on selective *Agrobacterium rhizogenes* strains growth: A deterrent to hairy root induction in *Andrographis paniculata* (Burm.f.) Wall. ex Nees

Mohd Zaheer and Charu Chandra Giri
Centre for Plant Molecular Biology (CPMB), Osmania University, Hyderabad-500007, Telangana State, India

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Abstract

Andrographis paniculata (Burm.f.) Wall. ex Nees is an important source of diterpene lactone andrographolide with a wide range of medicinal properties. The present study was initiated to induce hairy roots through *Agrobacterium rhizogenes* mediated genetic transformation. Maximum frequency of 12% hairy root induction was obtained following co-cultivation with *A. rhizogenes* 2364 strain by cut method using cotyledon explants. Further, growth of roots was not observed on subsequent subculture in medium containing MS nutrients without PGRs. A detailed study on the influence of *A. paniculata* cotyledon and hypocotyl tissue extracts was carried out to assess its effect on *A. rhizogenes* bacterial growth. The antibacterial activity was studied against selective *A. rhizogenes* strains such as 532, 2364, 8196 and A4T using methanolic tissue extracts of *A. paniculata*. The antimicrobial activity was determined by agar diffusion method. The diameter of inhibition zone on bacterial growth using methanolic extracts from cotyledon and hypocotyl of *A. paniculata* revealed inhibitory effect on *A. rhizogenes* culture. The highest zone of inhibition 0.65 ± 0.04 mm was observed with *A. rhizogenes* strain 2364 from hypocotyl tissue extracts. The effects on *A. rhizogenes* growth by methanolic tissue extracts were also comparable with the authentic andrographolide treatment which showed inhibition in all the strains tested. The present findings suggested that the inhibitory effect of tissue extracts and andrographolide may be a possible reason for the inability of inducing hairy roots in *A. paniculata* following genetic transformation with *A. rhizogenes*.

Key words: *Andrographis paniculata* (Burm.f.) Wall. ex Nees, *Agrobacterium rhizogenes* strains, andrographolide, growth inhibition, transformed roots

1. Introduction

Plants produce various biologically active compounds in the form of secondary metabolites, and many of these phytochemicals are used as medicines, or are currently under development for pharmaceutical purposes (Pan, 2014; Raomai *et al*., 2015). Plant secondary metabolites are used as potential drugs, chemopreventive agents, nutraceuticals and food additives (Ramawat *et al*., 2009; Kumar *et al*., 2017). The target organism for hairy root syndrome in plants is a gram-negative soil bacterium, *Agrobacterium rhizogenes*. It has the ability to transfer its T-DNA from root-inducing Ri-plasmid to host genome and thereby inducing hairy root at the site of infection in natural condition. The *in vitro* cultures of conventional roots are slow growing and have short life span compared to transformed roots (Giri and Narasu, 2000; Giri *et al*., 2003; Guillon *et al*., 2006). Further, certain secondary metabolites are synthesized exclusively in the roots. There are several reports on the induction of hairy root cultures for the production of secondary metabolites (Syklowska-Baranek *et al*., 2015; Hashemi and Naghavi, 2016; Giri and Zaheer, 2016; Banihashemi *et al*., 2017).

A. paniculata is an erect annual herb belongs to the Acanthaceae family and source of bioactive compounds. It is native to India, Pakistan, Sri Lanka, China, Thailand, Indonesia and neighbouring Asian countries (Kumar *et al*., 2012; Neeraja *et al*., 2015). *A. paniculata* is commonly known as king of bitters/kalmegh which has a bitter taste due to the presence of bioactive diterpene lactones (Kumar *et al*., 2012). The plant produces diterpenoids, flavonoids and polyphenols (Chao and Lin, 2010; Radhika *et al*., 2010). It is one of the mostly used medicinal plants in Ayurvedic and Unani system of medicines for its pharmacological activities (Akbar, 2011; Kumar *et al*., 2012). The plant is used for the treatment of upper respiratory-tract and urinary tract infections and disorders such as common cold, fevers, rheumatism, diabetes, arthritis, hepatoprotective, dysentery, diarrhea, hepatitis, skin infection, peptic ulcer, snake bites, sore throat, hypertension and central nervous system disorders (Negi *et al*., 2008; Sareer *et al*., 2014; Wang *et al*., 2014; White *et al*., 2014). *A. paniculata* plant extract is known to possess a variety of pharmacological activities. *A. paniculata* herb is widely used for therapeutic purposes (Joselin and Jeeva, 2014). Plant possess anti-inflammatory (Salaga *et al*., 2014; Wang *et al*., 2014), antimalarial (Mishra *et al*., 2011), antibacterial (Banerjee *et al*., 2017), antiviral (Niranjan *et al*., 2010),

Author for correspondence: Dr. Charu Chandra Giri
Professor, Centre for Plant Molecular Biology (CPMB), Osmania University, Hyderabad-500007, Telangana State, India
E-mail: giriccin@yahoo.co.in
Tel.: +91-040-27098087

immune-stimulatory (Kumar *et al*., 2004), anxiolytic (Malik and Malik, 2015), antidiabetic and antioxidant properties (Premanath and Nanjaiah, 2015). Anticancer activity was also studied from leaf extracts of plant by using spectrophotometric MTT assay method (Rajeshkumar *et al*., 2015).

In our laboratory, we have an inclusive research programme on distribution of *A. paniculata* and related species, pharmacological study and yield enhancement of bioactive compounds, using biotechnological approaches (Neeraja *et al*., 2015; Parlapally *et al*., 2015; Zaheer and Giri, 2015; Zaheer and Giri, 2017). The present study was aimed at genetic transformation of *A. paniculata* for hairy root induction, using different strains of *A. rhizogenes*. In addition, a study on the influence of *A. paniculata* cotyledon and hypocotyl tissue extracts on *A. rhizogenes* bacterial growth was investigated.

2. Materials and Methods

2.1 Collection of plant material

Seeds were harvested from the net-house plants of *A. paniculata*, grown at Centre for Plant Molecular Biology (CPMB), Osmania University, Hyderabad, India. Seed sterilization method was followed as per Zaheer and Giri (2015). Collected seeds were initially washed with teepol solution and subsequently under running tap water for 15 - 20 min. Further, surface sterilized using 0.1 % (w/v) mercuric chloride for 2 - 3 min and washed with sterile double-distilled water (SDDW) for 4 - 5 times. The surface-sterilized seeds were inoculated on to semi-solid MS (Murashige and Skoog, 1962) basal medium. Seeds were incubated for germination at 25 ± 1ºC in dark.

2.2 Genetic transformation studies

2.2.1 Collection of *A. rhizogenes* strains and maintenance

A. rhizogenes strains were collected from India and evaluated for genetic transformation studies. Wild strains of *A. rhizogenes* such as 532, 2364, 8196 and A4T were evaluated for genetic transformation studies. *A. rhizogenes* strains were procured from Microbial Institute of Technology (MIT), Chandigarh, PG Department of Botany, Utkal University, Bhubaneswar and JNT University, Hyderabad.

Different strains of *A. rhizogenes* were cultured and maintained in Luria-Bertani (LB) agar media at 29ºC. Bacterial suspensions of above mentioned strains were initiated by transfer of single bacterial colony grown on LB agar media to LB liquid media. These bacterial cultures were maintained in an incubator shaker at 120 rpm. Bacteria growth pattern was studied by taking the optical density (OD) after 24, 48 and 72 h using spectrophotometer at 600 nm.

2.2.2 Methods of co-cultivation

Genetic transformation using wild strains of *A. rhizogenes* were tested to evaluate the susceptibility of *A. paniculata* infection and hairy root induction. Different strains of *A. rhizogenes* were used for co-cultivation experiments. Cotyledons and hypocotyls were used as initial explants from 2-weeks old *in vitro* seedlings for co-cultivation experiment. Minimum of 20-30 seedlings were used for co-cultivation with each strain in different experiments. Filter sterilized acetosyringone was incorporated in the liquid bacterial culture media at the concentrations of 100 and 150 µM for the induction of *vir* genes in *A. rhizogenes*. Bacterial culture after 24, 48 and 72 h. with an O.D of 1.0-1.21 was used for co-cultivation experiments.

Method I

Co-cultivation of explants with *A. rhizogenes* bacterial suspension was carried out. Cotyledons and hypocotyls obtained from 2-week old germinated seedlings were cut into smaller halves and dipped in bacterial suspension for duration of 5, 10 and 15 min. Further, after incubation, the explants were placed over water absorbent soft tissue paper to remove excess bacterial suspension from the surface of different explants. Subsequently, the explants were inoculated on to MS medium without plant growth regulators (PGRs) for induction of hairy roots.

Method II

Cotyledons and hypocotyls from seedlings were used as explants. Injury and cut was made on explants with sterile scalpel blade. The explants with injury were dipped in bacterial suspension for co-cultivation. Further, the explants were soaked on water absorbent soft tissue paper to remove excess bacterial suspension from the surface of explants. The explants were then inoculated on to MS medium without PGRs for induction of hairy roots.

Method III

Pricking of explants such as cotyledons and hypocotyls was carried out by bacterial suspension dipped sterile needle. Further, the excess bacterial suspension from the surface of different explants was removed by placing the explants on water absorbent soft tissue paper. The explants were further inoculated on to MS medium without PGRs for induction of transformed hairy roots.

Method IV

Silicon carbide (0.5 % w/v) mediated injury of explants such as cotyledons, and hypocotyls was carried out in eppendorf tubes by vortexing the explants for 5 min using Cyclo Mixer (Remi Equipments, Pvt. Ltd., India). Subsequent to the injury, the explants were co-cultivated in bacterial suspension for duration of 5, 10 and 15 min. Further, the water absorbent soft tissue paper was used to remove excess bacterial suspension from the surface of different explants. The explants were subsequently, inoculated on to MS medium without PGRs for induction of hairy roots. The explants were also injured/pricked with sterile needle and scalpel blade without dipping in bacterial suspension. These explants were inoculated on to the MS basal medium as untreated control. All the cultures were incubated in dark at 25 ± 2ºC in culture room conditions for 24, 48 and 72 h. of co-cultivation period for the visible growth of *A. rhizogenes* bacterial strains.

2.2.3 Transfer of co-cultivated explants to cefotaxime media for control of bacterial growth

After 24, 48 and 72 h of co-cultivation, the explants were transferred in the MS basal medium, supplemented with various concentrations of cefotaxime, *i.e*., 200, 250, 300 and 350 mg/l. The concentration of cefotaxime showing best in terms of complete control of growth was selected. The observations of the cultures were taken after four to six weeks of culture.

2.3 Effect of *A. paniculata* crude extracts (cotyledon and hypocotyls) and authentic andrographolide on bacterial (*A. rhizogenes*) growth

2.3.1 Preparation of plant extracts

Cotyledons and hypocotyls were oven-dried at 65ºC for 24 h. Dry powdered material of cotyledon and hypocotyl samples weighing 50 mg DW each was placed in two separate vials containing 5 ml of high performance liquid chromatography (HPLC) grade methanol and incubated for 24 h at room temperature. The resulted extract was ultra-sonicated using an ultrasonic cleaning bath (Spectra Lab., Model UCB 30, and India). After sonication for 30 min, the extract was initially filtered using Whatman filter paper No. 41. Further, the filtrate was finally passed through 0.45 µM membrane (Millex HV, Millipore, Ireland). The methanolic extracts of cotyledon and hypocotyl were tested for bacterial growth study.

2.3.2 Effect of *A. paniculata* crude extracts and authentic standard andrographolide on *A. rhizogenes* growth

Antibacterial assay by growth inhibition study was performed by agar well diffusion method using *A. paniculata* extracts. The strains of *A. rhizogenes* such as 532, 2364, 8196, and A4T were grown overnight at 29ºC and used for testing of antibacterial activity. LB agar medium was used for the growth of *A. rhizogenes* strains. LB agar medium was dissolved in distilled water and the volume was made to 100 ml using 250 ml conical flasks. The pH was adjusted to 7.5 to 7.8 and was sterilized by autoclaving at 121ºC and 15 lbs pressure for 15 min.

The *A. rhizogenes* bacterial culture (50 µl) was added aseptically to 25 ml of melted agar medium, mixed well and poured immediately in sterile disposable petri plates. After gelling of LB media, four wells were made in each petridish, using sterile glass tube (7-8 mm diameter). HPLC-grade methanol, methanolic extracts and authentic standard andrographolide (Sigma-Aldrich, USA) were filter sterilized using 0.22 µm membrane filter in bacterial filtration unit before loading into the wells. About 50 µl of cotyledon and hypocotyl, methanolic extracts were poured into each well separately. One of the well was filled with HPLC-grade methanol and it was considered as negative control. Authentic standard andrographolide was added to another well and acted as positive control. After the addition of plant extracts, methanol and standard andrographolide the plates were incubated at 29ºC for 24 h.

Antibacterial activity and growth inhibition was estimated by measuring the diameter of inhibition zone of the tested *A. rhizogenes* bacteria around the well. Each experiment was carried out in triplicate and the mean diameter of inhibition zone was recorded. The inhibition zones produced by the test solutions were compared with the inhibition zone observed using standard andrographolide and HPLC-grade methanol.

2.4 Statistical analysis

All the data on growth inhibition in different strains of *A. rhizogenes* were recorded for analysis after 24-48 h of culture. Statistical analysis was carried out to calculate the mean and the standard error. Genetic transformation and antibacterial activity experiments were carried out in triplicate and average mean diameter of the inhibition zone was recorded.

3. Results

3.1 Genetic transformation studies and induction of hairy roots in *A. paniculata* using different strains of *A. rhizogenes*

Four strains of *A. rhizogenes* (532, 2364, 8196 and A4T) were compared for their transformation competence using cotyledon and hypocotyl explants for hairy root induction. Genetic transformation studies for hairy root induction using different strains of *A. rhizogenes* and co-cultivation methods showed variable culture responses (Figure 1 a, b, c, d). *A. rhizogenes* strains 2364 and 532 promoted hairy root induction in both cotyledon and hypocotyl explants using cut (CM) and incubation (Inb) method. The highest frequency of hairy root induction in the strains of 2364 on the cotyledon explants with 12 ± 0.13 was observed (Table 1). Only in hypocotyl explants the frequency of hairy root induction was 4.1 ± 0.083 using 8196 strain by CM. Additional incorporation of 100 µM acetosyringone in *A. rhizogenes* (2364, 532, 8196 and A4T strains) culture and co-cultivation media showed similar response as observed without acetosyringone. Induced hairy roots did not grow further in subsequent culture passage.

Table 1: Hairy root induction from explants of *A. paniculata*

A. rhizogenes strains	Methods	*% frequency of response (cotyledon)	*% frequency of response (hypocotyl)
2364	CM	12 ± 0.13	4.1 ± 0.083
	Inb	BE	4.1 ± 0.083
	Inj	BE	BE
	PWN	NR	NR
	VSC (5 %)	NR	BE
	C+VSC (5 %)	BE	SwE
532	CM	BE	4.1 ± 0.083
	Inb	BE	4.1 ± 0.083
	Inj	NR	NR
	PWN	NR	NR
	VSC (5 %)	NR	BE
	C+VSC (5 %)	BE	BE
8196	CM	SwE	4.1 ± 0.083
	Inb	BE	BE
	Inj	NR	NR
	PWN	NR	NR
	VSC (5 %)	NR	BE
	C+VSC (5 %)	NR	SwE
A4T	CM	4.1 ± 0.083	NR
	Inb	BE	BE
	Inj	NR	NR
	PWN	NR	NR
	VSC (5 %)	NR	BE
	C+VSC (5 %)	NR	SwE

C + VSC co-cultivation along with vortexing in silicon carbide, CM cut method, BE browning of explants, Inb incubation, Inj injection, VSC vortexing in silicon carbide, NR no response, PWN pricking with needle, SwE swelling of explants. Each treatment consists of 6 replicates and calculated % mean ± SE. *Observations were taken after one month of culture

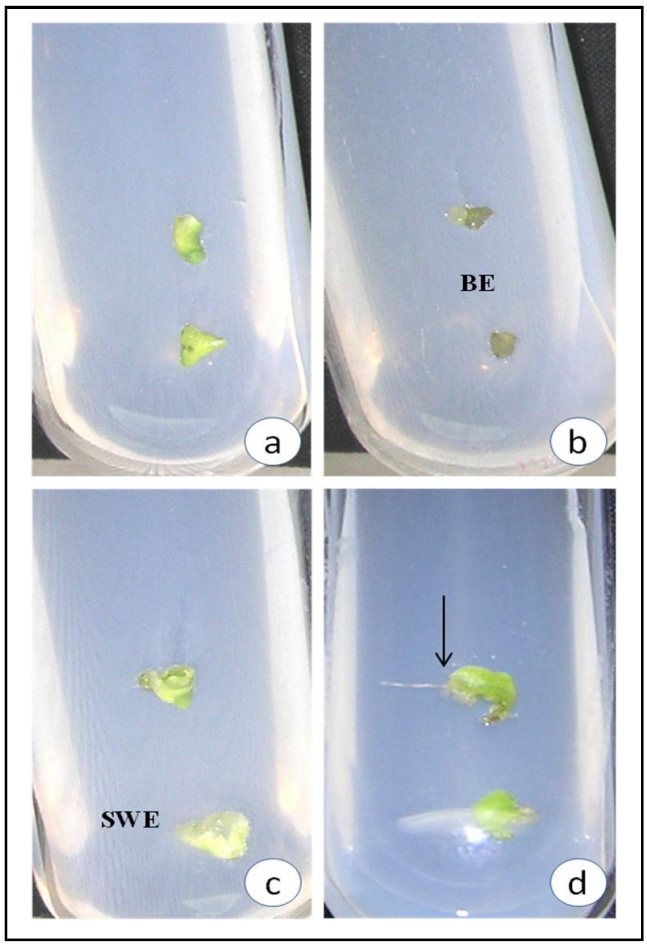

Figure 1: Co-ocultivation and culture responses of *A. paniculata* cotyledon explants using different strains of *A. rhizogenes* for induction of hairy roots: (a) Un-cocultivated control cotyledon explants, (b) Browning of explants (BE) after co-cultivation, (c) Swelling response of explants (SWE) following co-cultivation and (d) Root induction (arrow) response from co-cultivated cotyledon.

3.2 Effect of *A. paniculata* crude extracts (cotyledon and hypocotyls) and authentic andrographolide on bacterial growth

To elucidate the cause of inability of different *A. rhizogenes* strains to induce hairy roots and its sustained growth, the effect of *A. paniculata* crude extracts on bacterial growth was studied (Table 2). The diameter of inhibition zone on bacterial growth using methanolic extracts from cotyledon and hypocotyl of *A. paniculata* revealed inhibitory effect on *A. rhizogenes* culture (Figure 2a, b, c, d). The lowest zone of inhibition 0.35 ± 0.04 to highest of 0.65 ± 0.04 mm was observed with *A. rhizogenes* strain 2364 from crude extracts of cotyledon and hypocotyl, respectively. Agar well diffusion method against some of the selected *A. rhizogenes* strains such as 532, 2364, 8196 and A4T were tested. Both the examined extracts showed antibacterial activity against *A. rhizogenes* strains. Figure 2a, b, c, d depicted the antibacterial activity of cotyledon, and hypocotyl extracts of *A. paniculata* against *A. rhizogenes* strains.

The methanolic extracts from cotyledon and hypocotyl of *A. paniculata* showed considerable antibacterial activities. The methanolic extracts of *A. paniculata* hypocotyl showed maximum zone of inhibition (0.65 ± 0.04, 0.57 ± 0.07 mm for 2364 and 8196, respectively) compared to cotyledon and standard andrographolide for all the strains of *A. rhizogenes* evaluated (Table 2).

Table 2 : Effect of *A. paniculata* (cotyledon and hypocotyl) methanolic extracts against different strains of *A. rhizogenes**

A. rhizogenes strains	Zone of inhibition (mm)			
	Cotyledon	Hypocotyl	Authentic	Control
2364	0.35 ± 0.04	0.65 ± 0.04	0.47 ± 0.02	0
532	0.45 ± 0.04	0.40 ± 0.04	0.52 ± 0.12	0
8196	0.45 ± 0.04	0.57 ± 0.07	0.45 ± 0.04	0
A4T	0.52 ± 0.02	0.42 ± 0.02	0.55 ± 0.14	0

*Observations on zone of inhibition of different strains using methanolic extracts (50 µl) of cotyledon and hypocotyl, authentic and control were taken after 24 h. of incubation.

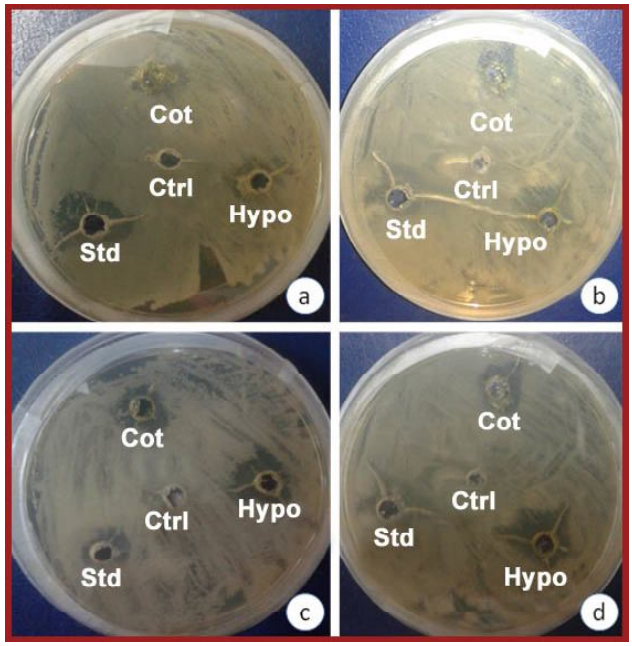

Figure 2: Influence of *A. paniculata* cotyledon and hypocotyl extracts and authentic andrographolide on growth of different *A. rhizogenes* strains. (a) Growth inhibition pattern of 532 strain, (b) Growth inhibition pattern of 2364 strain, (c) Growth inhibition pattern of 8196 strain and (d) Growth inhibition pattern of A4T strain. Cot: cotyledon, Hypo: hypocotyl, Std: standard and Ctrl: control.

4. Discussion

Different strains of *A. rhizogenes* showed hairy root induction response in cotyledon and hypocotyl explants of *A. paniculata*. Although, hairy root induction was observed but there was no further growth and proliferation of induced roots on subsequent subculture. A minimum of more than hundreds of experiments were conducted involving four strains of *A. rhizogenes*. However, we were not successful in inducing hairy roots from different explants of *A. paniculata*. In literature survey, only two reports are available where they claimed that hairy root can be induced in *A. paniculata*. These includes, the induction of hairy roots from cotyledon explants

of *A. paniculata* using two strains of *A. rhizogenes* such as ATCC and 15834 (Marwani *et al*., 2015). Further, the growth of hairy roots was improved on liquid half strength MS medium with an additional supplement of 5.0 µM IBA. In another report, hairy root cultures induced from *A. paniculata* was used for determining the effect of MeJA and SA on enhanced andrographolide production (Sharmila and Subburathinam, 2013). In the present work, cotyledon explant was found best for transformation; in contrast previous studies showed that hypocotyl explant were successful for transformation experiments (Wahby *et al*., 2013; Sudha *et al*., 2013). Leaf explants were used for the induction of hairy roots in *Persicaria minor* (Ashraf *et al*., 2015) and *Salvia castanea* (Li *et al*., 2016).

In the present study, keeping in view the unsuccessful outcome in the induction of hairy roots in *A. paniculata*, we conducted experiments to find out the possible reasons. The methanolic extracts of cotyledon and hypocotyl showed considerable antibacterial effect restricting the growth of *A. rhizogenes*. In a recent report, methanol was the best solvent for *A. paniculata* extracts against different strains of human pathogens (Shalini and Narayanan, 2015). The seedling (cotyledon and hypocotyl) methanolic extracts was studied for minimal inhibitory concentration. The antibacterial activity of *A. paniculata* hypocotyl methanolic extract showed maximum zone of inhibition (mm) compared to cotyledon and standard andrographolide for all the strains of *A. rhizogenes*.

5. Conclusion

Different strains of *A. rhizogenes* (2364, 532, 8196 and A4T) were employed for the induction of hairy roots. Hairy root induction from cotyledon and hypocotyl explants of *A. paniculata* was observed. However, the induced hairy roots did not grow in subsequent culture passages. The effect of *A. paniculata* crude extracts on bacterial growth was studied to test the inability of different *A. rhizogenes* strains to induce hairy roots and its sustained growth. The inhibition zone on bacterial growth using methanolic extracts from cotyledon and hypocotyl of *A. paniculata* revealed inhibitory effect on *A. rhizogenes* growth. The presence of chemical compound andrographolide in cotyledon and hypocotyl extracts possibly had an inhibitory/detrimental effect on *A. rhizogenes* growth and subsequent transfer of Ri-TDNA for induction of hairy roots in *A. paniculata*.

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Conflict of interest

We declare that we have no conflict of interest.

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