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Standardization of process for hairy root production using *Agrobacterium rhizogenes* for monoxenic culture of *arbuscular mycorrhizal* fungi

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ABSTRACT

Arbuscular mycorrhizal fungi are ubiquitous and form symbiosis with roots of plants and which cannot be pure cultured as it is an obligate fungi but can be cultured *in vitro* under root organ culture. Cowpea (*Vigna unguiculata* L. Walp) and tomato (*Solanum lycopersicum* L. Mill) explants were selected for hairy root production and conditions were standardized for media, incubation time and light based on the induction frequency of Ri T-DNA transformed hairy root through *Agrobacterium rhizogenes*. Different *A. rhizogenes* strain, viz 532, 2364 were used to induce hairy roots. *A. rhizogenes* 532 induced maximum hairy roots in cowpea, whereas 2 364 induced more hairy roots in tomato. Out of 3 different media evaluated, Modified White's medium supported more induction of hairy roots in cowpea and tomato. Manual wounding of explants with the addition of 100 µM acetosyringone in the media resulted better induction of hairy roots in both the explants with both the strains of *A. rhizogenes*. Also 48 hr of incubation in dark conditions produced maximum hairy root frequency for both strains using cowpea and tomato explants. These transformed hairy roots can serve as potential host for *in vitro* culturing of *Arbuscular mycorrhizal* fungus.

Key words: *Agrobacterium rhizogenes*, Explants, Hairy roots, Manual wounding, Modified White's medium

Root-organ cultures were first developed by White and coworkers (Butcher and Street 1964, Butcher 1980). *In vitro* propagation of root organ cultures consists of excised roots that proliferate under axenic condition on a synthetic nutrient media supplemented with vitamins, minerals and carbohydrates. Continuous cultures of vigorous root organ cultures have been obtained through transformation of roots by the gram negative soil bacterium *Agrobacterium rhizogenes*.Conn (Tepfer 1989).

The soil bacterium *Agrobacterium rhizogenes* infects the plant tissues and leads to the formation of adventitious roots or it is called as hairy roots. The hairy root harbours the T-DNA segment of Ri-plasmid within its nuclear genomes (Chilton *et al*. 1982). The endogenous production of auxin and or an increase in auxin sensitivity could lead to the formation of hairy roots at the site of infection (White *et al.* 1985).

Different culture media have been used for growing such Ri-T DNA transformed roots, viz Murashige and Skoog medium (Mugnier 1988), White's medium and Modified White's medium (Becard and Fortin 1988). Wounding is the pre-requisite for the genetic transformation process through *Agrobacterium* and may enter and aid in the production of signal phenolics (Gelvin 2000) and enhance the accessibility of putative cell-wall binding factors (Gelvin 2000) to bacterium. The infection of dicotyledonous plants by *A. rhizogenes* caused roots to proliferate rapidly at the infection site. It is mainly due to the transfer of T-DNA from the bacterial cells. Hairy roots can serve as potential host for *in vitro* culturing of *Arbuscular Mycorrhizal* fungi. Hence an experiment was conducted to assess the effect of different media, incubation time and light on production and proliferation of hairy roots using different explant sources.

MATERIALS AND METHODS

Two *in vitro* experiments were conducted on hairy root production for the standardization of factors involved. First experiment is on the effect of different media on *Agrobacterium rhizogenes* mediated hairy root induction.

Media consist of both macro element and microelement necessary for growth and development. Composition of the microelement varies with media. Since microelement varies with media, it plays an important role in production of hairy roots.

Treatments, T_1 control (without *A. rhizogenes*), T_2, manual wounding + *A. rhizogenes* and T_3 manual wounding +

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acetosyringone 100 µM + *A. rhizogenes* were subjected to explant segments of tomato and cowpea prior to co-cultivation with *A. rhizogenes* on 3 different tissue culture media, namely *A. rhizogenes* 532 and *A. rhizogenes* 2364.

The media used were Murashige and Skoog medium (Murashige and Skoog 1962) and Modified White's medium and Minimal Medium (Becard and Fortin 1988).

Totally 18 treatments were made with each of the explant sources, viz tomato and cowpea and replicated thrice.

The explant (leaves or stem) of *in vitro* propagated plants (10–12 days-old cowpea, tomato) were cut into 1–2 cm bits. Then the explants were pricked with needle and soaked in the overnight grown pelletized *A. rhizogenes* culture suspension for 15 min for infection. Then, the explants were placed in to hormone-free medium with Acetosyringone (ACS) at 100 µM concentrations without antibiotics and incubated in dark at 25°C for 72 hr. After 72 hr the explants were checked for the production of hairy roots and growth of bacteria on the media. If the growth was traced then immediately the explants were transferred to a hormone-free medium containing the antibiotic cefotaxime 500 mg/litre. The explants were transferred to the fresh medium as above till the bacterial growth was completely arrested. The concentration of cefotaxime was reduced in every transfer to fresh medium by 50 m g/litre. After the bacterial-free growth was observed, the transformed roots were sub-cultured at an interval of 7–10 days in hormone and antibiotic free solid medium for further growth of hairy roots.

The transformation rate was calculated as below

$$= \frac{\text{Number of explants inducing hairy roots}}{\text{Total number of explants infected with } A.\ rhizogenes} \times 100$$

The second experiment is on the effect of incubation time and light on hairy root production of *A. rhizogenes*.

The explants (tomato and cowpea), after soaking in overnight grown pelletized *A. rhizogenes* strains (532 and 2364) culture suspension for 15 min. were placed in to hormone free Modified White's medium with acetosyringone at 100 µM concentrations without antibiotics and incubated in dark as well as in light at 25°C for 24, 48 and 72 hr. Effect of the incubation time and light was studied. The same treatments were used for inducing hairy roots in cowpea as well as tomato explants, using Modified White's medium.

Two strains of *A. rhizogenes* namely, *A. rhizogenes* strain 532 and *A. rhizogenes* strain 2364 were employed. The treatment duration of the *A. rhizogenes* strain was 15 min. under an incubation temperature of 25° for about 24, 48 and 72 hr in dark as well as under light source.

RESULTS AND DISCUSSION

Effect of different media on Agrobacterium rhizogenes mediated hairy root induction

Medium is the important component in the process of hairy root development. Most of the media are having the ability to support the hairy root growth. Their potential only differs with the strain of *A. rhizogenes* used and the conditions employed. Hence to study the effect of 3 different media as well as *A. rhizogenes* strains on hairy root production with the addition of acetosyringone, an experiment was conducted.

By observing the plates, it was noticed that the formation of hairy roots occured only from the wounded regions. Each type of infection and wounding method showed unique pattern of hairy root induction with varying per cent of transformation frequency. Infection of leaf explants by manual wounding resulted an induction of hairy roots originating from the mid vein region. Application of acetosyringone resulted in the formation of hairy roots, all over the surface of explant source.

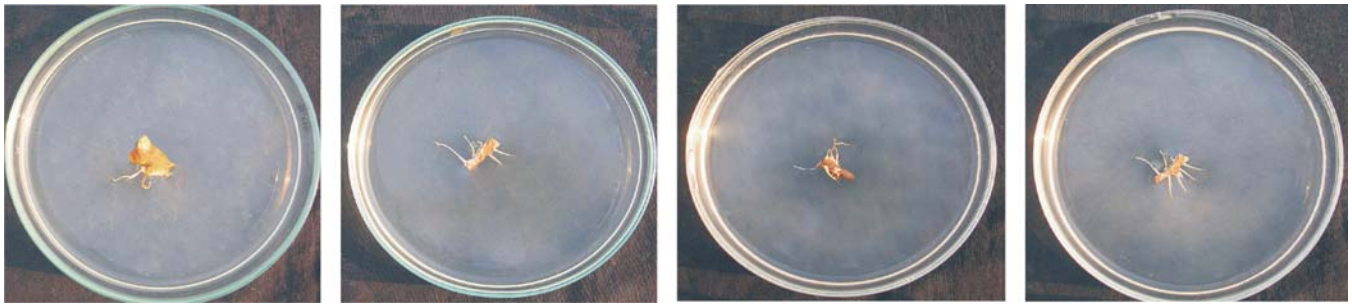
In cowpea explants hairy root induction was higher with

Table 1 Effect of different media on *Agrobacterium rhizogenes* strain 532 mediated hairy root induction frequency in cowpea explants

Treatment	Hairy root induction frequency (%)			
	Murashige and Skoog medium	Modified White's medium	Minimal medium	Mean
Control (without *A. rhizogenes)*	0^{f}	0^{f}	0^{f}	0^{c}
Manual wounding + *A. rhizogenes*	25^{e}	60^{c}	45^{d}	43.30^{b}
Manual wounding and Acetosyringone 100 µM + *A. rhizogenes*	50^{d}	100^{a}	75^{b}	75.00^{a}
Mean	40.00^{c}	80.00^{a}	55.00^{b}	
	SE M± (d)			CD (*P*=0.05)
Media (M)	2.30			5.00
Treatment (T)	2.30			5.00
M×T	4.10			8.60

Table 2 Effect of different media on *Agrobacterium rhizogenes* strain 2364 mediated hairy root induction frequency in cowpea explant

Treatment	Hairy root induction frequency (%)			
	Murashige and Skoog medium	Modified White's medium	Minimal medium	Mean
Control (without *A. rhizogenes*)	0^{f}	0^{f}	0^{f}	0^{c}
Manual wounding + *A. rhizogenes*	20^{e}	50^{c}	30^{d}	33.30^{b}
Manual wounding and Acetosyringone 100 µM + *A. rhizogenes*	60^{b}	80^{a}	60^{b}	66.70^{a}
Mean	40^{b}	65^{a}	45^{b}	
	SE M± (d)			CD (*P*=0.05)
Media (M)	2.01			4.20
Treatment (T)	2.01			4.20
M × T	3.48			7.30



Manual wounding-Strain 532 | Manual wounding-Strain 2364 | Manual wounding + 100 µMACS -Strain 532 | Manual wounding +100 µMACS -Strain 2364

Fig 1 Hairy root induction in cowpea (after 20 days)

Manual wounding-Strain 532 | Manual wounding-Strain 2364 | Manual wounding + 100 µMACS -Strain 532 | Manual wounding +100 µMACS -Strain 2364

Fig 2 Hairy root induction in tomato (after 20 days)

the use of *A. rhizogenes* 532 (Table 1) (100% Modified White's medium and 75% in Minimal Medium) than *A. rhizogenes* 2364 (Table 2). In tomato explants, *A. rhizogenes* 532 showed only 80% induction (Table 3; Fig 3) whereas *A. rhizogenes* 2364 recorded 100% induction in Modified White's medium (Table 4) with the addition of 100 µm acetosyringone. In both explant sources manual wounding registered lesser induction frequency in all the 3 media evaluated with out the addition of acetosyringone. Further, with acetosyringone addition, the time taken for hairy root induction was reduced by 7–10 days. Similar results were reported in tobacco in which hairy root induction was enhanced with the addition of 100 µm acetosyringone (Kumar *et al*. 2006).

Wounding is a prerequisite for the genetic transformation process through *A. enters* and may aid in the production of signal phenolics (Gelvin 2000) and enhance the accessibility of putative cell-wall binding factors to the bacterium. Acetosyringone is one such compound used successfully to enhance transformation in various plant species through *A. tumefaciens* mediated genetic transformation (Gelvin 2000) which induce the transcription of '*vir*' region. Bacterial cultures and explants cocultivated on Murashige and Skoog medium and Basal medium with acetosyringone addition reduce the time for hairy root induction by a week compared to the explants infected with *A. rhizogenes* without '*vir*' region (Archana *et al*. 2001). Acetosyringone has also known to enhance the transformation efficiency due to activation of 'ags' gene in *A. rhizogenes* (Aoki *et al.* 1997).

In both explants sources, Modified White's medium supported higher hairy root induction. Hence the Modified White's medium was preferred to Murashige and Skoog

Table 3 Effect of different media on *Agrobacterium rhizogenes* strain 532 mediated hairy root induction frequency in tomato explants

Treatment	Hairy root induction frequency (%)			
	Murashige and Skoog medium	Modified White's medium	Minimal medium	Mean
Control (without *A. rhizogenes*)	0^{f}	0^{f}	0^{f}	0^{c}
Manual wounding + *A. rhizogenes*	20^{e}	45^{b}	30^{d}	31.60^{b}
Manual wounding and Acetosyringone 100 µM + *A. rhizogenes*	50^{c}	80^{a}	60^{b}	63.30^{a}
Mean	35^{c}	62.5^{a}	45^{b}	
	SE M± (d)			CD (*P*=0.05)
Media (M)	2.01			4.23
Treatment (T)	2.01			4.23
M×T	3.48			7.33

Table 4 Effect of different media on *Agrobacterium rhizogenes* strain 2364 mediated hairy root induction frequency in tomato explants

Treatment	Hairy root induction frequency (%)			
	Murashige and Skoog medium	Modified White's medium	Minimal medium	Mean
Control (without *A. rhizogenes*)	0[f]	0[f]	0[f]	0[c]
Manual wounding + *A. rhizogenes*	40[d]	60[c]	30[e]	43.30[b]
Acetosyringone 100 µM + *A. rhizogenes*	80[b]	100[a]	100[a]	93.30[a]
Mean	60[b]	80[a]	65[b]	
	SE M± (d)			CD (*P*=0.05)
Media (M)	2.80			5.80
Treatment (T)	2.80			5.80
M×T	4.80			10.20

medium even when diluted because it allows significantly better growth of the roots. Specifically the presence of ammonium in Murashige Skoog medium causes a rapid (less than 2 week) drop in the *p*H of the culture medium that is important to the root growth. On White's medium, nitrogen exclusively in the form of nitrate is assimilated, which counteracts the acidification of the culture medium following root growth. In this way, the culture medium is buffered and maintains *p*H at 6 for several months. Also, as that of strain variation, use of different media can also contribute for the higher transformation frequency (Danesh *et al*. 2006). Here, Modified White's medium supported higher frequency of transformation than the other media evaluated. Probably it may be due to presence of a particular amino acid glycine in the media.

The transformation ability of *A. rhizogenes* strains was also found to be different. In tobacco, the performance was reported to be in the order of LBA 9402, 9340, 9365, 15834 and A4 (Archana *et al.* 2001). Similarly, in the present studies, transformation efficiency of *A. rhizogenes* differed with respect to host plants and media used. *A. rhizogenes* strain 532 was highly efficient in transformation of cowpea explants, and *A. rhizogenes* 2364 was efficient in the transformation of tomato explants. This may be due to the interacting factor between *A.rhizogenes* strain and the host explant source.

Effect of incubation time and light on Agrobacterium rhizogenes mediated hairy root induction

To optimize the incubation time and to study the effect of light on hairy root induction, an experiment was conducted

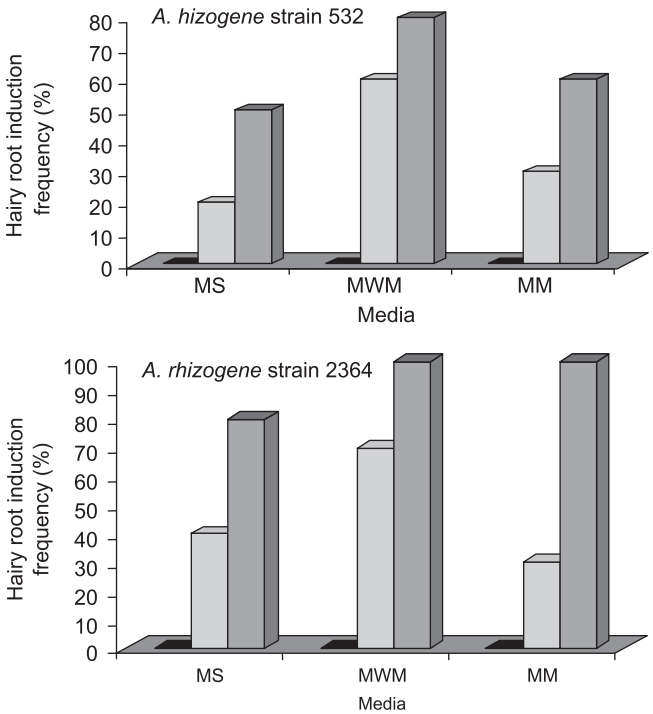


Fig 3 Effect of different media on *Agrobacterium rhizogenes* mediated hairy root induction frequency in tomato explants

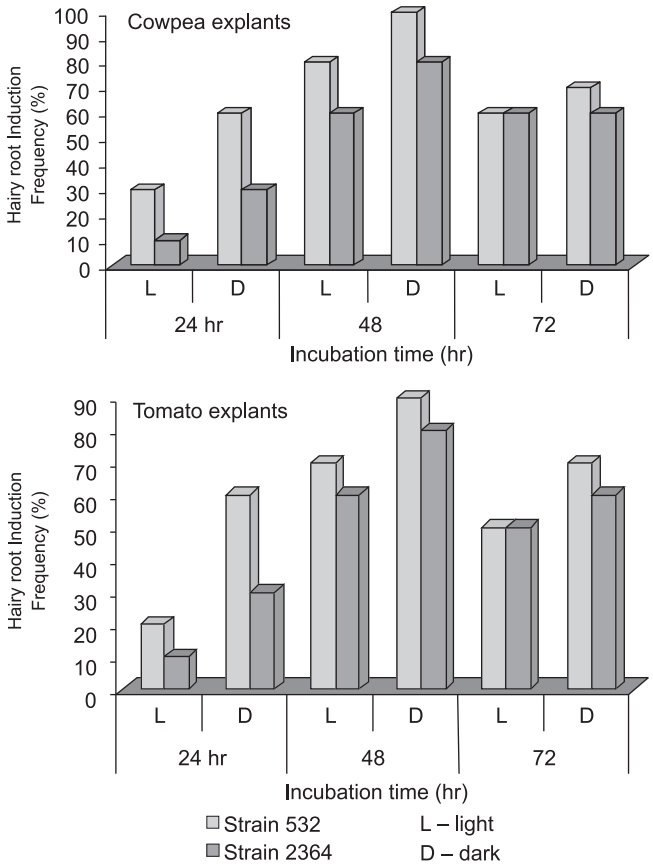


Fig 4 Effect of incubation time and light on hairy root induction by different strains of *A. rhizogenes*

with 3 different incubation time at light and dark condition.

Cowpea and tomato explants, used as explant source were tested for the production of hairy roots using 2 different strains of *A. rhizogenes (*strain 532 and 2364) with different incubation time (24,48 and 72 hr) in light as well as dark conditions.

At 48 hr of incubation, cowpea explants produced highest frequency of hairy roots with both the strains of *A. rhizogenes* 532 and 2364 under dark. Significant variation was observed between 2 strains of *A. rhizogenes*, while 532 strain recorded higher frequency of hairy roots. At 24 and 72 hr, the maximum induction was observed as 60 and 70% respectively with 532 strain. Here also 2364 recorded lesser values. Incubation under light recorded significantly lower induction than dark. The interaction effect of incubation time and condition is observed significant on hairy root production (Fig 1).

In case of tomato explants, hairy root induction was observed lower at 24 hr and it was observed higher with 48 hr incubation. Maximum frequency of 90% was recorded at 48 hr incubation with the strain 532. Induction frequency was observed higher with the incubation under dark than light by both the strains (Fig 4).

Incubation of inoculated explants for 48 hr resulted maximum induction of hairy roots (80–90%) in dark with the both the strains of *A. rhizogenes.* There was a significant reduction in induction with further increase in incubation period. This is in accordance with earlier results of Danesh *et al*. (2006), who reported the higher induction of hairy roots in carrot disc with the incubation of 48 hr, at dark may provide conducive environment for the insertion of copies of Ri-T DNA into the host. Prolonged incubation may have the possibilities of drying of explant sources as well as slowing down activities of Ri-TDNA on the host plant with lesser hormonal imbalance. Light is also an influencing factor for the production of hairy roots. The negative effect on hairy root production was eliminated with the incubation in dark at 25°C, which resulted higher induction of hairy roots (65–85%) (Fig 2).

Thus it was concluded that source of explants, triggering up of phenolics, strains of *A. rhizogenes* above all the growth medium and growth conditions may very much affect the hairy root formation and development. These factors created variation in hairy root induction frequency. Hence the optimization of these factors is considered prerequisite for getting efficient transformation. The importance of this study lies with the utilization of hairy roots as the host for the *in vitro* multiplication of AM fungi as if they are obligate for the presence of host roots.

REFERENCES

Aoki T, Masumoto H, Asako Y, Matsunaga Y and Shimomura K. 1997. Variation of alkaloid productivity among several clones of hairy roots and regenerated plants of *Atropa belladonna* transformed with *Agrobacterium rhizogenes* 15834. *Plant Cell Reports* **16**: 282–6.

Archana G, Sarish T, Dhingra R V and Narasu M L. 2001. Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and artemisinin production in *Artemisia annua*. *Current Science* **81**: 378–82.

Batra J, Dutta A, Singh D, Kumar S and Sen J. 2004. Growth and terpenoid indole alkaloid production in *Cartharanthus roseus* hairy root clones in relation to left- and right-termini linked Ri T-DNA gene integration. *Plant Cell Report* **23**: 148–54.

Becard G and Fortin J A. 1988. Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytologist* **108**: 211–8.

Butcher D N. 1980. The culture of isolated roots. (*in*) *Tissue Culture Methods for Plant Pathologists*, pp 13–7. Ingram D Sand Helgelson J P. (Eds) Blackwell Scientific, Oxford.

Butcher D N and Street H E. 1964. Excised root culture. *Botanical Review* **30**: 513–86.

Chilton M D, Tepfer D A, Petit A, David C, Casse-Delbart F and Tempe J. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. *Nature* **295**: 432–4.

Danesh Y R, Mohammadi Goltapeh E, Alizadeh A and Modarres Sanavy M. 2006. Optimizing carrot hairy root production for monoxenic culture of arbuscular mycorrhizal fungi in Iran. *Journal of Biological Sciences* **6**(1): 87–91.

Gelvin S B. 2000. Agrobacterium and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**: 223–56.

Kumar V B, Sharma C N, Prasad H, Gururaj B and Ravishankar G A. 2006. *Agrobacterium rhizogenes* mediated genetic transformation resulting in hairy root formation is enhanced by ultrasonication and acetosyringone. *Electronic Journal of Biotechnology* **9** (4): 349–57.

Mugnier J. 1988. Establishment of new axenic hairy root lines by inoculation with *Agrobacterium rhizogenes*. *Plant Cell Report* **7**: 9–12.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiology* **15**: 473–97.

Tepfer D. 1989. Ri t-DNA from *Agrobacterium rhizogenes*: a source of genes having applications in rhizosphere biology and plant development, ecology and evolution. *Plant-Microbe Interactions*, Vol. 3. pp 294–342: Kosuge T, and Nester E W, (Eds) McGraw-Hill Publishing, New York.

White F F, Taylor B H, Huffman G A, Gordon M P and Nester E W. 1985. Molecular and genetic analysis of the transferred DNA region of the root-inducing plasmid of *Agrobacterium rhizogenes*. *Journal of Bacteriology* **164**: 33–44.