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HAIRY ROOT INDUCTION IN ADAPATHIYAN (*HOLOSTEMMA ADA-KODIEN* K. SCHUM.)

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Abstract: *Holostemma ada-kodien*, commonly known as adapathiyan is a laticeferous climber belonging to the family Asclepiadaceae. The root tubers of the plant are useful to cure various ailments of eye and many other human diseases. Due to the indiscriminate collection of root tubers, the plant population in the natural habitats has declined drastically and consequently it has been listed out as vulnerable and rare in the FRLHT red list of medicinal plants. The present study reports the hairy root induction in *Holostemma* useful in conservation of the plant and also to explore possibilities for *in vitro* production of the active chemicals in *Holostemma*, which would be a good alternative to meet its ever-increasing demand. The procedure for induction of hairy roots is given in detail.

Key words: Adapathiyan, hairy roots, *Holostemma*, transformation

INTRODUCTION

Western Ghats comprise of one of the important areas of plant diversity in India. A large variety of medicinal plant species are found here. *Holostemma ada-kodien* K. Schum. is a medicinal plant of common occurrence in the tropical Western Ghats. It is used in the traditional system of medicine for maintaining youthful vigour and potentiality. The plant is a laticiferous climber belonging to the family Asclepiadaceae (Kolammal, 1979). The root tubers of this plant are medicinally important and are useful in ophthalmopathy, orichitis, cough, fever, burning sensation, stomachalgia and also as expectorant, tonic, stimulant and galac'tagogue (Warrier *et al*., 1995). The plant is widely distributed in the tropical rain forests of the world (Sivarajan and Balachandran, 1994). The indiscriminate and ruthless collection of the root tubers in recent times has led to acute scarcity of the plant and is listed out as vulnerable and rare in the Foundation for Revitalization of Local Health Traditions red list of medicinal plants (FRLHT, 1997). Therefore the plant needed immediate attention to sort out alternative means of its conservation.

Hairy roots formed after the insertion of the T-DNA of the *Agrobacterium rhizogenes* (Ackermann, 1977) are capable of regeneration, show rapid growth, are capable of producing the secondary metabolites of the mother plant and also useful in germplasm conservation (Rhodes *et al*., 1987). Keeping in mind the importance of conservation of this plant and also to explore the possibilities of *in vitro* secondary metabolite production, the study was conducted to induce hairy roots in *Holostemma*.

MATERIALS AND METHODS

In vitro grown seedlings, shoot bud cultures and callus cultures were raised as per the standard procedure (John and Keshavachandran, 1996). The leaf segments, shoot buds, internodal segments, seedling hypocotyls and callus obtained from *in vitro* grown plantlets, seedling hypocotyls and callus cultures were tested for their ability to induce hairy roots. Five strains *of Agrobacterium rhizogenes viz.*, PcA4, 15834 (obtained from C.B.Patel Research Centre, Mumbai, India), A4 (obtained from Dr. Eugene Nester, University of Washington, Seattle, USA), 8196 and 2659 were tested for their transforming ability. The bacterial strains were grown on yeast extract mannitol (YEM) medium at 25±2 °C.

Leaf margins were cut with a sterile blade and 10 pricks were made on the lower side of the leaf on the lamina and the midrib. The shoot buds, internodal segments and seeding hypocotyls were freshly pricked with a sterile needle. The callus was cut into small pieces with a sterile blade. *Agrobacterium rhizogenes* strains were grown on YEM medium for 48 h and were used for infection. The cells obtained from single cell colonies were either applied directly on the wounds of the explants or were suspended in Murashige and Skoog (MS) liquid medium (Murashige and Skoog, 1962) (10^8 cells ml^{-1}) and applied on the wounds. The wounded explants were also co-cultured in liquid MS medium containing bacteria (10^8 cells ml^{-1}) for 24, 36, 48 and 72 h

Table 1. Effect of explant types and the *A. rhizogenes* strains on transformation of *Holostemma ada-kodien*

Explants	Percentage transformation				
	A. rhizogenes strain				
	PcA4	15834	A4	8196	2659
Leaf	-	-	-	-	-
Shoot bud	11.65	18.18	12.14	-	-
Internodal segment	-	-	-	-	-
Seedling hypocotyl	22.52	13.80	13.52	-	-
Callus	-	-	-	-	-

- No transformation achieved

Table 2. Influence of bacterial inoculum on transformation of *H. ada-kodien* explants

Bacterial inoculum	Percentage transformation				
	Explants				
	Leaf	Shoot bud	Internodal segment	Seedling hypocotyls	Callus
Single cell colonies	-	-	-	-	-
Bacterial suspension	-	-	-	7.14	-

- No transformation achieved

Table 3. Effect of co-culture time on transformation of seedling hypocotyls of *Holostemma ada-kodien* co-cultured with less initial bacterial population (10^3 cells ml^{-1})

A. rhizogenes strains	Percentage transformation				
	Co-culture time (h)				
	12	24	36	48	72
PcA4	0.00	20.83	7.14	0.00	0.00
15834	0.00	13.94	11.11	0.00	0.00
A4	0.00	8.35	0.00	0.00	0.00
8196	0.00	0.00	0.00	0.00	0.00
2659	0.00	0.00	0.00	0.00	0.00

and in MS medium containing 10^3 bacterial cells ml^{-1} for 5, 10, 15, 30, 60 and 120 min. The inoculated explants were placed on 1 per cent agar medium for 24 h and then transferred on MS solid medium containing 250 mg ml^{-1} cefotaxime. The explants were immediately sub-cultured on the same medium when bacterial growth was seen on the media. After obtaining bacteria free cultures, they were sub-cultured on MS solid medium without antibiotics. All the cultures were maintained at 25±2°C temperatures. One set of cultures was kept in dark and other set in a day/night regime (16/8 h) in each experiment to test the effect of photoperiod on hairy root induction.

Induced hairy roots were placed on full and half strength MS solid and liquid medium to test its effect on root growth. The transformation was confirmed by opine analysis by the modified procedure given by Dessaux *et al.* (1991).

RESULTS AND DISCUSSION

Among the different *A. rhizogenes* strains tested, the strains PcA4, 15834 and A4 induced hairy roots. The strains 8196 and 2659 did not induce hairy roots (Table 1). The *A. rhizogenes* strains differ in their host range. The strains PcA4, 15834 and A4 are agropine strains and are classified as wide host range (WHR) strains whereas the strains 8196 and 2659 are classified as limited host range (LHR) strains (Rhodes *et al.,* 1987). They differ in the plasmids they harbour (Rhodes *et al,* 1989). WHR strains possess TR-DNA fragment of T-DNA harbouring genes for auxin synthesis (tms 1 and tms 2). These genes trigger cellular division by auxin synthesis due to which these strains are able to transform a wide range of species. On the contrary, the LHR strains are deficient in TR-DNA fragment, hence they cannot trigger auxin synthesis and so can infect only a limited number of species. Spencer and Towers (1989) reported that the WHR strains are more sensitive to the wound induced compounds than the LHR strains. So the lack or low levels of inducer molecules from wounded plant cells inhibit the virulence of LHR strains resulting in inhibition of transformation. So also, in addition to the *vir*-inducing compounds certain inhibitory compounds are induced from the wounds. Not all the *A. rhizogenes* strains are capable of degrading the inhibitory compounds. So they differ in their transformation ability. The

Table 4. Effect of co-culture time on transformation of *Holostemma ada-kodien* explants co-cultured with higher initial bacterial population (10^6 cells ml^{-1})

A. rhizogenes strains	Explant	Percentage transformation				
		Co-culture time (min)				
		5	10	30	60	120
PcA4	SB	0.00	12.50	18.18	0.00	14.28
	SH	0.00	17.64	16.52	16.64	0.00
15834	SB	0.00	0.00	18.18	0.00	0.00
	SH	0.00	0.00	9.82	0.00	0.00
A4	SB	0.00	0.00	0.00	0.00	10.00
	SH	0.00	0.00	11.25	0.00	0.00
8196	SB	0.00	0.00	0.00	0.00	0.00
	SH	0.00	0.00	0.00	0.00	0.00
2659	SB	0.00	0.00	0.00	0.00	0.00
	SH	0.00	0.00	0.00	0.00	0.00

SB = Shoot bud; SH = Seedling hypocotyl

present observation that hairy roots were induced only by WHR strains is similar to the observation in the case of strains A4 and 232 (both WHR types) of Patena *et al.*, 1988.

Among the different explants tested, only the seedling hypocotyls and shoot buds induced hairy roots whereas the internodal segments, leaf segments and callus did not induce hairy roots at all (Table 1). Juvenility and nature of explant influence the *Agrobacterium* mediated transformation process (Yonemitsu *et al.*, 1990 and Trypsteen *et al.*, 1991). Nin *et al.* (1997) have reported that specificity of *Agrobacterium* transformation is closely connected with the age and hormonal balance of the host tissue. Potrykus (1990) stated that wound response was the most important factor for the successful transformation. He reported that explants with pronounced wound response develop larger populations of wound adjacent competent cells for regeneration and transformation. It is quite evident that different explants vary in their wound response i.e., produce the number of competent cells for transformation. The explant cells differ in their DNA synthesis and cell division ability due to the difference in physiological maturity of the cells. The present observation that seedling hypocotyls and shoot buds induced hairy roots may be due to their ability to produce greater number of wound adjacent competent cells for regeneration and transformation.

The nature of bacterial inoculum influenced the transformation frequencies when bacteria were directly applied on the wounded explants (Table 2). The bacterial cells picked from single cell colonies when applied on the wounds did not induce hairy roots. On the contrary, the bacterial suspension (10^8 cells ml^{-1}) when applied on wounds induced hairy roots. The superior performance of the bacterial suspensions in the present study may be due to the optimum concentration of bacteria in suspensions as compared to the cells picked from single cell colonies. During co-culture of bacteria and wounded explants, the co-culture time and the bacterial population at the time of co-culture influenced the transformation frequencies. Only the seedling hypocotyls induced hairy roots when less population (10^3 cells ml^{-1}) of bacteria was present during co-culture whereas both shoot buds and seedling hypocotyls induced hairy roots when more bacterial population (10^8 cells ml^{-1}) was present at the time of co-culture (Table 3). When the initial population of bacterial cells was less, the co-culture time required for hairy root induction was more and vice versa. Hairy roots were induced only when seedling hypocotyls were co-cultured for 24 and 36 h. At 48 and 72 h co-culture, no hairy roots were induced. Similarly, hairy roots were induced from seedling hypocotyls and shoot buds when the co-culture time was 10, 30, 60 and 120 min (Table 4). No hairy roots were induced at 5 min co-culture. Xu *et al.* (1997)

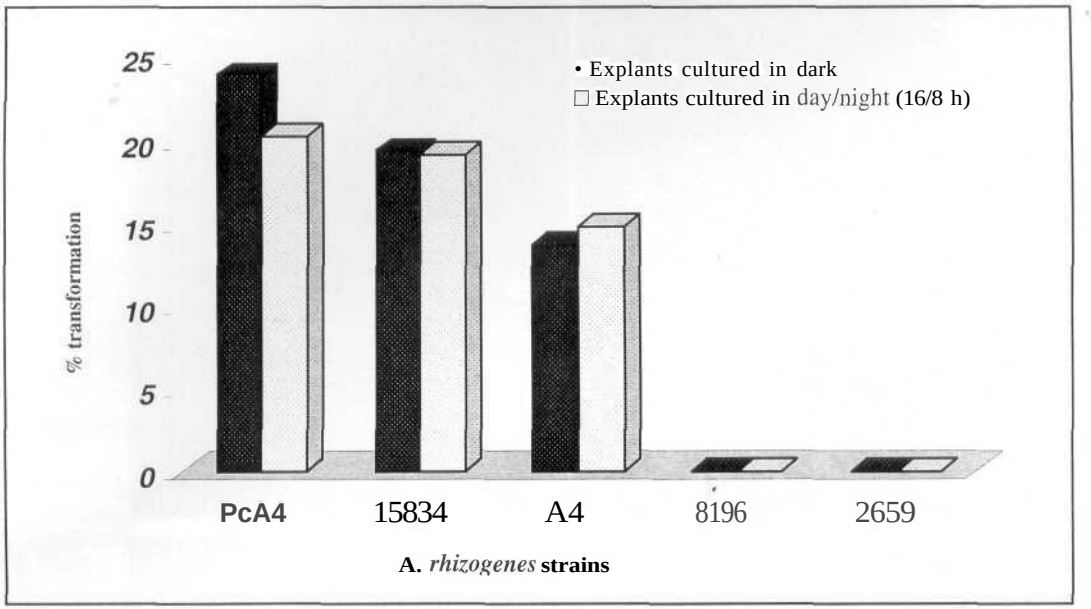

Fig 1. Effect of photoperiod on transformation of *H. ada-kodien* seedling hypocotyls on infection with *A. rhizogenes* strains

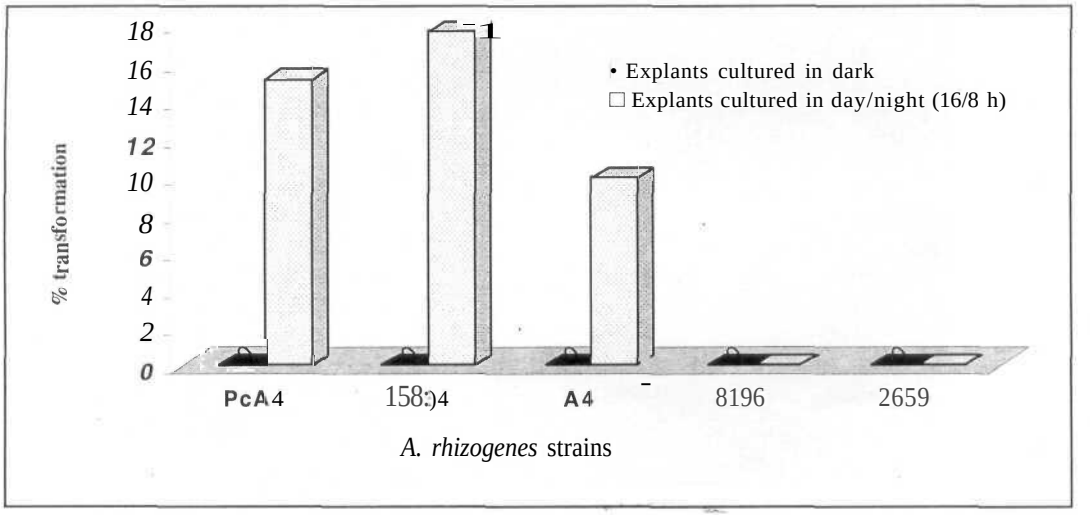

Fig 2. Effect of photoperiod on transformation of *H. ada-kodien* shoot buds on infection with different *A. rhizogenes* strains

reported that co-culture time affects the transformation frequencies of alfalfa suspension cultures. Kumar *et al.* (1991) reported that optimum concentration of bacterial cells is essential for transformation with *Agrobacteriurn.* In this study, during 24 hour co-culture, the bacterial cell concentration reached an optimum level, so the highest transformation

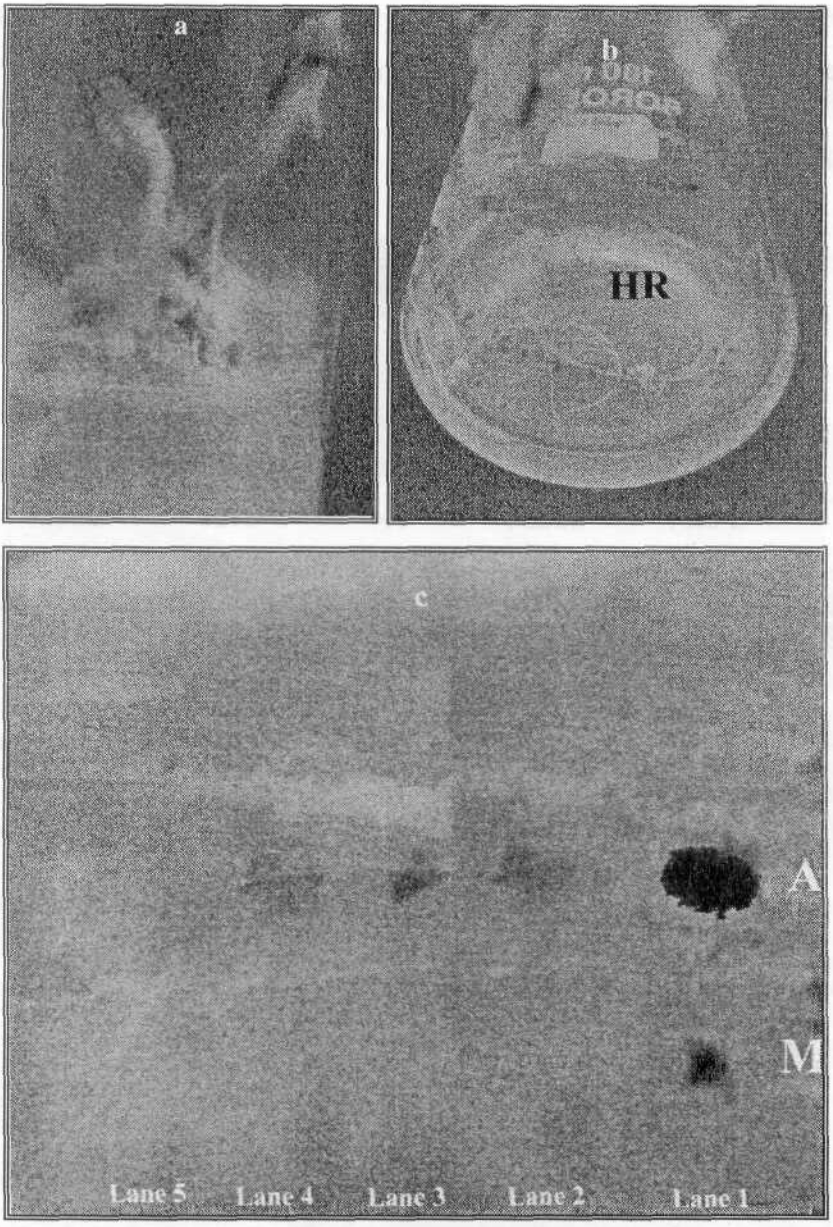

Plate 1. (a) Hairy roots emerging from seedling hypocotyl explants (b) Growth of hairy roots in "MS liquid medium (c) Hairy roots (lane 2, 3 and 4) showing presence of agropine (shown as A in lane 1) while normal roots (lane 5) showing absence of agropine

frequencies were achieved. After 36 h, the concentration of bacterial cells reached the supra-optimum level and the competitive inhibition of competent bacterial cells resulted in inhibition of transformation. Photoperiod affected the transformation frequencies (Fig 1 and 2). Seedling hypocotyls induced hairy roots both under continuous dark and in a day/night regime whereas shoot buds induced hairy roots only in day/night regime.

Thin whitish roots with numerous hairs emerged from the wounds in a period of 2-4 weeks (Plate la). These roots grew well in MS liquid medium (Plate 1b). The root growth was satisfactory on liquid MS medium but was not satisfactory on solid MS medium or in 1/2 MS liquid medium.

Opine analysis showed the presence of agropine in the roots obtained on infection with the strains PcA4, 15834 and A4 (Plate 1c). No opines were detected in the normal roots. This is a firm indication that the roots are transformed.

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