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IN VITRO STUDIES AND *AGROBACTERIUM RHIZOGENES* MEDIATED TRANSFORMATION OF *CISSAMPELOS PAREIRA* – A HIGHLY RECALCITRANT SPECIES

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ABSTRACT

An attempt was made to induce callus mediated regeneration of *Cissampelos pariera* plants using leaf explants along with induction of hairy root for increasing the secondary metabolites. A4, MSU 440, K599, NCIM 5140 and 9402 strains of *A. rhizogenes* was used for hairy root induction. Transformation using liquid co-cultivation, direct introduction of cultures into wound site and also sonication assisted co-cultivation methods. The leaf explants cultured on MS medium supplemented with NAA (2mg/l) showed proliferation of callus. But the callus failed to show organogenesis on MS media enriched with any growth hormones. The plant species were highly recalcitrant. Among the methods and strain used for hairy root culture, the leaves co-cultured with *A. rhizogenes* strain MSU440 using sonication-assisted method alone showed callus formation on MS Medium devoid of growth hormones. The callus subjected to HPLC analysis showed the increase in Berberine content.

Keywords: **Cissampelos pariera, Agrobacterium rhizogenes, Co-cultivation, hairy root culture, *in vitro* cultures**

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INTRODUCTION

Cissampelos pareira is a species of the flowering plant belongs to the family Menispermaceae. The common name of this plant is Patha, velvet leaf, ice vine, patindu and abuta. It is also called Laghu patha in the Ayurvedic medicine. In the Tamil medicine (Siddha medicine) it is called " Malaith thaangki" and used for several purposes. It has been use by the Tamil's for more than 3000 years and is still used by the tribals in Tamil Nadu. It is distributed throughout India. It is a sub-erect or climbing herb used in the Indian traditional medicine[1]. It reaches 3 to 6 m along the ground or into the crowns of the trees. The stem is woody, flexible, and slender (to 1 cm) and twines for support. Alternate leaves are usually softly pubescent on both the surfaces.The plant is reported to exhibit antidiarrheal activity[2], arthritis[3], antileukemic activity[4], cooling effect and heart complaints[5], antifertility effects[6], Antiplasmodial effect[7] and antifungal effect[8]. It is highly rich in alkaloids namely as curine, curine-4-Methyl-Ether, 4"-0-Methyl curine, alkaloids, Berberine, Cissamine, Cissampareine, Cyclanoline, Cycleanine, D-Quercitol, Hayatidine, Hayatine, Hayatinine, Isochondodendrine, Menismine, Perierine, Quercitol, Saponin, Tetrandrine and Tetrandrine-N-2'-oxide[9]. Berberine is reported as the major alkaloid from the *Cissampelos pareira*. Berberine possesses both anti-adipogenic and anti-inflammatory effects, anti-inflammatory and induced antiproliferative activities in human promonocytic U937 cells[10]. It protects against endothelial injury and enhances the endothelium- dependent vasodialation which is mediated through activation of the AMPK signaling cascade. Berberine and its derivatives may be useful for the treatment or prevention of endothelial dysfunction associated with diabetes and cardiovascular disease[11]. It exerts anticancer activities both in vitro and in vivo through different mechanisms. Regeneration of plants through tissue culture is an alternate technology applied to prevent collection and loss of useful medicinal plants from the natural environment. Rapid clonal propagation and mass propagation techniques are used to produce more number of plants that can be used as source medicinal compounds to treat many diseases. *Agrobacterium rhizogenes* mediated hairy root cultures are reported in many medicinal plants, which is a promising alternate for production of pharmaceutically important secondary metabolites. So far there was no attempt to induce hairy roots from stem and leaf of *Cissampelos pareira* and there are very few attempts on tissue culture aspects of this plant as it is reported to be highly recalcitrant. In the current study an attempt was made to induce callus from leaf and stem explants and callus mediated hairy root induction using A4, MSU 440, K599, NCIM 5140 and 9402 strains of *A. rhizogenes*.

MATERIALS AND METHODS

The plant *Cissampelos pareira* (L.) (Menispermaceae) was collected from the herbal garden of FRLHT, Bangalore and planted in the greenhouse for further use. The plant material was identified and authenticated taxonomically at the Life Science Department of Kristu Jayanti College, Bangalore. A voucher specimen of the collected sample was deposited in the departmental herbarium for future reference.

Sterilization

Leaf and stem explants collected from the mother plants were washed thoroughly with running tap water. The explants were treated with teepol for 3 min to remove surface dust and dirt and washed 4-5 times in distilled water. Then the explants were surface sterilized with 10% Sodium hypochlorite for 5 min and a thorough wash in sterile distilled water for 7-8 times.

Callus Induction

Stem, whole leaves and the trimmed leaves that were wounded using sterile blades were inoculated on Murashige and Skoog (1962) medium fortified with various combinations of 2,4-D, NAA and IAA at different concentrations for inducing callus. pH of the media was maintained between 5.6-5.8. 0.8% agar was used for solidification. The cultures were incubated at 25±2°C.A4, MSU 440, K599, NCIM 5140 and 9402 strains of *A. rhizogenes* obtained from MTCC (Microbial Type Culture Collection, Chandigarh) and TERI, were used for hairy root induction. Leaf explants were used for co-cultivation. Yeast Mannitol Broth was used for enriching the *A. rhizogenes* strains. After 48 hours of incubation in 50 ml of media, optical density was measured at 660 nm (OD660=1.0). The exponentially grown cultures were pelleted by centrifugation at 3500rpm/ 15 min at 20°C. The cultures were resuspended in 10 ml of MS medium and used for co-cultivation.

Standardization of Cefotoxime concentration

Sensitivity to Cefotoxime sodium was studied using different concentrations such as 2.5 mg, 5 mg to 300mg/l. It was found that 250mg/l of Cefotoxime is the best concentration in which there is no growth of *Agrobacterium rhizogenes* over the explant.

Hairy Root Induction

Transformation by Co-cultivation

The explants (whole leaves and trimmed, wounded leaves) and the callus obtained were punctured using a sterile scalpel and were placed into 50 ml conical flasks containing different strains of bacteria and 50 mM and 100 mM acetosyringone and kept for 24hr and 48hr incubation into shaker incubator. After 24hr and 48hr incubation the leaves were transferred onto MS broth containing cefotaxmine 250 mg/l and again incubated in a shaker incubator for 24 hr and 48 hr. After incubation, the leaves were subcultured on MS solid medium for further growth.

Sonication-assisted Agrobacterium-mediated transformation method

The sterilized leaves (very young, mid age leaves and old leaves, wounded and as a whole explant) were transferred into a 50 ml conical flask containing 10 ml of resuspended *Agrobacterium* cultures. The conical flask was placed in a Styrofoam float at the center of a bath sonicator which was controlled with an electronic photographic timer. The leaves were treated for different time intervals (from 60 sec, 90 sec and 120 sec) using both horizontal and vertical wave generator output. The leaves were incubated in the same condition on the

shaker incubator for variable time intervals (1hr, 2 hr and 3 hr). The leaves were removed from the conical flasks and placed on a sterile filter 50µM papers to blot off excess bacteria and then transferred onto MS medium containing and 100 µM acetosyringone for 24 hr, 48 hr, 72 hr. After 24 hr, 48 hr and 72 hr incubation the leaves were transferred to MS medium containing cefotaxmine 250 mg/l and again incubated at 25°C.

Transformation by direct inoculation of Agrobacterium on explant

The sterilized leaves (very young, mid age leaves and old leaves, wounded and whole explants) were infected with different strains of *Agrobacterium rhizogenes* with the help of sterilized needle. With the help of sterilized needle single bacterial colonies were picked up used to puncture the midrib of the leaves. After introducing the bacterial culture, injured leaves were placed on MS medium containing 50 mM and 100 mM acetosyringone and incubated for 24hr and 48 hr. After incubation the leaves were washed with MS broth containing cefotaxmine 250mg/l and again incubated onto MS solid medium containing cefotaxmine 250mg/l and incubated for 24hr and 48hr. After this the leaves were transferred to MS solid medium for further growth.

RESULTS AND DISCUSSION

Callus Induction

After 2-3 weeks of culturing on MS media supplemented with various growth regulators, explants showed growth response. Callus formation occurred from the cut ends and the mid rib regions of the leaf explants on MS medium fortified with IAA (2 mg/l). NAA at lower concentrations alone or in combination with other growth hormones did not show any callus induction from leaves. In most of the combinations the leaves showed an initial bulging and after a few days explants turned brown in colour and dead. The leaf explants cultured on MS medium supplemented with IAA (2 mg/l) showed profuse callusing (Table 1). The callus was friable and white[12]. It also reported that *Tinospora cordifolia* leaf explants cultured on NAA and 2,4-D alone could initiate callusing. But in the present study NAA was not found to induce callus from leaf explants of *Cissampelos pareira.*The stem explants cultured on MS media fortified with NAA (0.5-3 mg/l) showed initiation of callus within 28 days. NAA (2 mg/l) was found to be optimum concentration required for inducing profuse callusing from stem explants. The induction of callus from stem explants of *Cyclea peltata* on MS medium fortified with NAA (2 mg/l) was reported earlier[13]. Also the induction of callusing from the nodal, inter-nodal and leaf explants of *Tinospora cordifolia* on the medium supplemented with different concentrations of BAP and NAA was reported[14].The callus when sub cultured on MS medium enriched with IAA, NAA and 2,4-D at various concentrations and combinations failed to show organogenesis in any of the combinations of growth regulators used in the current study.

Table 1
Effect of growth regulators on callus induction from leaf explants

Growth hormones				Callus colour/texture	Wet weight of the callus	Dry Weight of the callus
IAA (mg/l)	NAA (mg/l)	2,4-D(mg/l)	Coconut water			
0.5	-	-	-	White friable	0.93±0.4159	0.33±0.0276
1				White friable	0.51±0.2298	0.35±0.0109
2	-	-	-	White friable	1.74±0.8836	1.02±0.0042
2.5				Creamish soft	1.20±0.5375	0.86±0.0042
3				Creamish soft	0.46±0.2075	0.35±0.0109
3.5	-	-	-	White soft	0.44±0.1976	0.29±0.0084
-	1-3.5	-	-	-	-	-
-		0.5-3	0.5-15%	-	-	-

Standardization of Cefotoxime and Acetosyringone concentration

Among the various concentrations of cefotoxime tested 250mg/l was found to be the suitable concentration in which there is no growth of *Agrobacterium rhizogenes* over the explants was noticed with less necrotic lesions on the explant. 50µM of Acetosyringone was found to be best in controlling the bacterial growth in the previous studies[15]. Similarly in the current study 50µM of acetosyringone effective controlled the over growth of bacteria.

Transformation by Co-cultivation

In the current study all the three methods, viz, direct stabbing of bacteria on explants, co-cultivation in 50 ml of MS media and ultra sonication assisted transformation failed to induce hairy roots from *Cissampelos parieria* leaf explants (both wounded and whole leaves) and callus. All the six different strains of *Agrobacterium rhizogenes* 532, A4, MSU 440, K599, NCIM 5140 and 9402 did not induce hairy roots in the leaf explants and leaf callus during the current study. Earlier studies also reported that there was no hairy roots induced in *Aegel marmelos*, by any of three methods viz, by stabbing the leaves with the bacteria, applying the bacteria at the cut-end, and by co-culturing the cut explants in 50 ml liquid MS medium along with 200µl of fresh bacterial culture used in the study[16]. The possible explanations given was it may be due to the absence of receptors for the*A. rhizogenes* strains on the plants cells and/or insusceptibility of *Aegle marmelos* to *A. rhizogenes* MTCC 532 strain used for transformation in the study. They also explained that it may be due to the fact that the plants of Rutaceae family have been

regarded as recalcitrant to *Agrobacterium* mediated transformation and give only low, *in vitro* regeneration efficiency which is highly genotype dependent. The genetic transformation with *Agrobacterium* in the *Citrus sinensis* (Rutaceae) species has been considered as recalcitrant due to the low transformation efficiency[17]. The reason for non induction of hairy roots in *Cissampelos pareira* also may be similar to citrus species which is recalcitrant as these are not natural hosts of *Agrobacterium* and also due to absence of specific phenolic compounds primarily acetosyringone which activates the virulence genes (Vir A protein) of the Ri plasmid to initiate the transfer of TDNA and also there are problems in the selection and rooting of shoots from transformed cells. But in the present study even after the addition of acetosyringone at 2 different concentrations, hairy roots were not induced. This shows the recalcitrant nature of *Cissampelos pareira.* In contrast it was previously reported that addition of Acetosyringone during co-cultivation enhanced the transformation rates with *Agrobacterium tumefaciens* in *Cissampelos pareira* which showed gus positive results[15]. The leaf explants of *Cissampelos pariera* showed induction of callus when transformed with *A. rhizogenes* strains 440 and A4 when transformed using all the three methods. Explants co-cultivated with all the other 4 strains in all the three methods of transformation, did not show callusing. The explants subsequently turned dark and dried. The leaf explants transformed with all the strains of *A. rhizogenes* except 9402 strain showed initial bulging within 5-10 days of post transformation period. Strain 440 and A4 further resulted in callusing from the explants within 25 days of post transformation period. In the rest of the cultures explants turned brown and eventually died.

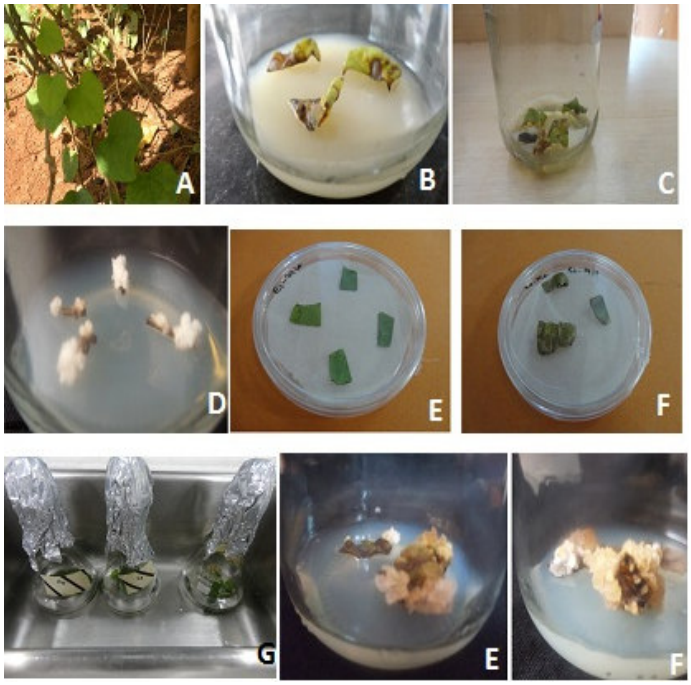
Table 2
Transformation of leaf explants using various strains of Agrobacterium rhizogenes

Strain used	Methods Used	Transformation frequency	Response	No. of days to respond
Agrobacterium rhizogenes 532	Direct inoculation of culture	No response	No response	-
	Co-cultivation			-
	Ultrasonication assisted co-cultivation			-
Agrobacterium rhizogenes 440	Direct inoculation of culture	No response	Callusing	20
	Co-cultivation			25
	Ultrasonication assisted co-cultivation			25
Agrobacterium rhizogenes A4	Direct inoculation of culture	No response	Callusing	25
	Co-cultivation			25
	Ultrasonication assisted co-cultivation			25
Agrobacterium rhizogenes K599	Direct inoculation of culture	No response	No response	-
	Co-cultivation			-
	Ultrasonication assisted co-cultivation			-
Agrobacterium rhizogenes 5140	Direct inoculation of culture	No response	No response	-
	Co-cultivation			-
	Ultrasonication assisted co-cultivation			-
Agrobacterium rhizogenes 9402	Direct inoculation of culture	No response	No response	-
	Co-cultivation			-
	Ultrasonication assisted co-cultivation			-

The previously induced callus in MS medium with IAA (2mg/l) when subjected to transformation also did not show any induction of hairy roots. The callus showed further profuse proliferation instead of inducing hairy roots. The callus after co-cultivation was collected, dried and processed for extraction and analysis of Berberine accumulated (Table 3).

Table 3
HPLC Quantification of Berberine in callus after transformation

Strain used	Explant	**Concentration of Berberine**
Agrobacterium rhizogenes 440	Leaf Callus	0.054%
Agrobacterium rhizogenes A4	Leaf Callus	0.063%



A: Cissampelos pareira Plant; B- Leaf explants cultured on MS media devoid of growth hormones; C- Leaf callus induced on MS media fortified with IAA (2mg/l); D- Stem showing callusing on MS media supplemented with NAA (2mg/l); E- leaves subjected for transformation; F- Callus induction from the leaves after Transformation; G- Sonication assisted co-cultivation of leaf explants; H & I- Profuse callusing from the leaf explants after co-cultivation with A. rhizogenes 440 and A4 strains.

CONCLUSION

The current study aimed at developing a protocol to induce hairy roots from the leaf and stem explants to increase the production of berberine content in the *in vitro* conditions. The study concluded that in accordance in with previous studies in many plants, *Cissampelos pariera* is also a recalcitrant species and a challenging species to induce hairy root cultures. Further studies are being attempted to successfully induce hairy roots.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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