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Establishment of Adventitious and Hairy Root Cultures from *in vitro* Leaves of *Lagerstroemia speciosa* (L.) Pers. for Corosolic Acid Production

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ABSTRACT

To develop a feasible production system for corosolic acid (antidiabetic triterpene) and study its biosynthesis and enable metabolic engineering, we established adventitious and hairy root cultures of *Lagerstroemia speciosa*. Best result for adventitious root culture establishment and corosolic acid (CRA) production was obtained for 0.5 mg l^{-1} Naphthalene Acetic Acid (NAA). Hairy roots initiated from the *in vitro* leaf explants co-cultivated with *Agrobacterium rhizogenes* strains. Transformation was confirmed by polymerase chain reaction (PCR) and Reverse Transcription PCR. Maximum transformation efficiency (60%) was observed for A4 strain. The influence of bacterial strain, leaf explant portion and media composition on hairy root induction was investigated. CRA content of adventitious and hairy roots was determined using HPLC. The root cultures established could synthesise CRA under *in vitro* conditions and hence they can be used for sustainable production of corosolic acid.

Key words *adventitious root culture; corosolic acid; hairy roots; Lagerstroemia speciosa; Agrobacterium rhizogenes*

Lagerstroemia speciosa (Lythraceae), is a deciduous tropical flowering tree that grows in South East Asian countries. It has a long history of folkloric medical applications that include blood pressure control, urinary dysfunctions (helps ease urination), controls the cholesterol levels, treatment of diarrhea, facilitates bowel movement, diabetes and as analgesic (Ashnagar, *et al*., 2013). The tree harbours many bioactive compounds like ellagitannins (lagerstroemin, flosin B and reginin A), valoneic acid, dilactone, ellagic acid etc of which corosolic acid (CRA) assumes significance as it is reported to have anti-inflammatory, anticancer and hypoglycemic activities. It showed statistically significant anti-diabetic activity in animal models and human clinical trials (Kazama, 2002; Judy, *et al*., 2003).

Corosolic acid (2á,3b-dihydroxyurs-12-en-28-oic acid) is a pentacyclic triterpene. At present the demand for corosolic acid and *L. speciosa* extract as medicine and dietary supplement is on the increase. Leaves of *L. speciosa* are reported to contain 0.005-0.868% d.wt of corosolic acid (Jayakumar, *et al*., 2014). Moreover secondary metabolite production being highly dependent on biotic and abiotic factors, a feasible and consistent supply of CRA can be ensured through development of an *in vitro* production system for CRA.

Like shoot cultures, root cultures are also valuable sources of secondary metabolites (Rao and Ravishankar, 2002). Adventitious and hairy root technologies may provide industrial-scale, plant sources for the production of possible plant-based anti-diabetic, antioxidant and other therapeutic molecules. To ensure sustainable CRA availability a production system which can incorporate the genetic modification and ensure genetic and biosynthetic stability is required for which hairy root culture induced by *Agrobacterium rhizogenes* is an excellent option. This technique can be extended to the genetic modification of the tree species under study, which otherwise is slow growing due to long generation cycles with inherent difficulty in introducing useful genes by parental line crossing, through conventional methods. Hairy roots resemble normal roots in terms of differentiated morphology and biosynthetic machinery, producing similar or more secondary metabolites compared to wild-type roots (Sharma, *et al*., 2013).

The present study deals with the development of root cultures of *L. speciosa* for production of CRA. To the best of our knowledge this is the first report of establishment of hairy roots in *L. speciosa*. It is expected that the insights generated from this study will certainly help to meet the increased demand for corosolic acid by the development of an *in vitro* system which could ensure high, uniform and year round production of corosolic acid.

MATERIALS AND METHODS

Plant material and bacterial strains

In vitro leaves for the study were from shoot cultures developed as described early (Vijayan *et al*. 2015). Bacterial strains used for hairy root induction were A4, TR105, LBA-9402, and R1022 (obtained from Dr. K. Satheeshkumar, JNTBGRI, India).

Establishment of adventitious root culture

Adventitious root cultures were established by inoculating *in vitro* leaves in MS medium supplemented with different concentrations (0.1, 0.2, 0.5, 1 and 2 mg l^{-1}) of NAA or Indole Acetic Acid (IAA) or Indole Butyric Acid (IBA). Subculturing of the developed roots was done in the same medium devoid of gelling agent. Afterwards 100mg of fresh roots were transferred to liquid MS medium with optimum concentration of auxin (0.5 mg l^{-1} NAA) and used for CRA analysis.

Establishment of hairy root culture

Agrobacterium rhizogenes strains were grown for 48 hour in yeast extract mannitol (YEM) medium. Leaf (1cm^2) explants from *in vitro* raised plants were pre-cultured on MS basal medium for 24 hour prior to infection. The explants

were stabbed with bacterial strain using surgical blade and co-cultivated on solidified hormone free MS medium with 30 g l^{-1} sucrose and 0.13% phytagel for 2 days in dark conditions. After co-culture, the explants were rinsed with sterile water, blotted dry and transferred to fresh MS medium containing 500 mg l^{-1} antibiotic (Cefotaxime- from M/s Sigma-Aldrich, USA) and kept under complete darkness at 25 ± 2^0C. After 4 weeks, hairy roots appeared on cut ends of the explants were detached and cultured onto fresh MS medium. The induced roots were subcultered several times on medium containing decreasing concentrations of antibiotic to get the bacteria removed from the hairy root culture lines. The roots were washed with 250 mg l^{-1} antibiotic to kill the residual *Agrobacterium* and the cultures were then transferred to MS medium containing antibiotic, as and when required. The obtained hairy root lines, which are bacteria free, were then maintained by subculture of 3–5 cm long pieces of roots, on the same medium without the gelling agent. The cultures were kept on a rotary shaker at 100 rpm rotation under an 8 hour day^{-1} photoperiod at photon flux intensity of 50-60 ìEm^{-2}s^{-1}by cool white fluorescent tubes. Hairy roots obtained from a single clone were used for further analysis. Twenty five explants were used per experiment and each experiment was carried out in triplicate.

Hairy root confirmation

PCR

PCR amplification of the genomic DNA from hairy root was carried out using specific primers for *rol* and *vir* genes. Sequences of the primers were given in Table 1. PCR was carried out in 0.025 cm^3 reaction mixture containing 0.2 mM dNTP's, 10mM Tris-HCl, 1.5mM $MgCl_2$, 50 mM KCl, 0.1%Triton X-100, 1.0 U Taq DNA polymerase (M/s Finzymes, Helsinki, Finland), 15 pmol primers (M/s IDT, Coralville,USA) and 50 ng of genomic DNA. The amplification was performed in a thermal cycler. After an initial denaturation at 94º C for 5 min, a total of 35 cycles of 30 sec at 94º C, 30 sec at 55 º C and 1 min at 72 º C were performed. The final extension step was at 72 º C for 10 min. Reaction mixture containing genomic DNA from adventitious (non-transformed) root was used as negative control. Positive control contained plasmid DNA from the bacterial strain A4. Amplified products where resolved in 1.4% agarose gel (1XTBE) followed by EtBr staining and the gel was visualized and photographed under UV light using gel documentation system.

Reverse Transcription PCR

Total RNA was extracted from hairy root lines using the 'Spectrum plant total RNA kit' according to the manufacturer's instructions (M/s Sigma-Aldrich USA). Approximately 100 mg hairy root of each lines were used to isolate total RNA. Total RNA isolated from *in vitro* roots that had developed without *Agrobacterium* infection was subjected to RT-PCR under similar conditions to serve as negative control. The primers for the *rol* genes were then used for PCR amplification with the same conditions. The amplified fragments were resolved using electrophoresis in 1.4% agarose gel with DNA marker and visualized with ethidium bromide staining under UV light.

Fresh Growth Index Measurement

Fresh weight of the harvested roots was determined after washing with distilled water to remove the medium and blotting with filter paper. Root growth was then expressed in Fresh Growth Index calculated using the formula given by Kittipongpatana *et al*. (1998).

$$\text{Growth Index (GI)} = \frac{\text{Final Fresh Weight-Initial Fresh Weight}}{\text{Initial Fresh Weight}}$$

Extraction and analysis of CRA

Sample preparation

The dried root powder of *L.speciosa* was accurately weighed (0.5 g), fluxed with methanol for 5hour at 65ºC. The extract was then transferred into a 25 ml volumetric flask which was made up to its volume with extraction solvent. The resultant solution was centrifuged (10000 g) for 15 minutes under 20ºC and the supernatant was filtered through 0.22 ìm Millex GP Filter Unit (M/s Millipore, Ireland) prior to injection into the HPLC system.

Thin Layer Chomatography (TLC)

Thin Layer Chromatography was used to confirm the presence of CRA in the extract. Extracts were spotted in a line on the readymade silica gel (TLC Silica gel 60 F254, M/s Merck) TLC plate (20cm×20cm) to develop in a developer. The developer used for TLC separation was mixture of $CHCl_3$: Methanol in 9.5:0.5 ratio. Subsequently, the plate was sprayed with the anisaldehyde reagent and then heated at 105! for 15minutes to visualise CRA.

High Performance Liquid Chromatography (HPLC)

HPLC determinations were carried out using LC-

Table 1. List of primers used for confirmatory PCR and RT-PCR of hairy roots

Sl. No	Gene	Sequence	Product size (in bp)
1	*rol* A	5' CAT GTT TCA GAA TGG AAT TA 3' 5' AGC CAC GTG CGT ATT AAT CC 3'	304
2	*rol* B	5' TCA CAA TGG ATC CCA AAT TG 3' 5' TTC AAG TCG GCT TTA GGC TT 3'	797
3	*rol* C	5' ATG GCT GAA GAC GAC CTG TGT 3' 5' TTA GCC GAT TGC AAA CTT GCA 3'	550
6	*vir* D1	5' ATG TCG CAA GGA CGT AAG CCG A 3' 5' GGA GTC TTT CAG CAT GGA GCA A 3'	450

2010CHT integrated system (M/s Shimadzu) equipped with quaternary gradient, autoinjector and UV- Visible detector in combination with Lab solution software. HPLC grade methanol, acetonitrile, orthophosphoric acid (M/s Sigma-Aldrich, USA) and water (M/s Sartorius Arium water purification system, Germany) were used for the analysis. CRA reference standard was purchased from M/s Sigma-Aldrich, USA**.** Analyses were carried out using the above system equipped with C-18 column (250mm x4.6mm) and UV detector set at wavelength ë=205 nm. The mobile phase with 0.1 % v/v orthophosphoric acid and acetonitrile were filtered through 0.45ìm nylon membrane filter (Millipore) and degassed by sonication for 30 minutes. CRA standard and *L. speciosa* extract were injected into the column at a flow rate of 1.6ml per minute. Identification of CRA in the extract was carried out by comparing their retention time with the standard. The estimation of CRA in the individual samples was calculated from the calibration curve prepared in the range of 0.5 to 100 µg/ml (r2=0.999). Each sample was analyzed thrice and mean value was taken for estimation of yield (w/w).

RESULTS AND DISCUSSION

Adventitious root culture development

According to Wang *et al.* (2013), adventitious root culture is the most attractive system for the production of biomass and commercially important metabolites. Since the concentration of auxin hormone is critical for organizing the root apical meristem for adventitious root growth, leaf explants were cultured on MS medium supplemented with various auxins such as IAA, IBA and NAA (Table 2). The

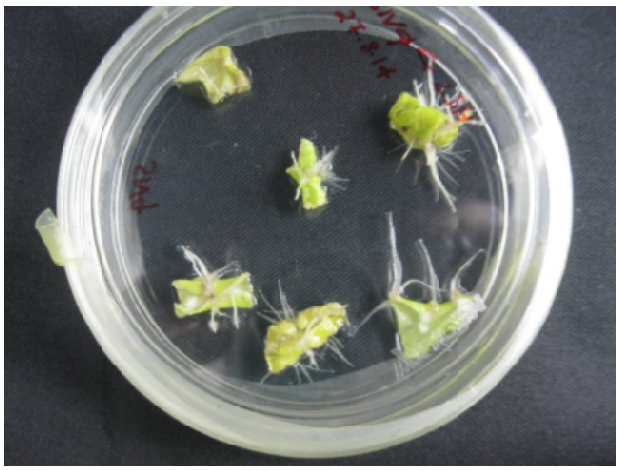


Fig. 1. Adventitious roots developed from *in vitro* leaves of *Lagerstroemia speciosa* on MS medium with 0.5 mg l^{-1} NAA

medium devoid of auxins failed to induce root formation. Explants cultured on MS medium containing auxins induced roots from the mid rib region within two weeks. NAA induced roots in all explants for all the concentrations tried except 0.1 mg l^{-1}. Maximum number of roots produced at a concentration of 1 mg l^{-1} i.e. 21 roots/explants. However, the number of roots induced, decreased when concentration of NAA became 2 mg l^{-1}. Callusing was observed when leaf explants were cultured on MS medium with IAA. However, IAA when supplemented at a concentration of 1 mg l^{-1} induced highest percentage of response and maximum number of roots. Above this optimum concentration,

Table 2. Effect of different concentrations of auxins on root induction from *in vitro* leaves of *L. speciosa* in MS medium

Growth regulators	Concentration (mg l^{-1})	% of response	No. of roots	Length of roots (cm)
0	0	0	0 .00±0.00^{i}	0 .00±0.00^{e}
NAA	0.1	60	6.33±1.45^{efg}	4.00±0.58^{bcd}
	0.2	100	10.67±0.88^{d}	3.67±0.33^{bcd}
	0.5	100	10.00±0.58^{de}	5.00±0.58^{bc}
	1.0	100	21.33±1.76^{b}	9.00±0.58^{a}
	2.0	100	5.67±0.88^{fgh}	2.33±0.33^{d}
IAA	0.1	10±5.8	1.33±0.33^{i}	3.67±0.88^{bcd}
	0.2	20±11.55	1.67±0.88^{i}	3.00±1.58^{cd}
	0.5	16.67±12.01	2.33±0.88^{hi}	4.00±0.58^{bcd}
	1.0	91.67±4.41	20.00±2.88^{b}	3.00±0.58^{cd}
	2.0	53.33±8.82	2.00±0.58^{hi}	2.67±0.33^{d}
IBA	0.1	6.67±0.33	1.33±0.33^{i}	2.33±0.88^{d}
	0.2	88.33±6.00	3.67±0.88^{ghi}	3.00±0.58^{cd}
	0.5	100	14.33±2.33^{c}	5.00±0.58^{bc}
	1.0	100	17.67±0.88^{def}	5.67±0.33^{b}
	2.0	100	**29.00±0.58^{a}**	**3.67±0.33^{bcd}**

Values represent mean values ± SE

Means followed by the same letters in each column are not significantly different at P < 0.05 according to Duncan's multiple range test (DMRT).

percentage of response and number of roots decreased. When IBA was supplemented with MS medium, the number of roots and percentage of root induction increased with an increase in the concentration from 0.1 to 2.0 mg l^{-1}. When the roots were subcultured to the same medium, maximum growth in terms of fresh weight was observed with 0.5 mg l^{-1} NAA. Beyond these concentrations of NAA, root growth was less and cells were released to the medium resulting in reduced biomass. Therefore, for further experiments roots developed from medium supplemented with 0.5 mg l^{-1} NAA (Figure 1) were selected. Earlier, Sivakumar *et al.* (2011), developed adventitious root cultures of *L. speciosa* from primary roots and leaves in MS medium supplemented with IBA under dark condition for CRA production.

Hairy root culture development

In the hairy root induction process, the selection of *Agrobacterium* strains, plant species (their explants) and the growth medium plays important role (Chandran and Potty, 2008). Earlier, Sivakumar *et al.* (2011) failed to induce hairy roots in *L. speciosa* and hypothesized that the antimicrobial activity of the plant may be hindering the growth of *A. rhizogenes*. Contrary to this, in the present study, hairy root was induced from the leaves of *L. speciosa* using A4 strain of *A. rhizogenes* (Figure 2). The difference in the *Agrobacterium* strain and the source of explant (*in vitro* leaves) used could be the reason for the success.

Fig. 2. Hairy roots developed from *in vitro* leaves of *Lagerstroemia speciosa* on MS medium

Standardization of bacterial strain

During standardization of suitable strain of *Agrobacterium rhizogenes* for induction and proliferation of hairy roots in *L. speciosa* leaves, significant variation ($P<0.05$) was observed in terms of transformation efficiency *i.e.* number of days required for hairy root induction and transformation frequency, in all the strains used in the present study (Figure 3). Only A4 strain was able to induce hairy root from *in vitro* leaves at 8-10 days of incubation. Maximum transformation frequency of 60% was also obtained with A4 strain. This is in agreement with the earlier reports that among the different types of *Agrobacterium* strains, Agropine type has the strongest root induction ability (Thimmaraju, *et al.*, 2005). Even though, strain R1022 was able to produce hairy roots, its transformation efficiency and frequency was poor. It took 14-18 days to induce hairy roots. So A4 strain was selected for further experiments. Similarly, strains LBA9402 and TR105 were unable to produce hairy roots in the leaves of *L. speciosa*.

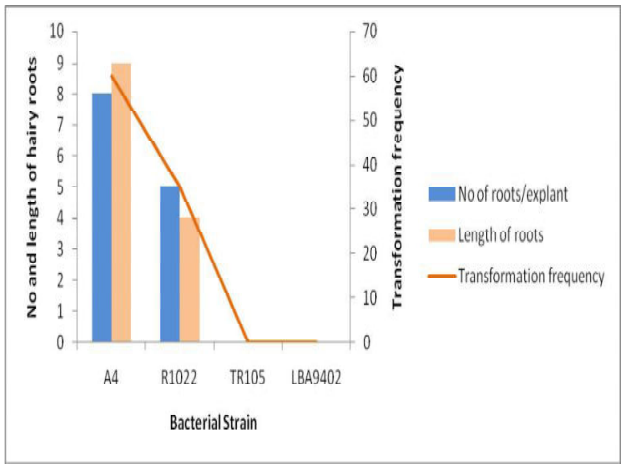


Fig. 3. Effect of different bacterial strain on hairy root induction from *in vitro* leaves of L. speciosa

Standardization of explant

Different parts of the *in vitro* leaves such as apical, middle and basal portions were used for transformation using *A. rhizogenes*. It was found that basal portion of the leaf with prominent midrib was more amenable for transformation than other parts (Figure 4). The possible explanation of this observation is that the phloem cells in midrib region which is supposed to have high sucrose and IAA might be the target for *A. rhizogenes*. Nilsson and Olsson (1997) hypothesized that the cells containing high level of auxin and sucrose are ideal targets for hairy root induction. Although middle portion with well developed midrib was also susceptible to transformation, hairy roots formed only from the wounded veins including midrib.

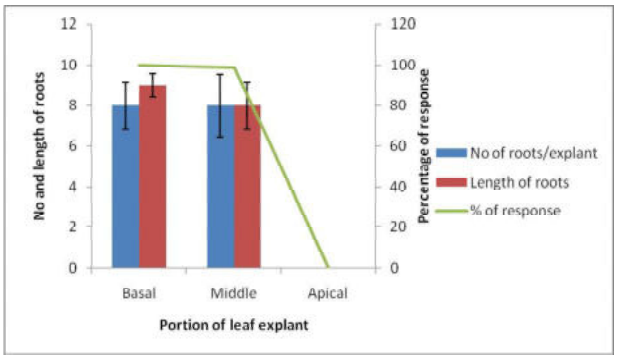


Fig. 4. Effect of leaf explant portion on hairy root induction from *in vitro* leaves of *L. speciosa*

Standardization of media

Different media composition comprising MS, B5 and SH media were tried to identify the best suitable medium for hairy root initiation. Transformation frequency and number of roots produced was found to be high in MS medium followed by SH and B5 media (Figure 5). Furthermore, MS medium sustained better growth than others. Azlan *et al.* (2002) showed that hairy root growth was strongly affected by medium formulation. MS medium with varying salt concentrations were checked for growth of hairy roots. After a period of 30 days, highest root length was observed in quarter strength MS medium, but branching was absent.

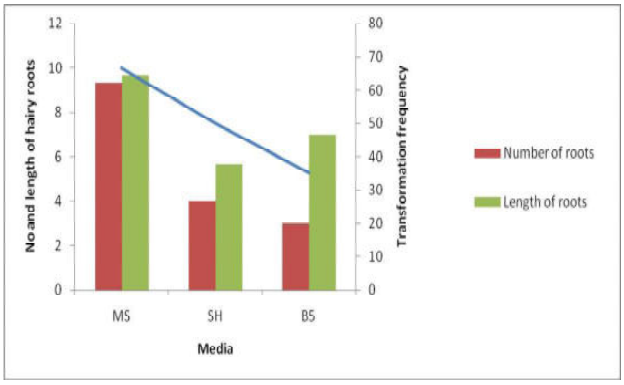


Fig. 5. Hairy root initiation in different media

However, remaining concentrations of MS like ½ MS, ¾ MS and full MS all triggered branching. As more branching was observed in MS medium with full strength salt concentration, it was selected for further experiments.

Hairy root confirmation

PCR

PCR analysis using the *rol* gene primers provided molecular evidence for the transgenic nature of the hairy root culture. Plasmid DNA and genomic DNA from hairy roots showed amplification of all the *rol* genes whereas no bands were observed in lane containing DNA from non-transformed roots. The PCR products for *rol*B regions yielded the expected 797 bp (Figure 6). Absence of *vir*D1 gene eliminated the possibility of false positive PCR due to *A. rhizognes* contamination on the hairy roots.

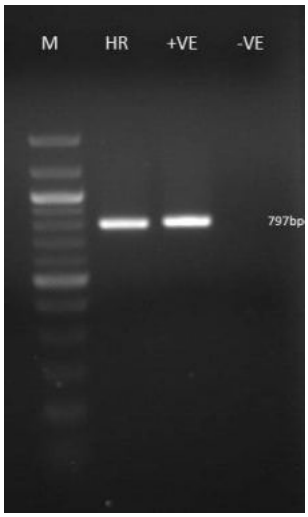


Fig. 6. PCR amplification obtained for *rol* B primer; M- DNA ladder, HR- DNA from hairy root, +VE- Plasmid DNA, -VE- DNA from adventitious root

Reverse Transcription PCR

cDNA from hairy roots and plasmid DNA showed amplification of the *rol* genes. PCR products for *rol* A, B and C were of expected size i.e. 304, 797 and 550 bp. This result indicated that the integrated genes from bacterial plasmid expressed in the hairy roots.

Comparison of Fresh Growth Index and CRA content of adventitious and hairy root cultures

Growth index (GI) of the roots was determined as explained in materials and methods, after 30 days of incubation. Growth was more in hairy roots than adventitious roots (Table 3). In both types of root cultures, presence of CRA was determined using TLC (Figure 7) and quantification was done by HPLC (Figure 8). The root cultures were found to be good sources of CRA, despite the fact that the content is negligible in field grown roots of *L. speciosa*. This is in line with the observation made on another member of Lythraceae family *viz., Lawsonia inermis* by Bakkali *et al*. (1997).

Table 3. Comparison of Growth Index and CRA content of hairy and adventitious root cultures of *L. speciosa*

Sample	Growth Index (Initial FW = 0.2 g)	CRA content (%)
Hairy root	1.6	0.127
Root from *in vitro* leaf in MS medium containing 0.5 mg l^{-1} NAA	1.125	0.169

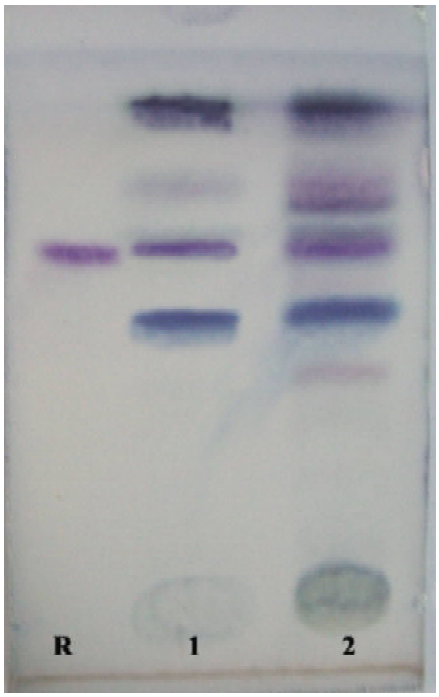


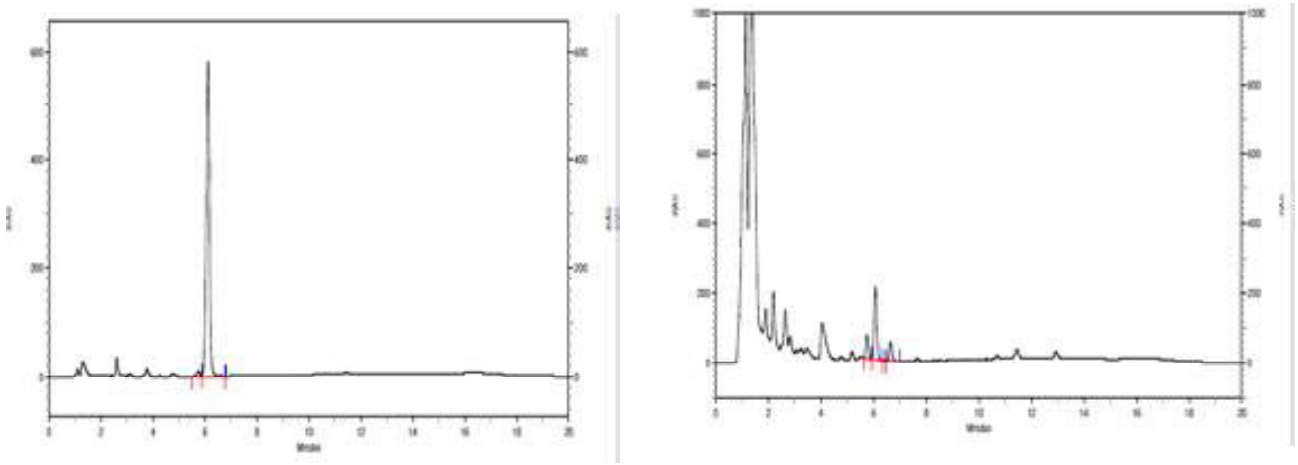
Fig. 7. TLC plate developed using anisaldehyde reagent

R- CRA Reference Sample

1. Extract from adventitious root 2. Extract from hairy root

CONCLUSION

The root cultures of *Lagerstroemia speciosa* developed herein could synthesise CRA. Hence they would be highly suitable as an *in vitro* production system for



2a. Chromatogram of CRA Reference sample

2b. Chromatogram of one of the test sample

Fig. 8 HPLC chromatogram of CRA reference sample and one of test sample

consistent year round supply of CRA. Further optimization for increased production of CRA is underway to find commercial usefulness.

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