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Effect of Media on The Production Of Oleanolic Acid in *Lantana camara* Hairy Root Cultures

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ABSTRACT
The present study was on the accumulation of Oleanolic acid (oleanolic acid) in hairy root culture of Lantana camara using leaf disc explants. Lantana camara hairy roots were induced using Agrobacterium rhizogenes strain A4. Effect of full and half strengths of (1/2 MS,1/2 B5) Morishige and Skoog's media (MS) and Gamborg media (B5) on biomass accumulation and oleanolic acid production were investigated. Biomass of Lantana camara hairy roots was the highest (2.93± 0.12 g/flask of fresh weight and 0.48±0.02 g/flask of dry weight) in the cultures grown on 1/2 MS medium. HPLC analysis also revealed the highest oleanolic acid content (30.24±0.02 mg/g dry wt) in roots cultured in ½ MS medium under dark conditions. The yield of oleanolic acid was about 10 times higher compared to the roots of field grown mother plants. This was the first report on the production of oleanolic acid in Agrobacterium rhizogenes strain A4 induced hairy roots of Lantana camara plants grown on ½ MS medium.
Key words: *Hairy roots, Oleanolic acid, Lantana camara, Agrobacterium rhizogenes, Ms medium, B5 medium*

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INTRODUCTION

Lantana Camara plant (Verbenaceae), is mostly native to subtropical and tropical America, but a few taxa are indigenous to tropical Asia and Africa. It is a weed and ornamental plant, can grow in any type of soil and climate. The plant is source for triterpenes and flavones and the roots are rich in Oleanolic acid (oleanolic acid). The root decoction of the plant is used to treat stomach ache and vomiting in infants in many parts of the world. Oleanolic acid (3β-hydroxy-olea-12-en-28-oic acid) is a pentacyclic triterpenoid an aglycone of triterpenoid saponins.[3]

Oleanolic acid exhibits anti-inflammatory, anti-hyperlipidaemic, anti-viral and anti-tumour properties. The demand for Oleanolic acid and its derivatives is very high, but Oleanolic acid from plant source is limited. Tissue culture technology is adopted as alternative source for Oleanolic acid [5]. Priyanka Srivastava *et al*., [1] reported Oleanolic acid production in cell cultureby continuous growth and root culture is more efficient compared to the cell culture due to fast growth and stable metabolite production. Genetically transformed hairy roots, produced by infection of *Agrobacterium rhizogenes* is an attractive option for the production of high biomass and secondary metabolites and genetic stability compared to cell culture technology[7]. Oleanolic acid production has not been reported in hairy root cultures of *Lantana camara*. The present study is aimed at development of hairy root culture of *Lantana camara* for enhanced production of Oleanolic acid.

MATERIAL AND METHODS

Establishment of Hairy root culture:

Lantana camara leaves were collected and sterilized used as explant for Agrobacterium mediated transformation. After making incisions mechanically with scalpel the leaves were infected by incubating with bacterial suspension on a shaker. Control explants were wounded similarly using scalpel. The inoculated and control explants were incubated in the dark at 26º C on MS agar medium without growth regulators. Leaf explants bearing hairy roots were excised and transferred to 25 ml of half strength MS basal media with Cefotaxime (500 mg/L) for the elimination of bacteria. The cultures were maintained in the dark on a rotary shaker at 100rpm. After fifth subculture (1 week each), the concentration of Cefotaxime was decreased to 250 mg/L. After the successive five transfers Cefotaxime was eliminated from the medium. After the subcultures, hairy roots were transferred separately in to 300

ml Erlenmeyer flasks containing 80 ml of liquid media: MS, B5 with full and half strengths. All the media contained 3% sucrose. The pH of the media was adjusted to 5.6-5.9 and the media were sterilized at 121ºC in an autoclave for min. The cultures were maintained in the dark on a rotary shaker at 100 rpm. Subcultures were carried out for every 4 weeks. After that hairy roots were collected and determine the fresh and dry weights of the hairy roots. Three flasks were used for each medium and experiments were performed in triplicate. Fresh and dry weights were expressed as g/flask [10-12].

Roots from the plant:

Lantana camara roots were harvested, washed with water and used for the extraction of oleanolic acid. The roots served as control for the comparison of oleanolic acid content in transformed roots of *Lantana camara* [15].

Preparation of plant Extracts:

Lyophilized and powdered plant material (150 mg) (transformed roots and roots of 6-months old intact plants grown in the field) were extracted thrice with methanol (25ml) for 10 min at room temperature using ultrasonic bath. After filtration, the extracts were combined and evaporated to dryness under reduced pressure [8].

The residue was dissolved in methanol (10 ml). About 1 ml of methanolic extract was centrifuged at 18,000 rpm for 3 min. The supernatant was analysed by using High performance liquid chromatography (HPLC).

Estimation of oleanolic acid:

Quantitative estimation of oleanolic acid was carried out on HPLC system using an, LC-20AD (Shimadzu corporation, Kyoto, Japan), equipped with SPD-20A, UV detector set at wavelength 210 nm and a 20 ul injection loop. Separation was performed on a C18 column (250 x 4.6 mm, 5µm) using methanol: water (80:20 v/v) as mobile phase at ml/min flow rate.

oleanolic acid in hairy roots was detected by comparing retention time of hairy root culture produced Oleanolic acid with that of the standard oleanolic acid. The experiment for production of oleanolic acid from hairy root cultures and untransformed roots was repeated thrice on each medium and at different culture conditions. oleanolic acid content of the analysed samples was expressed as milligram per gram of dry weight of biomass [17].

RESULTS AND DISCUSSION

In the present study, hairy roots of *Lantana camara* were generated by infecting the leaves with *Agrobacterium rhizogenes* strain A4. After 4 weeks growth the hairy roots were evaluated for the detection of oleanolic acid. A few hairy roots were induced from the petiole portion of the leaf explants following inoculation with A.rhizogenes A4. Cream coloured hairy roots were induced within 12-15 days after inoculation, the roots were incubated for 30 days [fig-1]. Hairy Roots were not observed in uninfected control explant. Transformed nature of culture was confirmed by typical morphological characters of hairy roots like faster growth on hormone independent medium after excision from explants, extensive lateral branching and plagiotropism.

Four weeks old hairy roots from liquid medium were harvested and dried. Roots excised from inoculated leaves of Lantana camara were transferred on to hormone free half strength MS medium with Cefotaxime (500 mg/l) initially, which was gradually reduced during subsequent subcultures. After 10 subcultures the produced roots were used for the estimation of oleanolic acid. Hairy roots were observed in transformed roots but not in untransformed roots, obtained from intact plants of *Lantana camara*. The effect of various culture media on root biomass of *Lantana camara* and oleanolic acid content were examined.

The media studied were full and half strength MS and B5. After four-week period in all tested media enhanced biomass production was observed. Therefore, the study was planned for 28 days. At the end of 4th week, the root biomass found to be highest in ½ MS medium i.e FW 2.93±0.12g/flask,DW 0.48 ±0.02 g/flask[Table 01].

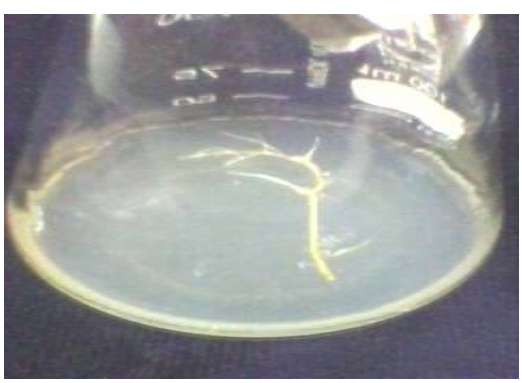
The methanolic extract was analysed for qualitative and quantitative analysis of oleanolic acid by RP-HPLC [fig 2-6]. oleanolic acid content found to be 30.24 ±0.02 mg/g dry wt in ½ MS media[Table]. Compared with field grown mother plant oleanolic acid content(3 mg/g dry wt) found in ½ MS media tenfold higher.

This implies that transformed cultures can be potential alternate for secondary metabolite production than untransformed culture systems and especially for root derived secondary metabolites. The present protocol could provide a sustainable alternative source for oleanolic acid.

Table 01.Effect of different media on the growth of biomass and Oleanolic acid production in *Lantana camara* hairy roots

Medium	Fresh wt(g/flask)	Dry wt(g/flask)	oleanolic acid content (mg/g dry wt)
MS	2.64±0.08	0.30±0.17	28.74±0.43
½ MS	2.93±0.12	0.48±0.02	30.24± 0.02
B5	1.16±0.03	0.15±0.11	16.56±0.12
1/2 B5	1.56±0.10	0.18±0.16	18.56±0.81

The values were expressed as mean of triplicates ± SE


A


B

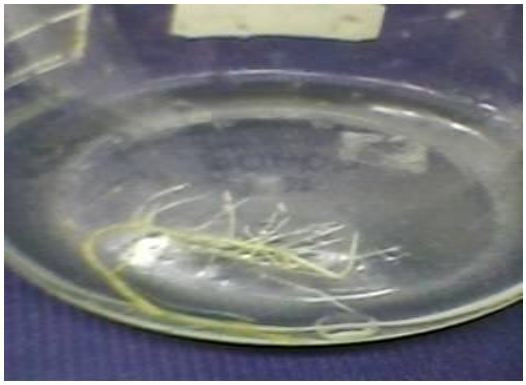

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Fig 1: Hairy roots induced from leaf explants.
(A) Excised hairy root on solid medium (1/2 MS medium)
(B) Proliferation of hairy roots on solid medium
(C) Hairy roots in liquid medium

a) HPLC chromatogram of oleanolic acid in methanolic extract of hairy roots of *Lantana camara* (MS media) fig-2
b)HPLC chromatogram of oleanolic acid in methanolic extract of hairy roots of *Lantana camara* (B5 media) fig-3
c)HPLC chromatogram of oleanolic acid in methanolic extract of hairy roots of *Lantana camara* (1/2 B5 media) fig-4

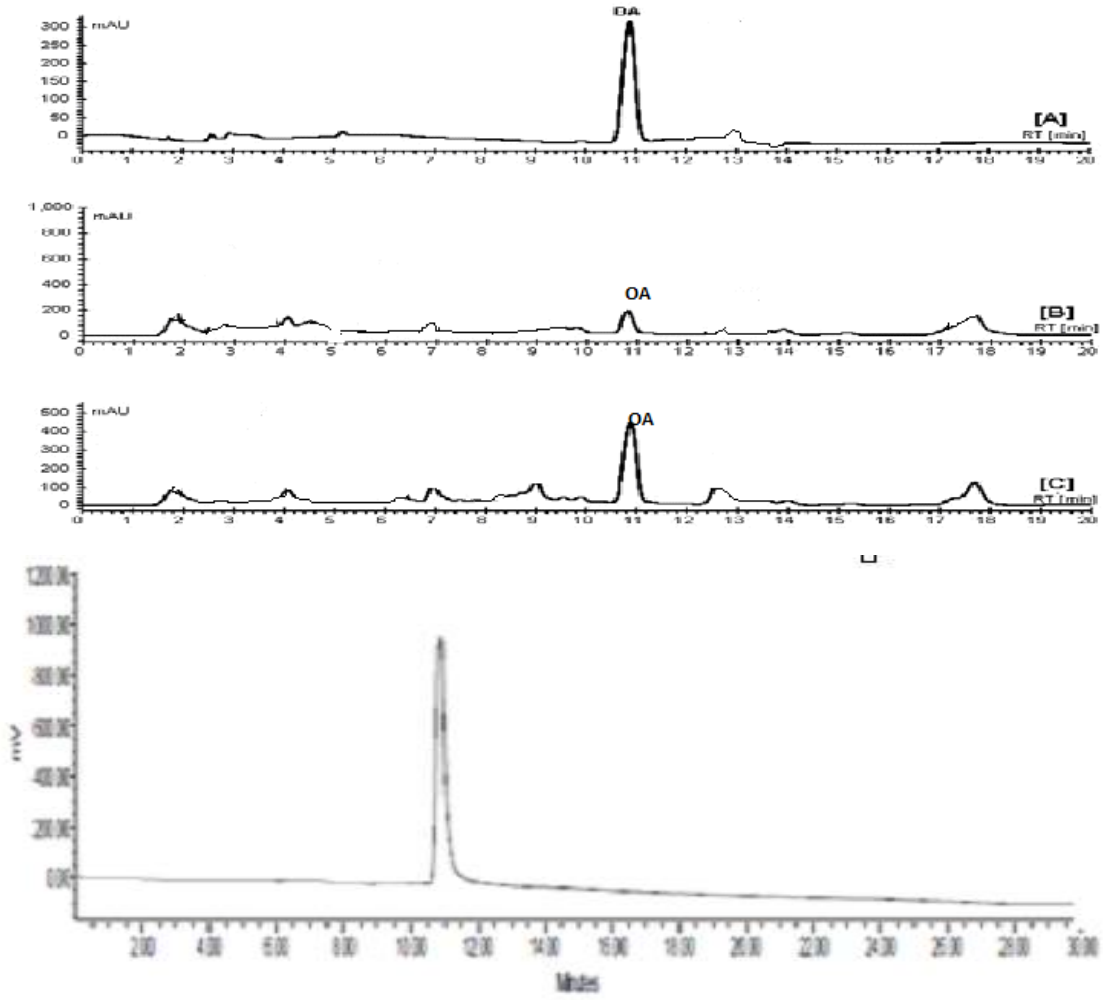

Fig-5 Standard HPLC chromatogram of oleanolic acid

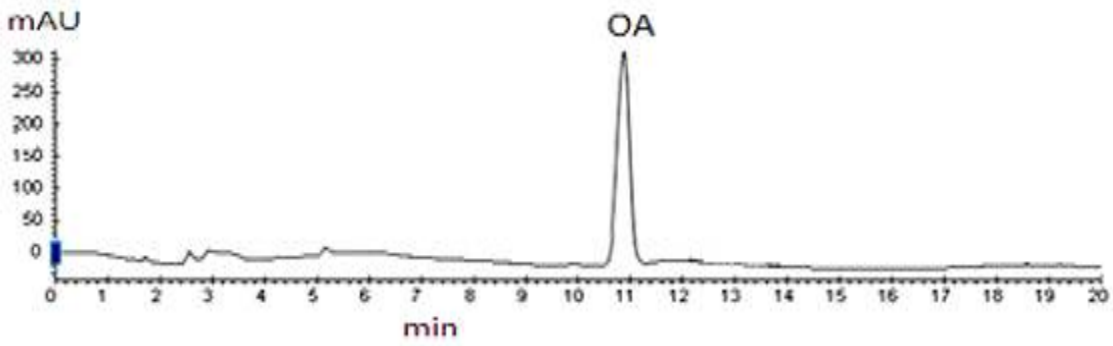

Fig-6 HPLC chromatogram of oleanolic acid in methanolic extract of hairy roots of *Lantana camara* (1/2 MS media)

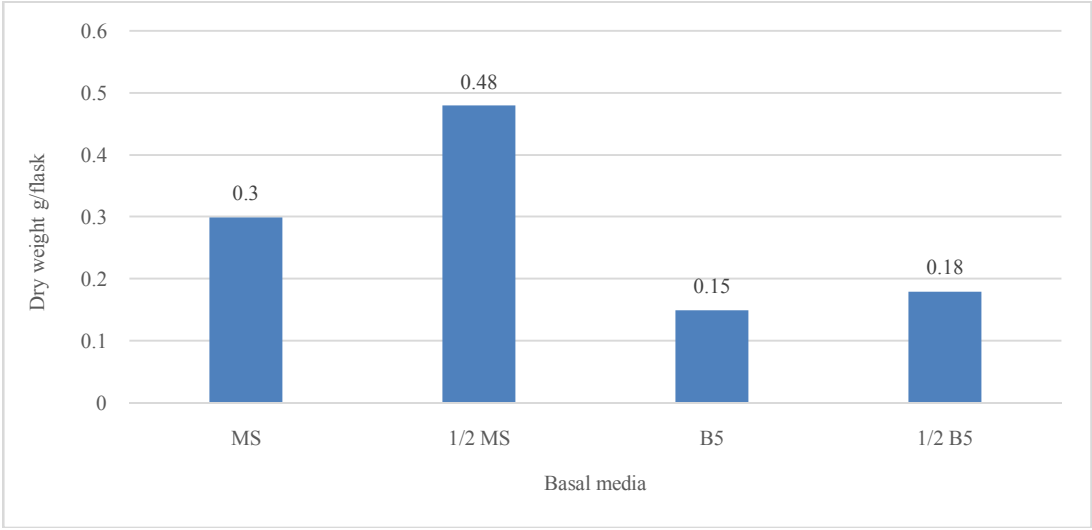

Fig-7 Hairy root growth, expressed as Dry weight(g),after 30-days culture on different basal media of Lantana camara

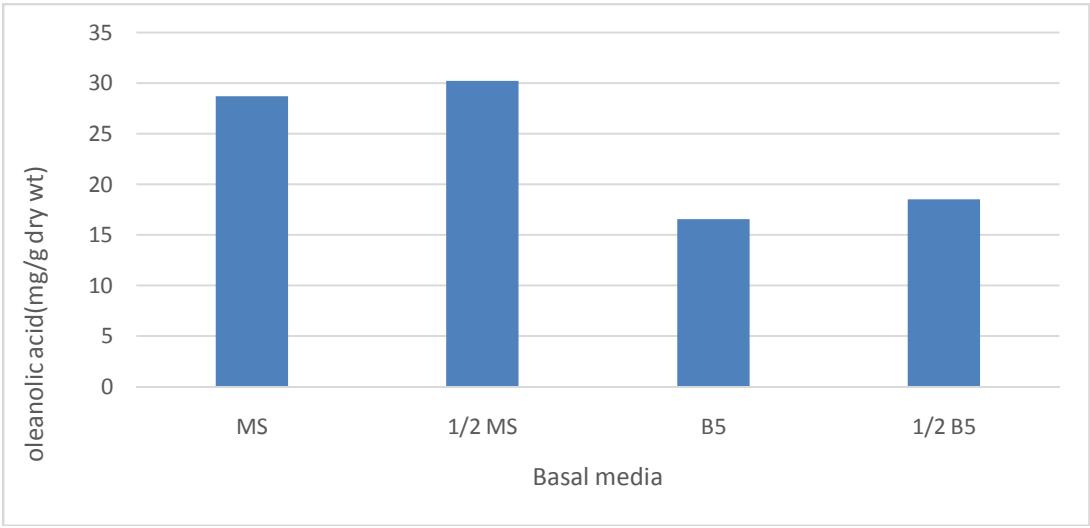

Fig-8 Different media effect on accumulation of oleanolic acid in hairy root culture of Lantana camara

CONCLUSION

The present investigation showed that 1/2 MS medium was best for enhanced biomass production as well as for increased oleanolic acid production in hairy root cultures of *Lantana camara*.

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