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Suspension Culture Studies To Observe Secondary Metabolite Content In *Hyptis suaveolens*

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ABSTRACT

In recent years plant cells isolated and cultivated *in vitro* have potential to produce and accumulate medicinally important secondary metabolites by the plant from, which they originate. In the present investigation an attempt was made to develop the protocol to study the accumulation of secondary metabolites in *in vitro* raised samples of *Hyptis suaveolens*. During the studies, different combinations and concentrations of growth hormones were standardized to induce callusing, to obtain friable callus and for suspension culture. Different combinations of NAA, BAP and kinetin were tried to obtain the results. The MS and LS medium containing 1 mg/l BAP and 1 mg/l NAA was used to obtain the suspension culture from the friable callus. © 2007 Trade Science Inc. - INDIA

KEYWORDS

Cotyledons;
Callus culture;
Hypocotyls;
Growth hormones.

INTRODUCTION

Hyptis suaveolens Poit., pounded and applied to parasitical cutaneous diseases. In Brazil infusion used as carminative and as sudorific in catarrhal conditions^[1]. Anti-inflammatory and free radical scavenging activity of *H. suaveolens* was studied and concluded that it is endowed with significant anti-inflammatory activity^[2].

Recently in Japan, aqueous and methanolic extracts of 39 Panamanian medicinal plants were tested for HIV-induced cytopathic effects in cultured cells, HIV- reverse transcriptase(RT) and HIV-protease(PR) enzymes; found that water extract of the aerial parts of *H. suaveolens* were found as potent inhibitors of HIV- RT^[3,4].

There is an increasing commercial interest in the large-scale tissue culture of certain plant species, which are difficult to obtain in sufficient amounts and quality.

Most important of all, production in the laboratory or factory can be geared to demand (in terms of quantity and timing), yielding product of assured, standard quality. Some promising results have been obtained in the production of glycosides, alkaloids, steroids and anthraquinones, but still there are many taxa for exploitation.

Hence considering today's increasing demands for biologically active constituents or natural products the present investigation is proposed.

MATERIAL AND METHODS

To raise seedlings aseptically, seeds were treated with 70% alcohol for 1 min., followed by thorough washing with distilled water. The seeds were surface sterilized with 0.1% (w/v) HgCl₂, for 2-3 min. after subjecting to repeated washing for 3-4 times with sterilized

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double distilled water; seeds were cultured in sterile petriplates with filter papers soaked in tap water. These were incubated for 16hr photoperiod. Within three days seedlings attained 3-4cm height, root tip, hypocotyls, cotyledon and apical buds were used as explants.

The explants obtained were sterilized using same method mentioned above for seed sterilization. The process was carried out in laminar air-flow cabinet after proper U.V. sterilization. Subsequently different explants were cultured on MS medium^[5] containing 3.0% sucrose(AR) as carbon source and 0.8% agar(Hi-Media Lab., Mumbai, India) for gelling. This served as a basal medium. MS medium supplemented with various growth regulators in various combinations were used for the study. The pH of the medium was adjusted to 5.8 using 0.1N NaOH or 1N HCl before autoclaving, 20ml of hot medium was dispensed into each rimless culture tube(25mm×150mm). Each tube was plugged with non-adsorbent cotton wrapped in cheese cloth and sterilized by autoclaving at 120° for 15min at 15lbs. cultures were maintained at 25±2°C under a 16hr photoperiod(2000lux) provided by cool white fluores-

cent lamps. Each set with different combination of growth hormone was performed in triplicates. The cultures obtained were subculture on the same medium.

Suspension cultures were established by suspending 2g of fresh callus in 100ml LS^[6] and MS media in 250ml Erlenmeyer flasks. Cells were sub cultured after every 18 days and grown on a gyratory shaker at 100rpm at 25°C in the dark. Cultivation was performed in a LS nutrient medium supplemented with 50g/l, 60g/l, 70g/l and 80g/l sucrose, under the conditions described above to study the effect of sucrose. The results were compared with those on cultivation on standard LS media with 30g/l sucrose(control).

The growth in a suspension culture was measured by determination of cells fresh and dry weights. The sample of cells was collected on pre-weighed nylon fabric in a Hartley funnel to determine fresh weight and washed with sterile water to remove the medium, then drained under vacuum and again weighed. To determine cell dry weight the nylon together with cells, was dried for 12hr at 80°C, cooled in a desiccators and weighed to constant weight and expressed as weight

TABLE 1 : Effect of different concentrations and combinations of auxins and cytokinins on different types of explants on the type of callus in *Hyptis suaveolens*

Media	Hormones			Cotyledons	Hypocotyl	Root	Apical bud
	NAA	BAP	Kn.				
1	0.1	-	0.5	Compact green callus	Compact green callus	No response	Compact green callus
2	0.3	-	0.5	do	do	do	do
3	0.5	-	0.5	do	do	do	do
4	1.0		0.5	Initially friability observed but after few days it becomes compact	do	do	do
5	1.0	0.1	-	Compact green callus	do	do	do
6	1.0	0.3	-	do	do	do	do
7	1.0	0.5	-	do	do	do	do
8	1.0	1.0	-	Beige colour friable callus observed with less response	Beige colour friable callus observed but only 10% growth	Greenish white callus	do
9	0.2	1.0	1.0	Compact green callus	Compact green callus	No response	Maximum multiple shoots
10	0.5	1.0	1.0	Dark green callus with few shoots	Callus with few shoots	No response	Regenerating callus with few shoots
11	1.0	1.0	1.0	Compact green callus with few shoots, after few days turns brown	Compact green callus with formation of few shoots	do	do
12	1.0	0.5	1.0	Compact callus with few shoots	Compact callus with few shoots	do	do
13	1.0	0.2	1.0	Compact callus with few shoots with rhizogenesis	Compact callus with few shoots with rhizogenesis	do	do

Note – In all combinations of 2, 4 – D and Kn. browning was observed

per ml culture. Fresh and dry weights of the cultures were measured.

RESULTS

In *H. suaveolens*, the better response for callusing was found in explants of cotyledonary and hypocotyls region of 3 days old seedlings. The callusing was induced after 10 days of inoculation. The explants from apical bud region showed shooting.

Browning of callus mass was observed in all combinations of 2,4-D and Kn. Therefore it was replaced with NAA and MS media with different combinations of NAA along with Kn. were tried.

In the three combinations of NAA and Kn. viz., 0.1mg/1NAA, 0.3mg/1NAA and 0.5mg/1NAA with 0.5mg/1Kn. formation of compact callus with induction of shooting was observed after three weeks. In MS medium containing 1.0mg/1NAA and 0.5mg/1Kn. initially slight friability was seen in the callus obtained from cotyledonary explants. Therefore, 1.0mg/1NAA concentration was kept constant and effect of different concentration of NAA and BAP along with NAA, Kn. and BAP was studied as tabulated in TABLE 1. Cotyledonary and hypocotyls explants inoculated on MS media containing 1.0mg/1 BAP and 1.0mg/1 NAA resulted in friable callus after two weeks with only 10% growth of callus (Plate 1).

On the MS medium containing 1.0mg/1 BAP and 1.0mg/1 NAA, friable callus was obtained. The callus obtained from hypocotyls and cotyledonary explant was subcultured after every twenty days and maintain under aseptic conditions. The yield as fresh weight from cotyledonary callus was 2g./10 tubes and callus from hypocotyls was 2g/15 tubes. While the yield from suspension on the same medium i.e. MS media containing 1.0mg/1 BAP and 1.0mg/1 NAA. The MS and LS medium containing 1.0mg/1 BAP and 1.0mg/1 NAA was used to obtain the suspension culture from friable callus mass of cotyledonary explants and successfully maintained for four months. Variations in colour of suspension cultures grown in MS and LS medium were observed (Plate 2).

After maintaining for six months the callus was harvested culture maintained in both MS and LS medium was more or less similar i.e. up to 24.76g. fresh weights.

During HPTLC analysis, small traces of PCA (chlorogenic acid) (Rf~0.4-0.5) was observed in the extract obtained from callus mass of *H. suaveolens* cotyledon

figure 1. Callus obtained from cotyledons of *H. suaveolens* show presence of scopoletin (Rf~0.31) in low concentration figure 2.

DISCUSSION

The work done by some scientists on this aspect of secondary metabolites production in the family lamiaceae are discussed below. Preliminary evidence of a rosmarinic acid peroxidase in cell cultures from lavender (*Lavandula × intermedia*) flowers was reported by^[7]. Spectrophotometric determination of rosmarinic acid in plant cell cultures by complexation with Fe²⁺ ions was done^[8]. Stimulation of accumulation of terpenoids by cell suspension of



Plate 1 : Friability induced in callus obtained from cotyledonary explant in *Hyptis suaveolens*



Plate 2 : Variation in color of suspension culture (MS and LS media) from cotyledonary callus of *Hyptis suaveolens*

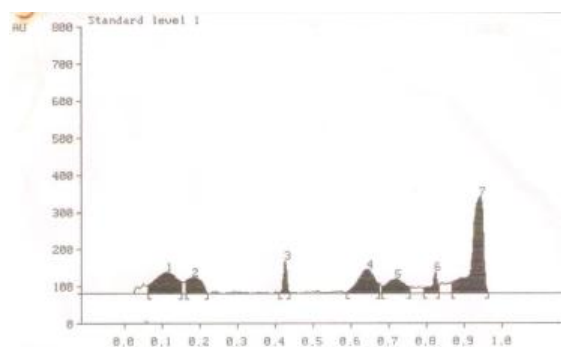


Figure 1 : Chromatogram of methanolic extract of callus from *hyptis suaveolens* cotyledon (for rutin, chlorogenic acid and ferulic acid)

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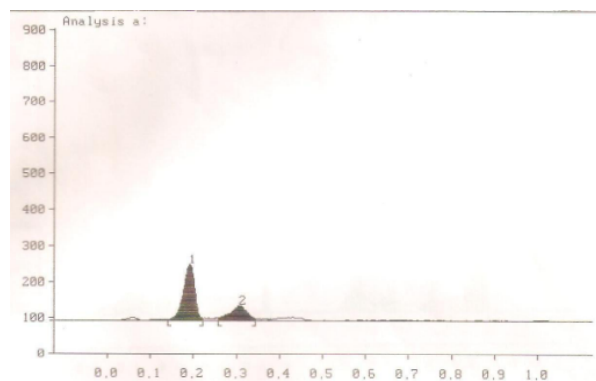


Figure 2 : Chromatogram of methanolic extract of *hyptis suaveolens* cotyledon (for emodin and Scopoletin)

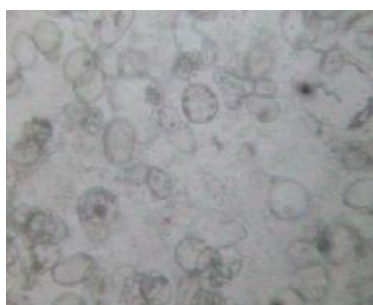


Plate 3 : Cells from suspension culture of *Hyptis suaveolens* (8 days old after inoculation in fresh medium)

Lavradula angustifolia following pre-treatment of parent callus was studied^[9]. The growth of *Lavradula vera* MM cell suspension and biosynthesis of rosmarinic acid were followed during their cultivation in LS media^[10].

The membrane bound cytochrome P450- dependent hydroxylase which are involved in the biosynthesis of rosmarinic acid have been characterized in microsomal preparations from cell cultures of *Coleus blumei*^[11]. Recently a review under the head of "Biotechnology to harness the benefits of phenolics: focus on lamiaceae" was presented^[12].

Benzothiadiazole enhances the elicitation of rosmarinic acid production in a suspension culture of *Agastache rugosa* O.kuntz^[13].

Recently, *in vitro* rosmarinic acid accumulation in leaf derived suspension cultures of sweet basil (*Ocimum basilicum* L.) was observed^[14]. Most of the earlier reports discussed above indicate accumulation of phenol carboxylic acid i.e. rosmarinic acid, carnosic acid and caffeic acid in suspension cultures of lamiaceae members, tentative evidence of accumulation of PCA in callus as well as suspension cultures were observed during the present investigation. Hence there remains need

for further work.

ABBREVIATIONS

NAA-naphthalene acetic acid, Kn.-kinetin, KR- killo radicals, HgCl₂-mercuric chloride, CO⁶⁰-cobalt 60, AR-analytical reagent, NaOH-sodium hydroxide, PCA-phenol carboxylic acid, etc.

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