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Research Article

In vitro Propagation and Comparative Phytochemical Analysis of Wild Plant and Micropropagated *Cleome rutidosperma* DC.

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ABSTRACT

Plants are widely used by all sections of the society either as folk medicines or as pharmaceutical preparation of modern medicine. *In vitro* propagation of plants holds great promise for conservation and enhancement of valuable medicinal plants. *Cleome rutidosperma* has been used in indian ayurvedic medicine for the treatment of a wide number of health disorders. The present study deals with the influence of different plant growth regulators (PGR) including kinetin (Kin), 6-Benzylaminopurine (BAP) and 2, 4-Dichlorophenoxyacetic acid (2,4-D) on the growth of plant and the identification and comparison of bioactive constituents of wild and *in situ* propagated *C. rutidosperma* plant using Gas Chromatography - Mass Spectrometry analysis (GC-MS). Nodal segments used as explants were cultured on Murashige and Skoog's medium (MS) supplied with different concentrations of PGRs. Multiple shoot generation was achieved after 28 days of incubation. The GC-MS analysis showed the presence of ten compounds of micropropagated and seven compounds of wild plants were identified. The result concluded that various concentration of PGR had a significant role in *in vitro* regeneration of plant and showed that the phytoconstituents of micropropagated plant is comparatively higher than that of wild plant.

Keywords: Micropropagation, Nodal Explant, MS Medium, Kinetin, Benzylaminopurine, *C. rutidosperma*.

INTRODUCTION

Medicinal plants are the important bio-resource in traditional systems of medicine; and are valuable source of industrially important natural products which includes flavors, fragrances, essential oils, pigments, sweeteners, feed stocks, antimicrobials and pharmaceuticals[1]. Utilizing natural products from medicinal herbs as phytoremedy in the treatment of many diseases and several infection is an age-old practice. The long-term availability of many herbs have become uncertain due to indiscriminate exploitation by human and other natural means resulting in possible threat of extinction[2] and the efforts made to cultivate these herbs have met with less fortune. Tissue culture offers the means for rapid and mass multiplication of existing stock of germplasm and also a method for conservation of important, elite endangered plants[3]. Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions, often to produce the clones of plants[4]. The development of tissue culture technology holds great promise for conservation and enhancement of valuable medicinal plants[5]. The decision to propagate medicinal plants using tissue culture depends on the plant part collected, level of threat in the wild, market demand and the quality of transplants for propagation[6]. Micropropagation is an alternative technique for large-scale production of disease-free plantlets. It has superiority over conventional method of propagation because of high multiplication rate. *C. rutidosperma* DC. belonging to the family Cleomaceae which is a low-growing herb, up to 70 cm tall with trifoliate roots and small violet-blue flowers, which turn pink as they age, found in marshy and grassy places[7,8]. The elongated capsules display the asymmetrical, dull black seeds. The plant is native to West Africa, from Guinea to Nigeria, Zaire and Angola. It has become naturalized in various parts of tropical America as well as Southeast Asia[7]. *C.rutidosperma* is traditionally used in the treatment of paralysis, epilepsy, convulsions, spasm, ear ache, skin disease and analgesic, antipyretic, anti-inflammatory, antimicrobial, diuretic, laxative, antioxidant, and antiplasmodial activities[9]. Mass spectrophotometry coupled with chromatographic separations such as Gas chromatography (GC-MS) is normally used for direct analysis of components existing in traditional medicines and medicinal plants[10]. Hence, the aim of the present study was to develop high frequency multiple shoots regeneration of *C. rutidosperma* utilizing the least number and various concentrations of PGRs under aseptic laboratory condition and to compare the phytochemical constituents between chloroform extract of micropropagated and wild plants to ascertain the rationale for its use in traditional medicine.

MATERIALS AND METHODS

Collection of Plant Material

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Figure 1a


Figure 1b


Figure 1c

Table 1: Effect of different concentrations of BAP, KIN and 2,4-D in MS medium on multiple shoot induction from nodal explants of *C. rutidosperma*.

Hormone Con.(mg/L) BAP + Kin + 2,4D	Shoot length (Mean ± S.D)	No. of shoots/explants (Mean ± S.D)
0.5 + 0.15 + 0.5	2.33 ± 0.57	4.52 ± 0.49
1.0 + 0.20 + 1.0	2.33 ± 0.57	7.44 ± 0.39
1.5 + 0.25 + 1.5	1.66 ± 0.57	6.45 ± 0.43
2.0 + 0.30 + 2.0	2.00 ± 1.00	4.36 ± 0.51
2.5 + 0.35 + 2.5	2.33 ± 0.57	5.43 ± 0.40
3.0 + 0.40 + 3.0	1.66 ± 0.57	7.02 ± 0.26

Medium: MS+ additives; mean± SD, n= 6 replicates

The healthy plants of *Cleome rutidosperma* DC. were collected during the month of January from the natural habitats of Kancheepuram district, Tamil Nadu, India. The plant specimen was identified and authenticated by Botanical Survey of India (BSI) Coimbatore, Tamil Nadu, India.

Sterilization of Explant

The nodal segment of the plant was chosen as explants for the present investigation. Actively growing shoots were selected as the source for explants. The explants were pre-sterilized by washing with running tap water to remove the dust particles from the surface. The explants were then wrapped in 25% (v/v) Clorox containing three drops of tween 20 solution for 10 min and again rinsed several times with sterile distilled water until all traces of Clorox were eliminated. Surface sterilization of explants were carried out by rinsing it with 0.01% mercuric chloride ($HgCl_2$) for 3 minutes and then washed 3 times with sterile distilled water[11].

Inoculation in culture medium

The nodal segments were cut into 5 mm in size and carefully transferred to the sterile MS basal medium (pH 5.8) supplemented with 3% (w/v) sucrose, 0.8% agar and different concentration [Table.1] of PGRs such as kinetin (Kin), Benzylaminopurine (BAP) and 2, 4-Dichlorophenoxyacetic acid (2,4D). The inoculated cultures were maintained in growth chamber with regulated temperature (26±2ºC), relative humidity (55±5%), light conditions 16/8 hours photoperiod and 3000 lux intensity of constant light was provided in culture shelves by cool-white fluorescent tubes. Data was recorded after 4-6 weeks[12].

Preparation of Solvent extraction

Wild and Micropropagated plant

The whole wild and micropropagated plants were washed thoroughly in sterile distilled water. The plants were shade dried and ground to fine powder using mortar and pestle. One gram (dry weight) of powdered extract was soaked in 10 ml of ethanol for 3 hours and sonicated in an Ultrasonic Sonicator at 20 pulses for 20 min. The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was freeze-dried and stored at 4ºC until further use[13].

Gas Chromatography- Mass Spectrometry Analysis

GC-MS analysis of the Ethanol extract of *C. rutidosperma* was performed in a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused a capillary column (30 × 0.25 µm ID × 0.25 µm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2µl was employed (a split ratio of 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min to 200°C, then 5 °C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC-MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and

Table 2: Phytocomponents identified in the ethanol extract of *C. rutidosperma* (wild).

S. No.	Retention Time	Name of the compound	Molecular Formula	Peak area	Molecular Weight
1.	15.35	Hexadecadienoic acid, methyl ester	$C_{17}H_{30}O_2$	2.48	266.41
2.	15.88	7,10-Hexadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	5.14	294.47
3.	17.02	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	3.92	296.48
4.	17.82	2-Cyclopentene-1-tridecanoic acid, methyl ester,(S)-	$C_{19}H_{34}O_2$	2.41	294.47
5.	18.8	n-Butyl myristste	$C_{18}H_{36}O_2$	1.27	284.27
6.	20.9	Octadecanoic acid, hexyl ester	$C_{24}H_{48}O_2$	61.08	368.63
7.	23.72	Octadecanoic acid, octyl ester	$C_{26}H_{52}O_2$	23.67	412.68

Table 3: Phytocomponents identified in the ethanol extract of *C. rutidosperma* (callus).

S. No.	Retention Time	Name of the compound	Molecular Formula	Peak area	Molecular Weight
1.	15.3	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	14.80	270.45
2.	15.85	2-Cyclopentene-1-undecanoic acid, methyl ester	$C_{17}H_{30}O_2$	13.59	266.41
3.	17.07	8-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	35.52	296.48
4.	17.63	(E)-9-Octadecenoic acid ethyl ester	$C_{20}H_{38}O_2$	3.91	310.51
5.	17.83	Ethanol,2-(9-octadecenyloxy)-,[Z]-	$C_{20}H_{40}O_2$	6.63	312.53
6.	18.85	[E]-13-Docosenoic acid		2.85	
7.	18.85	[1,1'-Bicyclopropyl]-2-octanoic acid, 2-hexyl-,methyl ester	$C_{21}H_{38}O_2$	19.44	322.52
8.	20.85	1-Docosanol, acetate	$C_{24}H_{48}O_2$	2.48	368.63
9.	21.43	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	0.75	390.55
10.	23.67	Octadecanoic acid, octyl ester	$C_{26}H_{52}O_2$	14.80	396.68

chromatograms was a Turbo-Mass ver-5.2[14].

Identification of compounds

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the known component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained[14].

RESULTS AND DISCUSSION

The nodal explants were incubated on MS medium fortified with different concentrations and combinations of PGRs for shoot induction of *C. rutidosperma* Fig.1a-c. Most of PGRs combinations tested for *in vitro* culture resulted in shoot production after 2 weeks of incubation. The results are summarized in Table 1. After 28 days of incubation, the average number of shoots were more in the medium supplemented with BAP + Kin + 2,4D in the concentration of 1.0mg + 0.20mg + 1.0mg/l and 3.0mg + 0.40mg + 3.0mg/l with an average number of shoots ranging 2.33 ± 0.577 cm and 2.00 ± 1.00, cm respectively. Further the medium containing PGR at 1.0mg + 0.20mg and 1.0mg/l also resulted with an elongated single shoot of mean length 7.44 ± 0.39 cm and 7.02 ± 0.26 cm, but the growth regulator type and concentration did not significantly affect shoot length. However, there was a tendency for shoot length to decrease with increasing BAP level after 28 days of culturing. Analysis of variance showed significant differences (α=0.01) between the different treatments. GC-MS analysis of ethanol extract of wild and micropropagated of *C.rutidosperma* contains seven and ten major peaks. The bioactive components in those peaks were identified and tabulated (table 2, figure 2, table 3 and Fig 3). The retention times (RT), molecular formula, molecular weight (MW) and peak area (%) of the identified compounds that could contribute the medicinal quality of the plant were summarized in Tables [2 and 3]. The major components in the wild extract were found to be Octadecanoic acid, hexyl ester and Octadecanoic acid octyl ester. In the micropropagated plant, the major compounds identified were 8- Octadecanoic acid, methyl ester and Pentadecanoic acid, 14-methyl-, methyl ester. Octadecanoic acid octyl ester was the only component found similar in both extracts though was recorded with higher percentage in the wild extract. The use of herbal medicine is a common practice in many countries, particularly in Asia and Africa. A simple and efficient micropropagation system was vital for development of *C. rutidosperma* obtained from mature field-grown plants. Even though the environmental conditions and nutrients were highly favorable for the shoot induction, considerable difference in concentrations of bioactive compounds was observed between wild plant and *in vitro* propagated plants[15,16]. Different concentration of plant growth regulators in MS medium showed a significant impact percentage of shoots which can be observed. In basal medium nodal explants were failed to regenerate shoots whereas, the medium supplemented with BAP induced shoot proliferation. Increased number of shoots with enhanced level of BAP[17]. The current study is similar to (Saini and Jaiwal, 2000) in *Peganum harmala* and (Walia et al., 2003) in *Crataeva nurvala* where no shoot buds developed on MS basal medium devoid of growth regulators as such medium without PGR did not induce shoot proliferation in *C. rutidosperma.* Hence it has been suggested that application of the growth regulators externally during *in vitro* studies might result in

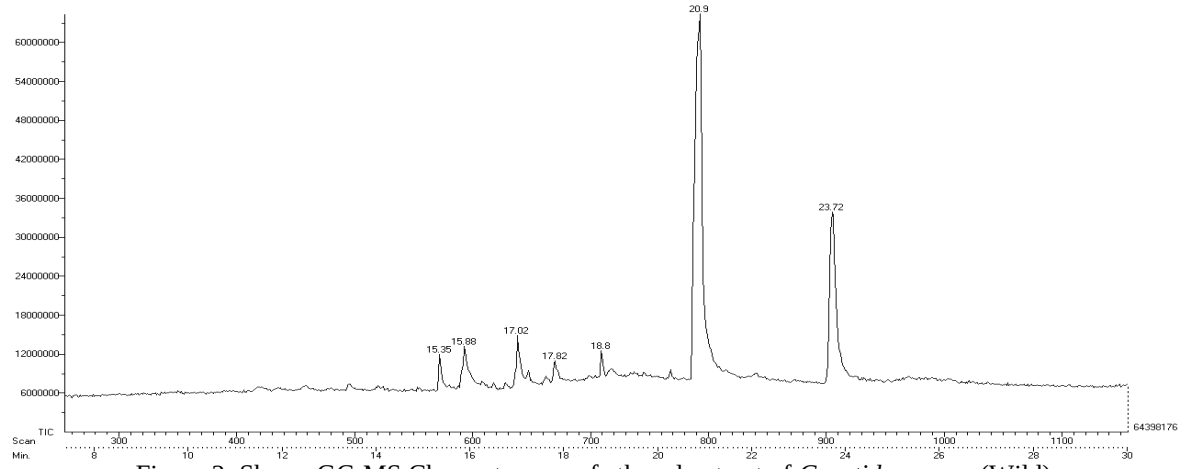

Figure 2: Shows GC-MS Chromatogram of ethanol extract of *C. rutidosperma* (Wild).

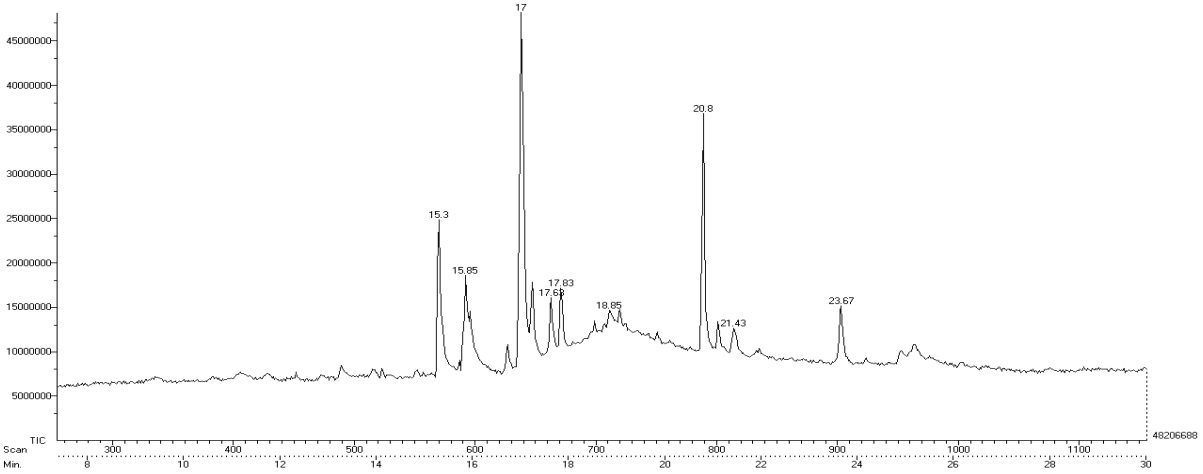

Figure 3: Shows GC-MS Chromatogram of ethanol extract of *C. rutidosperma* (Callus).

organogenesis of explant. Here the different concentrations of cytokinin BAP along with Kin and 2,4D were used for the multiplication of shoot in *C. rutidosperma*. Similarly (Sharma and Mohan, 2006) in *Chlorophytum borivilianum* and (Chakraborty and Roy, 2006) in *Cyphomandra betacea* reported BAP as efficient growth regulator for shoot multiplication. Present study deals with the investigation of the effect of three cytokinins (BAP, kinetin and 2,4D) on shoot formation. While BAP has been used widely for shoot regeneration Johnson et al. (1997) concluded that thidiazuron is more effective than BAP in shoot induction for *S. lappa*. The current study results showed that BAP is most appropriate for shoot multiplication of *C. rutidosperma*. The GC-MS analysis showed the presence of seven compounds in wild namely Hexadecadienoic acid, methyl ester, 7,10-Hexadecadienoic acid, methyl ester, 10-Octadecenoic acid, methyl ester, 2-Cyclopentene-1-tridecanoic acid, methyl ester,(S)-, n-Butyl myristste, Octadecanoic acid, hexyl ester, Octadecanoic acid, octyl ester and ten compounds namely Pentadecanoic acid, 14-methyl-, methyl ester, 2-Cyclopentene-1-undecanoic acid, methyl ester, 8-Octadecenoic acid, methyl ester, (E)-9-Octadecenoic acid ethyl ester, Ethanol,2-(9-octadecenyloxy)-,[Z]-, [E]-13-Docosenoic acid, [1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-,methyl ester, 1-Docosanol, acetate, Bis(2-ethylhexyl) phthalate, Octadecanoic acid, octyl ester, in micropropagated plant of *C. rutidosperma*. The results of ethanolic shoot extracts of *C. rutidosperma* clearly imply that the strength of active principle depends upon the use of solvent besides the type of plant species to achieve the positive results. The identified phytochemical compounds have biological properties. For instance, n-Butyl myristate, reported to contain plasticizers, antimicrobial activity and Octadecanoic acid, butyl ester reported on Antioxidant, pesticide, antimicrobial, lubricant activity[18]. The 13-Docosenoic acids are found in grape seed, wallflower seed, and mustard seed, making up 40-50% of their oils are used for skin disease[19]. Plants are natural source of bioactive compounds to treat many diseases. The plant *C. rutidosperma* has showed good phytochemical which means that it can be used for various pharmacological applications.

CONCLUSION

The study concludes that various concentrations and combinations of PGRs had significant consequence for shoot induction of the plant. This study also clearly indicates that environmental factors play a vital role in formatting the phytoconstituents of the plant. A noticeable decrease in the concentration of metabolites was observed in wild when compared to the *in vitro* propagated plants. The micropropagation protocol reported here was

characterized with a rapid proliferation of shoots, hence this is highly advantageous for the production of uniform source of *C. rutidosperma* plants for a range of further biotechnological applications.

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