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In vivo and *in vitro* studies in *Cymbopogon citratus* (DC.) Stapf for sustainable development

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Abstract
The Cymbopogon grasses belong to the family Poaceae yielding essential oil of commerce. The *Cymbopogon citratus* (DC.) Stapf presently investigated is commonly called as West Indian Lemongrass yielding Lemon grass oil. The essential oil is rich source of terpenoids having high Neral and Geranial content. During the study, the accessions of *Cymbopogon citratus* (DC). Stapf were collected from various geographical locations of south India and were maintained in the Departmental garden with an aim to restore the germplasm which existed as "cultigens". The collected germplasm were predominantly found in south canara regions in Karnataka besides Kerala and Tamil Nadu. The collections were assigned with accession numbers and were subjected to cytogenetic and essential oil studies. The accession collected from Bangalore with robust habitat possessing high essential oil yield was used for further investigations. The karyological analysis carried out using root tips showed chromosome number of 2n=6x=60 from which the ideogram was constructed. The essential oil extracted from the plant was analyzed using Gas chromatography and 1% (v/w) yield was obtained showing Citral (72%) and Geraniol (17%) as predominant compounds. The *in vitro* culture studies performed using leaf explant revealed organogenesis and embryogenesis callus formation which were cultured further to obtain plantlets. Further, the rooted plantlets were acclimatized and transplanted to field conditions. The field established plants were maintained for further investigations.

Keywords: *cymbopogon citratus*, karyomorphology, essential oil, organogenesis, somatic embryogenesis

Introduction
The genus Cymbopogon is a major aromatic plant genera belonging to the tribe Andropogoneae of the family Poaceae, comprising about 180 species, subspecies, varieties and subvarieties [1]. They are unique in possessing essential oil as a source of wide array of terpenes of enormous International potential in perfumery, flavoring and pharmaceutical preparations. The *Cymbopogon citratus* (DC.) Stapf is an important and oldest species of Cymbopogon commonly called 'West Indian lemongrasses or 'lemongrasses. Cymbopogon is derived from the Greek word 'kymbé', meaning a boat, and 'pogon', meaning a beard, referring to the beard like appearance of the inflorescences, and the boat-shaped spathes; 'citratus' in Latin means 'steeped in citrus-oil'. It is a tall tufted perennial grass with a short rhizome, which rarely flowers. Nowadays, *C. citratus* exist only as 'Cultigen' (not known in the wild form and presumably originated from cultivation).The origin of *C. citratus* is very vague as there are many causes which tend to obscure the history of this plant [2]. The ancient history details on the use of the lemongrass mostly in South India by the natives of Madras with subsequent popularity throughout the Carnatic regions and finally spreading all over India [3]. The properties of the plant which recommended this grass to the native gardner of India also contributed for its early introduction into the colonies of those European powers which then had colonized India. Further, the literature reveales that the Indian lemongrass has similar features with that of Malaysia lemon grass [2]. Along with fragrance and flavouring agent, the lemongrass oil has been used for a wide variety of ailments in folk medicine. In India, it has a long history (nearly 2000 yrs) of application against fever in Ayurvedic medicine. The lemongrass plant extract and its essential oil are approved for food use by the USFDA as 'generally recognized as safe' GRAS No.2624 and possesses the International Standardization Organization standard as ISO3217 since 1974 [4]. The oil is soothing to the nerves and also an invigorating agent. The essential oil and infusions of this grass is used to treat a number of human diseases like digestive disorders, menstrual disorder, inflammation, nervous disorder, rheumatism, sprain, cough, cold, fever and various other health problems [4]. It is also known to be a good laxative and anthelmintic. The oil is considered as useful in application for ringworms [5]. Studies have demonstrated antifungal and insecticide efficacy and anticarcinogenic activities. The essential oil of *C. citratus* and its terpenoids showing antimicrobial, antioxidant, anticancerous and antidiabetic activities has been studied [5].

Materials and Methods
1. Collection and maintenance of Germplasm:
A total of nine accessions of *C. citratus* were collected from various regions of south India, including Karnataka, Kerala and Tamil nadu were maintained in the Departmental garden of Microbiology and Biotechnology, Bangalore University, Bangalore under uniform conditions. (Table: 1)

2. Cytogenetic studies
Pre-treatment, fixation and storage of tissues
Healthy root tips were selected from the cultigens for karyological studies. The root tips were collected between 12 PM and 2 PM as maximum mitotic activity was observed during this period. The roots were washed with tap water and subjected to pretreatment with 0.002M 8-

hydroxyquinoline (8HQ) at 4^0C for 3 hrs. The root tips were washed in water and fixed in 1:3 acetic ethanol or propionic ethanol for 24hrs before squashing. The materials were stored in fixative for 4^0 C till the squashes were made. The squash preparations made within one or two weeks after fixation gave better staining and spreading of chromosomes enabling to study the morphology of chromosomes.

Squash preparation and photomicrography

The fixed root tips were treated with mordant of 4% iron-alum in 45% propionic acid for 15-30 min. The roots were transferred to 4% haemotoxylin stain in 45% propionic acid treating for 30-60min.Subsequently, the root tips were washed using 45% propionic acid. Temporary slides were prepared by sealing the edges of the cover glass using paraffin wax. The cytological observations were made from the temporary slides prepared. The photomicrograph of the chromosomes were taken using apochromatic lens system of Carl Zeiss microscope using 60X oil immersion objective and 15X eyepiece. The scale of the stage micrometer was also photographed at the said magnification for the purpose of measuring the chromosomes. The slides were made permanent by passing through 1:1 Butanol: Acetic acid and pure Butanol series and mounted using Euparal.

Karyotypic analysis

Karyotypic measurements were made using dial calipers and were expressed in microns with the aid of stage micrometer. The following characteristics of the karyotype were recognized and compared

a) Difference in absolute size of the chromosome
b) Difference in relative size of the chromosome
c) Differences in position of the centromere
d) Difference in the basic chromosome number.
e) Difference in number and position of satellites.

3. Essential oil studies

Extraction

The shade-dried leaves of *C. citratus* were collected and chopped into small (10cm long) pieces, weighed and subjected to hydro distillation for three hours using Clevenger's apparatus [7]. The essential oils obtained were collected and dried over anhydrous sodium sulphate and kept at 4 °C until analysis. The essential oil yield was calculated on the basis of dry weight of the material (V/W) using the formula,

$$\text{Essential oil content [V/W]} = a \times 100/b$$

Where,

a = volume of the oil (ml) collected
b = weight of sample (g) taken.

Analysis

Gas Chromatographic analysis of the essential oil samples were performed on an Agilent Technologies Gas Chromatograph Model 6890N equipped with dual FID and ACP Sil8CB column (30m X 0.25mm X 0.25 µm film thickness) coated with dimethylpolysiloxane with 5% diphenyl as the stationary phase. Helium was used as the carrier gas at flow rate of 1 ml per min (constant flow).Temperature programming was done from 50 °C (2 min.) to 280 °C at10 °C/min. Injector and detector temperature was maintained at 250 °C and 280 °C respectively. Samples of 1µL dissolved in hexane was injected using a split ratio of 10:1.

Identification of compounds

The component identification was done by comparison of linear retention indices of GC peaks with those of the standard compounds and literature [8].

4. *In vitro* culture studies

The *C. citratus* accession possessing higher essential oil yield was considered as an elite accession and used for further *in vitro* culture studies.

Sterilization and culture of the explant

The young leaf rolls (1.5 - 2 cm) were used as explant source. The leaf rolls were surface sterilized, disinfected with 0.01% mercuric chloride for 10 min and immersed in 50 mg/l potassium permanganate solution for 20-30 min. The outer whorl of the leaf rolls were removed aseptically and excised into small discs of 3-5mm size and cultured onto MS media [9] and B5 [10] solid medium supplemented with various concentrations and combinations of auxins and cytokinins. Coconut milk (5%) was added as adjuvant to the medium.

Acclimatization of *in vitro* regenerated plants to field

The *in vitro* regenerated plantlets via somatic embryogenesis and organogenesis were transferred to plastic pots containing 1:1 mixture of sterilized sand and peat moss. The plantlets were incubated at 25 °C and 16 h photoperiod for 3weeks and subsequently transferred to field conditions.

Results

Morphology

It is a perennial, tufted, aromatic grass with numerous erect culms arising from a short oblique, ring-shaped, sparingly branched rhizome. The culms (stem) grow up to 2-3m tall, smooth and glabrous. Leaves sheathing, coriaceous, terete, embracing the culm, glabrous, striate; blade linear, 50-100cm x 0.5-2cm, long attenuate at both ends, apex acuminate, drooping, glabrous, glaucous-green, midrib prominent below and white above, top part and margins often scabrid as the membranes of epidermal cells accumulate silica. The plant is essentially non-blooming and flourishes in well-drained sandy soil. An annual rainfall of 80-100 in. and an average temperature of 75-80°F are reported to be favorable for its growth. (Figure: 1)

Cytogenetic studies

The chromosome number of *C. citratus* determined from the root tip squashes were found to be 2n=6x=60. The details of karyological analysis are shown based on chromosomal analysis (Table: 2). The karyotype consisted of 11 pairs of M, 13 pairs of m and 6 pairs of sm chromosomes. The total chromatin length of the haploid complement was 53.73 micrometer. The longest and shortest chromosomes measured were 2.7 micrometer and 1.06 micrometer respectively. Two pairs of satellite chromosomes were recorded (Figure 2). The type of karyotype asymmetry was determined to be 2B type. The hexaploid race of the presently studied species did not flower.

Essential oil studies

Out of all, the Cc5 accession yielded highest percentage of essential oil (0.9±0.02d) (Table 3). Therefore, the essential oil of Cc5 was subjected to fractionation and analysis by GC and GC-MS. The oil showed good percentage of Citral (72%) with moderate amounts of Myrcene (8.8%) and Geraniol (2.2%) (Graph 1).GC-MS analysis of essential oil enabled the identification of compounds listed in (Table 4). Oil was found to be rich in monoterpenes (9.5%), oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes (84%), aliphatic compounds and phenylpropanoids. Oxygenated terpenes like Citral [Neral (30.4%) + Geranial (41.8%)], Geraniol (2.2%) and monterpene β- Myrcene (8.8%) were found to be major component of the oil. The gas chromatogram of the essential oil is represented in (Graph 2) in comparison with Standard Citral (Graph 3). Exo-IsoCitral was reported earlier in the oil of *C. citratus* from Colombia as a minor constituent. Identification of the compounds 2,4-octanediol, 6,7-epoxyneral and 6,7- epoxygeranial was tentative based on their mass spectral data. The monoterpene aldehydes (Z)-isoCitral and (E)-isoCitral, 6,7-epoxyneral and 6,7-epoxygeranial were not found earlier in *C. citratus* oil and are being reported for the first time in this oil. The mass spectral data of these compounds are presented in (Table 4).

***In vitro* culture studies**

Callus induction

MS medium (C4) when amended with growth regulators 2, 4-D (2.0 mg/l), Kn (0.5 mg/l) and CM (5%) for four weeks expressed hard, nodular and green and friable callus having regeneration potential. B5 medium (C9) although gave considerable amount of soft, nodular, green callus on 2,4-D (2.0 mg/l), Kn (0.5 mg/l) and CM (5%) containing medium, the callus did not show potential for regeneration as it lacked friability (Table: 5).

Shoot and root regeneration

For regeneration of shoots from the leaf callus MS medium (S4) supplemented with 2, 4 - D (2.0 mg/L) and BAP (2.0 mg/L) along with CM (5%) gave maximum shoots (approx. 28 Nos.) per gram of the callus (Table 6). The regenerated plantlets were transferred onto MS medium fortified with different concentrations and combinations of IAA and IBA (Table 7). Maximum roots were initiated on (R2) with IAA (1.0 mg/L) (Figure: 3& 4).

Discussion

Although claims of the lemongrass essential oil therapeutic value are rife and as such demand internationally is spiraling, but more information on functionality and efficacy of aroma compounds, which is more clinically studied to authenticate results, is needed to make health claims. Also in present scenario, the lemongrass oil it is not being adequately explored and feasible business opportunities in terms of essential oil industries are ignored as it is considered inferior to its competent East Indian lemongrass oil because the oil has tendency to polymerize due to high monoterpene content, and at the same time natural lemongrass market is facing tough competition from the synthetic market [11]. In contrast to this, unsustainable resource management due to complex environment and economic factors in essential oil trade has led to a decline of lemon grass cultivation and in turn oil production. In view of this, approaches directed towards enhancing the quality of *C. citratus* essential oil for its up gradation to be used at commercial level, thereby restoring back its national and international market is the need of the hour. For sustainable development of this species, conventional method (cultivation by suckering) and biotechnological methods (*in vitro* culture technique by tissue and cell culture) are required to harness essential oil and its isolates for economic usage.

Tables

Table 1: Collection of *C. citratus* accessions

Karnataka	Tamil Nadu	Kerala
Udupi-Cc1	Nadugani, Coimbatore-Cc6	Kasaragod-Cc8
Mangalore-Cc2	Yercaud, Salem-Cc7	Kochi- Cc9
Moodabidri-Cc3		
Puttur- Cc4		
Bangalore- Cc5		

Table 2: Data showing karyomorphological details

Chromosome pair	Short arm (µm)	Long arm(µm)	Ratio (1a/sa)	TCL (µm)	Relative length	Chromosome type
1	1.35	1.35	1.00	2.70	5.02	M
2	1.28	1.28	1.00	2.56	4.76	M
3	1.28	1.28	1.00	2.56	4.76	M
4	1.10	1.10	1.00	2.20	4.09	M
5	1.03	1.03	1.00	2.06	3.83	M
6	0.96	0.96	1.00	1.93	3.59	M
7	0.80	0.80	1.00	1.60	2.98	M
8	0.75	0.75	1.00	1.50	2.79	M
9	0.75	0.75	1.00	1.50	2.79	M
10	0.73	0.73	1.00	1.46	2.72	M
11	0.70	0.70	1.00	1.40	2.61	M
12	1.03	1.36	1.32	2.40	4.39	m
13	1.00	1.36	1.36	2.36	4.39	m
14	1.00	1.23	1.23	2.23	4.15	m
15	0.8+0.15(sat)	1.10	1.37	2.15	4.00	m
16	0.88	1.03	1.17	1.91	3.55	m
17	0.78	1.01	1.30	1.80	3.35	m
18	0.70	1.10	1.57	1.80	3.35	m

19	0.76	0.86	1.14	1.63	2.03	m
20	0.70	0.86	1.23	1.56	2.90	m
21	0.66	0.83	1.26	1.50	2.79	m
22	0.60	0.90	1.50	1.50	2.79	m
23	0.56	0.86	1.54	1.43	2.66	m
24	0.56	0.76	1.36	1.33	2.47	m
25	0.76	1.35	1.77	2.11	3.92	sm
26	0.60	1.30	2.16	1.90	3.54	sm
27	0.43+0.1(sat)	0.80	1.86	1.33	2.48	sm
28	0.43	0.76	1.78	1.20	2.23	sm
29	0.33	0.73	2.22	1.06	1.97	sm
30	0.33	0.73	2.22	1.06	1.97	sm

Total chromatin length: 53.73 µm
Karyotype formula: 11M+13m+6am
Karyotype asymmetry: 2B

Table 3: Percentage of essential oils in *C. citratus*

Accessions	Essential oil yield (%)*
C1	0.7±0.03a
C2	0.65±0.1b
Cc3	0.83±0.04c
Cc4	0.72±0.02a
Cc5	0.9±0.02d
Cc6	0.7±0.02a
Cc7	0.54±0.04e
Cc8	0.7±0.01a
Cc9	0.7±0.01a

*Data represented as mean ± SD. Mean followed by different letter are significantly different (n=8, P<0.05 Tuley's HSD Test)

Table 4: Chemical composition of *C. citratus* essential oil (Cc5)

RI	Compound	Percentage
937	α-Pinene	tr
986	6-Methyl5-hepten-2-one	0.6
990	β-Myrcene	8.8
1026	p-Cymene	0.1
1030	Limonene	0.5
1038	(Z)-β-Ocimene	0.1
1048	(E)-β-Ocimene	tr
1081	6-Camphenolone	0.7
1088	6,7-Myrceneepoxide	0.3
1098	Linalool	1.3
1103	2,2-Dimethyl3,4-octadien-1-al	0.4
1111	epi-PhotoCitralB	0.1
1143	*exo*-IsoCitral	1.0
1151	Citronellal	0.3
1165	(Z)-IsoCitral	0.9
1174	Rosefuranepoxide	0.1
1184	(E)-IsoCitral	1.4
1204	Decanal	0.1
1229	Citronellol +Nerol	0.5
1259	Neral	30.4
1260	Geraniol	2.2
1280	Geranial	41.8
1298	2-Undecanone	0.1
1305	Geranylformate	0.1
1344	6,7-Epoxyneralti+2,4Octanediolti	0.6
1358	Nericacid	0.3
1362	Citronellylacetate	0.1
1377	6,7-Epoxygeranialti	0.3
1385	Geranylacetate	0.4
1387	Geranicacid	0.3
1392	β-Elemene	0.1

RI = Retention Index on DB-5 column; nd = notdetected;
tr = trace(>0.05%), ti = tentativeidentification

Table 5: Effect of growth regulators on callus induction from leaf explants

Composition				
Species	**Media with Hormones**	**Conc. (mg/l)**	**Callus nature**	**Callus yield**
C1	MS (Basal)	-	-	-
C2	MS+2,4-D+Kn+CM	1.0+0.5+5%	Hard,nodular,green	+
C3	MS+2,4-D+Kn	1.0+1.0	Hard, nodular, green / yellow	++
C4	MS+2,4-D+Kn+CM	2.0+0.5+5%	Hard, nodular, green, friable	+++
C5	MS+2,4-D+Kn	2.0+1.0	Hard, nodular, green / yellow	++
C6	B5 (Basal)	-	-	-
C7	B5+2,4-D+Kn+CM	1.0+0.5+5%	Soft,nodular, green	++
C8	B5+2,4-D+Kn	1.0+1.0	Soft,nodular, white or green	+
C9	B5+2,4-D+Kn+CM	2.0+0.5+5%	Soft,nodular,green	+++
C10	B5+2,4-D+Kn	2.0+1.0	Soft,nodular, white / green	++

Each treatment with 5 replicates of 5 each Nil, +Low, ++Moderate, +++High

Table 6: Effect of growth regulators for shoot regeneration from callus

Composition				
Trial	**Media with hormones**	**Conc. (mg/l)**	**Shoot no / g callus***	**Average length(mm) of shoots(6 weeks)**
S1	MS+2,4-D+BAP	0.5+0.5	21	30.6
S2	MS+2,4-D+BAP+CM	1.0+1.0+5%	19	33.2
S3	MS+2,4-D+BAP	1.5+1.5	21	34.4
S4	MS+2,4-D+BAP+CM	2.0+2.0+5%	27	35.6
S5	B5+BAP+Kinetin	0.5+0.5	24	30
S6	B5+BAP+Kinetin+CM	1.0+1.0+5%	19	31.4
S7	B5+BAP	1.5	19	34.4
S8	B5+BAP+Kinetin+CM	2.0+2.0+5%	24	35.1

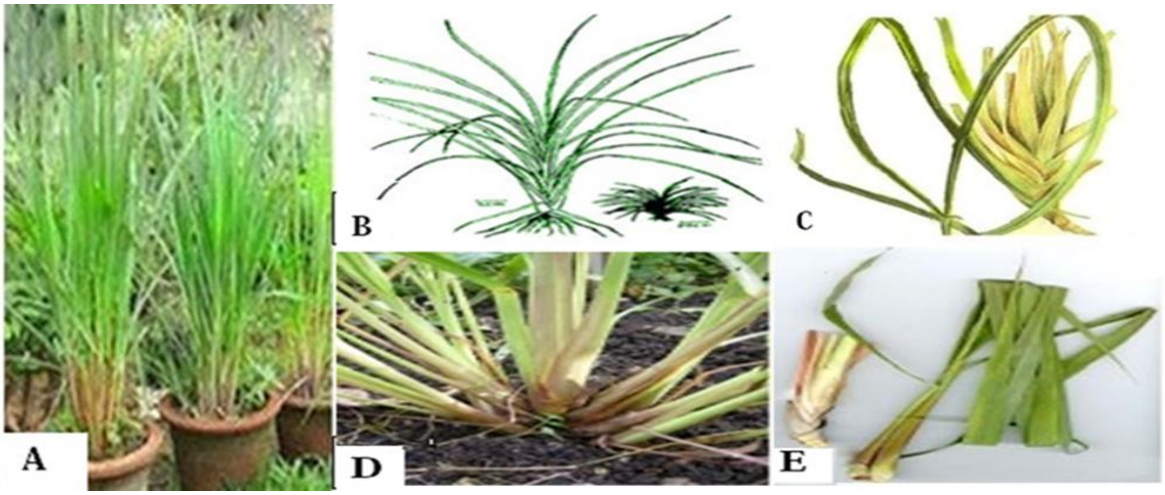
*Average of 8 experiments with 5 replicates each

Table 7: Effect of Auxins on rooting of shoots

Composition			
Trial	**Media with Auxins**	**Conc. (mg/l)**	**Average length(mm) of roots *(6 weeks)**
R1	MS+IAA	0.5	9.6
R2	MS+IAA	1.0	18.8
R3	MS+IBA	0.5	9.9
R4	MS+IBA	1.0	12.2
R5	B5+IAA	0.5	9.8
R6	B5+IAA	1.0	11.2
R7	B5+IBA	0.5	9
R8	B5+IBA	1.0	16

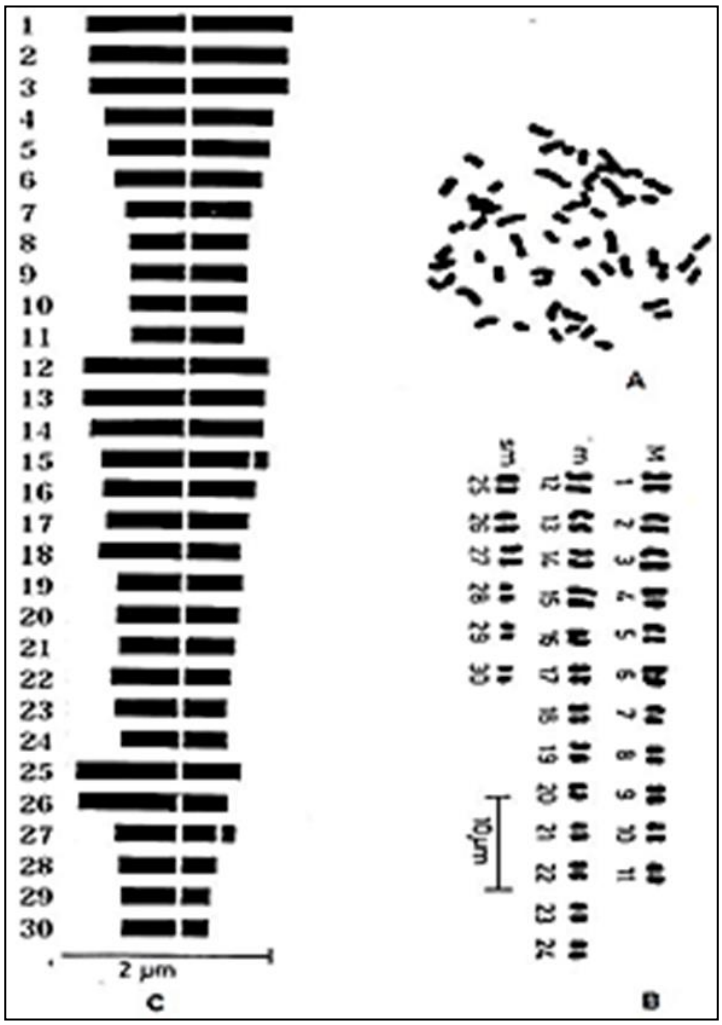
*Average of 8 experiments with 5 replicates each.

Figures



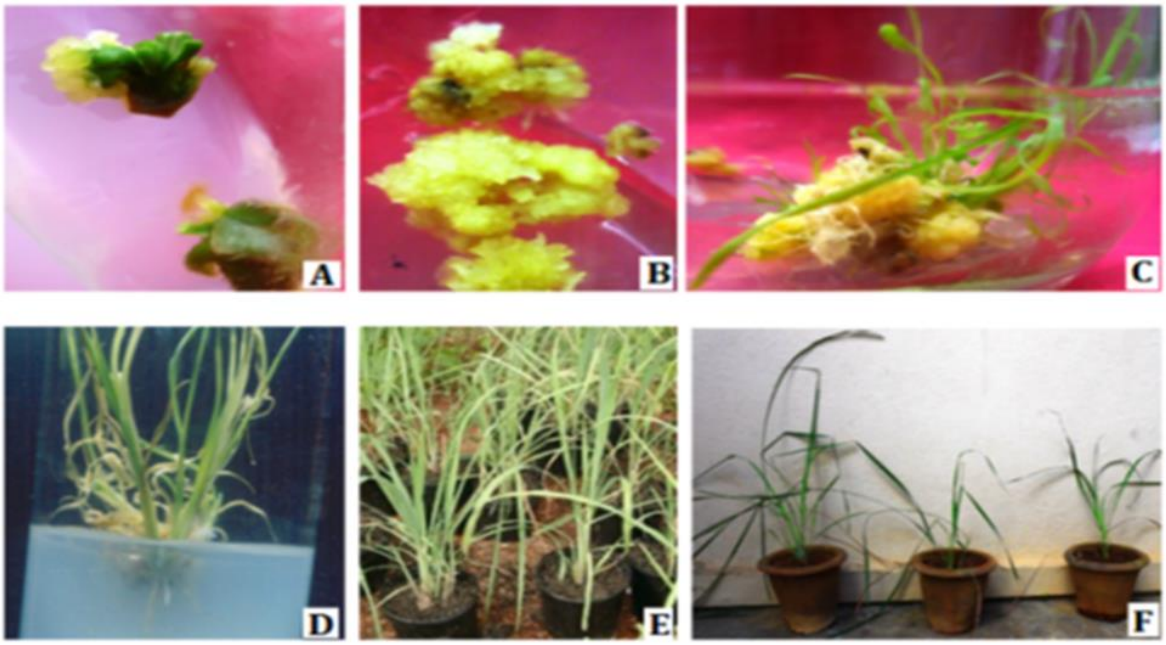
A) *C. citratus* maintained in Departmental garden. **B)** Tufted grass of *C. citratus* with numerous stiff stems arising from rhizomatous rootstock (Schematic diagram). **C)** Stout culms bearing linear drooping leaves (50-100cm long) tapering to long membranous acuminate tip (Schematic diagram). **D)** Tufted culms. **E)** Rhizome and leaf blades

Fig 1: Morphological details of *C. citratus*:



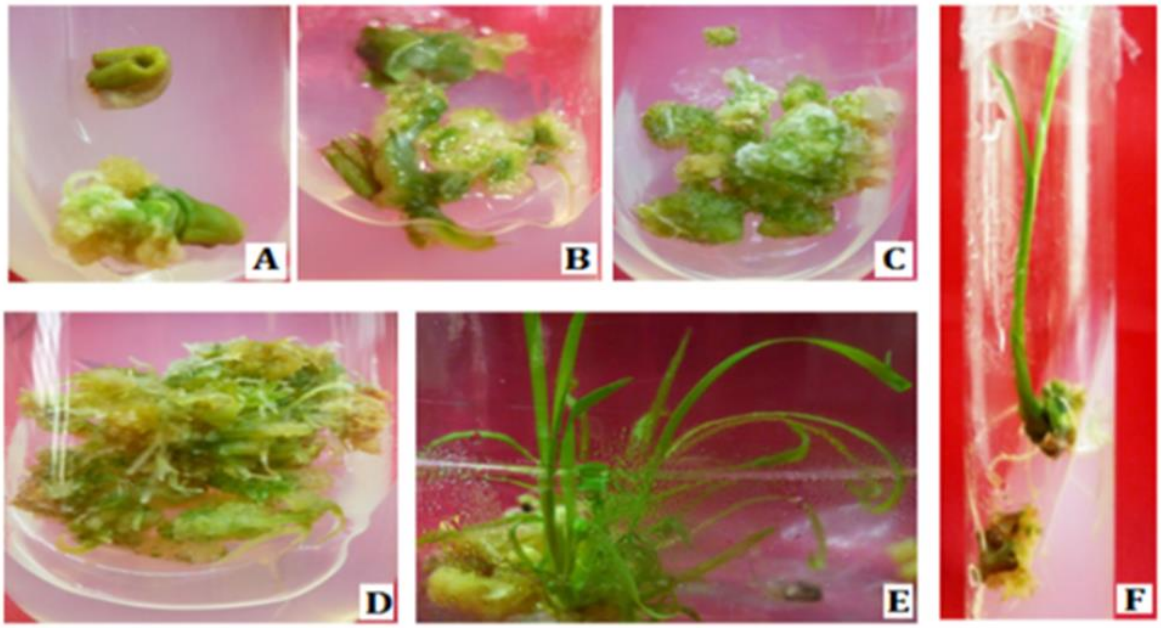
A=Mitotic metaphase B=Idiogram C= Karyotype

Fig 2: Chromosomal analysis showing mitotic metaphase, ideogram and karyotype



A) Initiation of callus from leaf explant. **B)** Callus induction. **C)** Shoot formation. **D)** Rooting of the individual shoot. **E & F)** Plantlets acclimatized under field conditions

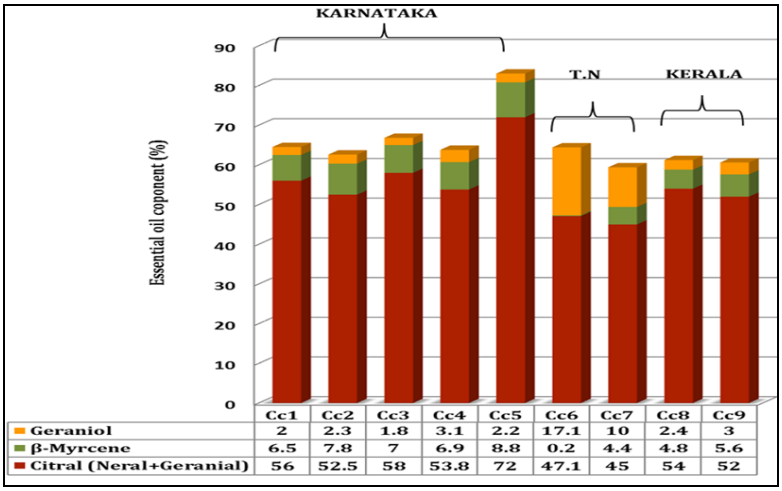
Fig 3: Organogenesis of *C. citratus* (Cc5),



A &B) Early formation of embryogenic callus from leaf base, **C & D**) Maturation and germination of somatic embryos, **E)** Multiple shoots and root regeneration, **F)** Individual somatic embryo developing intoplantlet.

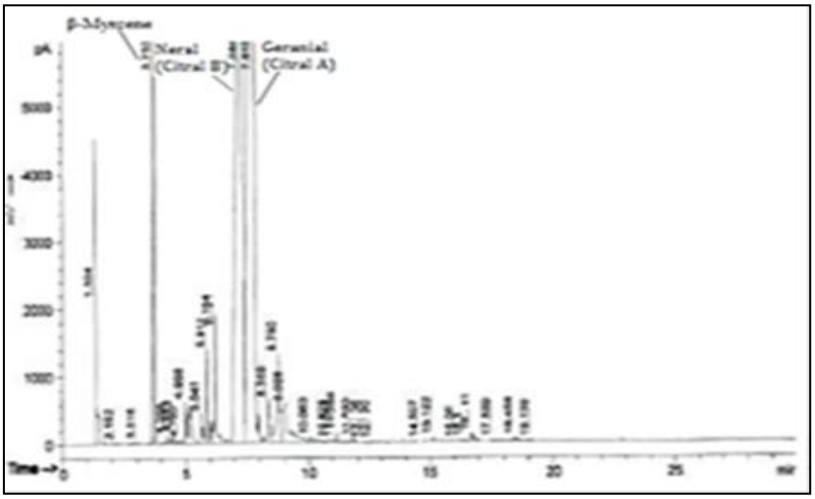
Fig 4: Somatic embryogenesis in *C. citratus* (Cc5),

Graphs

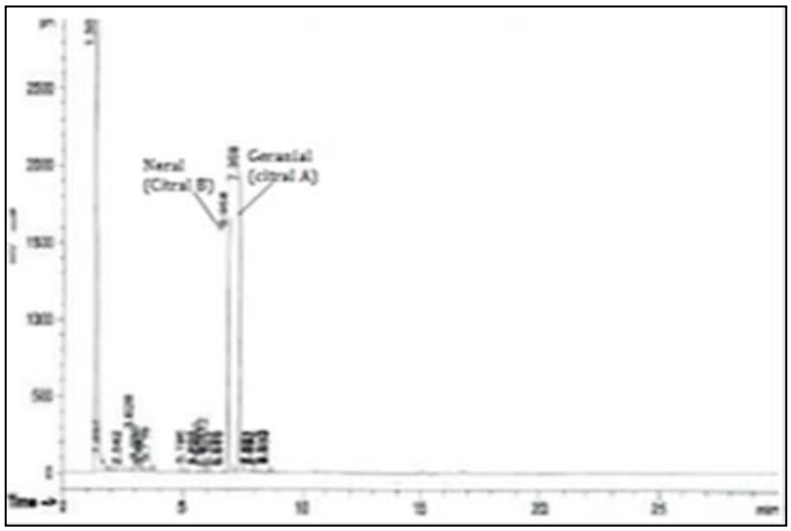


	Cc1	Cc2	Cc3	Cc4	Cc5	Cc6	Cc7	Cc8	Cc9
Geraniol	2	2.3	1.8	3.1	2.2	17.1	10	2.4	3
β-Myrcene	6.5	7.8	7	6.9	8.8	0.2	4.4	4.8	5.6
Citral (Neral+Geranial)	56	52.5	58	53.8	72	47.1	45	54	52

Graph 1: Percentage of major components of essential oil from *C.citratus* accessions



Graph 2: Gas Chromatogram of essential oil (Cc5)



Graph 3: Gas Chromatogram of Std Citral

Conclusion

As the essential oil of *C.citratus* is economically important and used extensively in aromatic and pharmaceutical industries, the present work on collection, conservation and *in vitro* propagation of the cultigens of species is important.. Hence it is of prime importance to restore the germplasm of these species through *in vitro* methods. The chemotypic variation expressed in the accessions presently investigated through essential oil studies could possibly be due to the outstanding adaptability of the individual species in the soil conditions.

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