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The essential oil of 'bhang' (*Cannabis sativa* L.) for non-narcotic applications

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***Cannabis sativa* L. (family Cannabaceae) is a medicinal and aromatic plant growing all over the world. The present study aims to investigate the essential oil composition and antimicrobial activity of *C. sativa* from the foothills of northern India. The hydrodistilled essential oil of *C. sativa* was studied by capillary gas chromatography/flame ionization detector (GC-FID) and GC-mass spectrometry (GC-MS) and evaluated against nine pathogenic bacterial strains using disc diffusion assay. A total of 57 constituents representing 90.5–93.1% of the total oil compositions were identified. Major constituents of the essential oil were (*E*)-caryophyllene (19.6–26.1%), limonene (4.1–15.8%), caryophyllene oxide (2.0–10.7%), (*E*)-β-farnesene (4.8–8.5%), α-humulene (5.4–7.8%), α-pinene (0.7–7.7%), myrcene (0.8–6.0%), terpinolene (0.2–6.0%) and β-selinene (1.8–5.4%). The oil showed moderate to good activity against most of the tested Gram-positive bacteria (*Staphylococcus aureus* (MTCC2940), *Staphylococcus aureus* (MTCC96) and *Streptococcus mutans*). The oil also showed moderate activity against a Gram-negative bacterium, *Salmonella typhimurium*. The chemical composition of the examined *C. sativa* essential oil was quite different from earlier reported compositions. The oil possessed moderate to good activity against most of the tested bacterial strains.**

Keywords: Antibacterial activity, *Cannabis sativa*, essential oil composition, non-narcotic applications.

CANNABIS sativa L., commonly known as 'bhang' or marijuana, an annual herb of the family Cannabaceae, is a hemp plant that grows freely throughout the world. In India, the plant is distributed throughout the Himalayan foothills and the adjoining plains, from Kashmir in the west to Assam in the east. It has become acclimatized in the plains of India and grows even in the warm climate of southern India[1]. It has been used to treat an array of ailments in the Indian traditional systems of medicine[1]. Bhang is being used as an anaesthetic and antiphlegmatic[2] since before to the 10th century BC. The *C. sativa* plant is known today as a potent psychoactive substance, but for many years it was harvested primarily for

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its fibre[3]. It is known for its hallucinogenic, hypnotic, sedative, analgesic and anti-inflammatory activities. Hemp derivatives are suggested for treating glaucoma and as an antiemetic in cancer chemotherapy[4]. Seeds of *C. sativa* contain 25–35% fatty oil, 20–25% protein, 20–30% carbohydrates and 10–15% fibre, along with an array of trace minerals. The fatty oil of *C. sativa* is well balanced considering the ratio of omega-3 to omega-6 fatty acids for human nutrition and can be used as a sustainable alternative to fish oil[5]. *C. sativa* is being used in treating patients with chronic debilitating diseases such as HIV/AIDS, chronic non-cancer pain, epilepsy, multiple sclerosis and amyotrophic lateral sclerosis for the management of symptoms such as pain, nausea, poor sleep, anxiety and stress[6–11]. The extracts of the seed and whole herb possess antibacterial activity[12]. *C. sativa* is complex in its chemistry; it contains more than 525 known chemicals[13]. The best known and most specific class of *Cannabis* constituents is the C_{21} terpenophenolic cannabinoids, with delta-9-tetrahydrocannabinol (THC) being the most psychologically active constituent[14]. THC is used for medical purposes to treat nausea and vomiting side effects in patients receiving chemotherapy[15].

In addition, *C. sativa* also possesses pleasant-smelling essential oil. The essential oil compositions of *C. sativa* differ considerably due to their origins and cultivars[16–19]. Based on the content of α-terpinolene, *C. sativa* cultivars have been divided in two distinct groups – an Eastern European group of cultivars having approximately 8% α-terpinolene and a French group of cultivars containing about 16% α-terpinolene[19].

On the basis of earlier studies, the hydro/steam distilled volatile oil of *C. sativa* is expected to be free from psychoactive substances; hence its potential could be explored for flavour, fragrance and cosmetic purposes. Although *C. sativa* is considered as a most studied plant, its potential as an aromatic plant is still under-explored. Therefore, chemical composition and antibacterial activity of *C. sativa*, collected from the foothills of Uttarakhand have been studied.

Fresh aerial parts of *C. sativa* were collected from CSIR-Central Institute of Medicinal and Aromatic Plants, Research Centre, Pantnagar, located in the foothills of Uttarakhand, India. The plant material was authenticated at Botany Department of the Centre by one of the authors (A.C.). The voucher specimen of the plant has been retained in the department. The fresh aerial parts of *C. sativa* were subjected to hydro-distillation in a Clevenger-type apparatus for 3 h for isolation of essential oil. The essential oil was measured directly in the extraction burette and content (%) was calculated as volume (ml) of essential oil per 100 g of fresh plant material. The oil samples were dehydrated over anhydrous Na_2SO_4 and kept in a cool and dark place until analysis.

Gas chromatography (GC) analysis of the oil samples was carried out on a Nucon gas chromatograph (model 5765) equipped with DB-5 (5% phenyl polysiloxane, 30 m length × 0.32 mm internal diameter; 0.25 µm film coating) fused silica capillary column and flame ionization detector (FID). Hydrogen was the carrier gas at 1.0 ml min^{-1}. Temperature programming was done from 60°C to 230°C at 3°C min^{-1} with final hold time of 10 min. Injector and detector temperatures were 220°C and 230°C respectively. Injection size was 0.02 µl neat (syringe: Hamilton 1.0 µl capacity, Alltech, USA) and split ratio was 1 : 40.

Gas chromatography-mass spectrometry (GC-MS) analysis of the essential oil was carried out on a Perkin-Elmer (PE) turbomass quadrupole mass spectrometer fitted with PE-5 fused silica capillary column (60 m × 0.32 mm; 0.25 µm film coating). The column temperature was programmed at 70°C, initial hold time of 2 min, to 250°C at 3°C min^{-1} with final hold time of 3 min, using helium as carrier gas at a flow rate of 1.0 ml min^{-1}. The injector and ion source temperatures were 250°C. The injection volume was 0.06 µl pure/undiluted with split ratio 1 : 30. Mass spectra were recorded at 70 eV (electron impact ionization) source over a mass scan range of m/z 40–400.

Essential oil constituents were identified on the basis of retention index (RI, determined with reference to homologous series of *n*-alkanes, C_8–C_{30}), co-injection with known compounds, MS Library search (NIST and WILEY), by comparing with the MS literature data[20]. The retention times of standards/marker constituents of known essential oils were also used to confirm the identities of constituents. The relative amounts of individual components were calculated based on GC peak area (FID response) without using correction factor.

Antibacterial activity of the *C. sativa* essential oil was determined by filter paper disc diffusion assay[21].

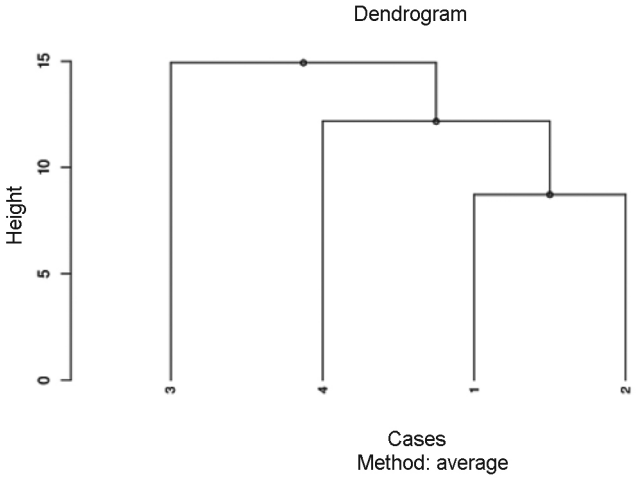

Figure 1. Hierarchical cluster analysis of the essential oil compositions of *Cannabis sativa*: 1: Vegetative stage (April); 2: Flowering stage (May); 3: Flowering stage (October); 4: Flowering stage (November).

Table 1. Essential oil composition of *Cannabis sativa* L. collected from the foothills of Uttarakhand, India

Compound	RI	Content (%) VS April	FS May	FS October	FS November	Identification method
α-Thujene	924	0.2	0.2	0.2	t	RI, MS
α-Pinene	932	3.7	4.2	7.7	0.7	RI, MS, Std
Camphene	945	0.1	0.1	0.1	t	RI, MS
Sabinene	971	0.1	t	t	0.1	RI, MS
β-Pinene	975	2.1	1.9	3.4	0.7	RI, MS
6-Methyl-5-hepten-2-one	981	–	–	t	t	RI, MS
Myrcene	988	0.8	1.6	6.0	1.0	RI, MS
α-Phellandrene	1002	0.2	0.1	0.2	t	RI, MS
δ-3-Carene	1008	–	–	0.2	–	RI, MS
α-Terpinene	1014	0.1	0.2	0.2	–	RI, MS
p-Cymene	1024	t	0.1	t	0.2	RI, MS
Limonene	1027	13.2	8.2	15.8	4.1	RI, MS, Std
1,8-Cineole	1029	0.3	0.3	0.5	1.6	RI, MS
(*Z*)-β-ocimene	1035	t	–	–	0.1	RI, MS
(*E*)-β-ocimene	1045	2.2	2.0	4.1	0.4	RI, MS
γ-Terpinene	1056	0.4	0.9	0.4	t	RI, MS
cis-Sabinene hydrate	1065	2.2	1.4	0.9	1.1	RI, MS
Terpinolene	1088	0.6	0.2	6.0	0.5	RI, MS
Linalool	1098	1.4	1.1	0.7	1.0	RI, MS
trans-Sabinene hydrate	1101	–	–	–	t	RI, MS
endo-Fenchol	1114	0.1	0.1	0.1	0.2	RI, MS
allo-Ocimene	1124	0.1	0.2	0.1	0.2	RI, MS
(*E*)-epoxy-ocimene	1137	–	–	–	0.1	RI, MS
Isoborneol	1158	t	0.1	t	0.2	RI, MS
Coahuilensol	1168	0.2	0.2	t	0.2	RI, MS
Terpinen-4-ol	1175	0.4	1.0	0.4	0.6	RI, MS
p-Cymen-8-ol	1180	0.1	0.1	t	0.9	RI, MS
α-Terpineol	1186	0.6	0.7	0.6	1.0	RI, MS
7-*epi*-Sesquithujene	1387	0.4	0.2	t	0.1	RI, MS
Sesquithujene	1404	0.2	0.2	0.1	0.4	RI, MS
(*Z*)-caryophyllene	1407	0.1	0.1	0.3	0.7	RI, MS
α-*cis*-Bergamotene	1416	t	t	0.1	0.7	RI, MS
(*E*)-caryophyllene	1425	26.1	21.1	19.6	24.5	RI, MS, Std
α-trans-Bergamotene	1435	3.8	3.6	2.1	3.9	RI, MS
Aromadendrene	1441	–	0.2	0.1	0.2	RI, MS
(*Z*)-β-farnesene	1442	0.1	t	–	0.4	RI, MS
α-Humulene	1455	7.8	5.4	5.7	6.0	RI, MS
(*E*)-β-farnesene	1458	6.3	8.1	4.8	8.5	RI, MS
allo-Aromadendrene	1462	0.1	–	t	0.5	RI, MS
γ-Gurjunene	1475	0.4	0.2	0.1	0.4	RI, MS
γ-Curcumene	1480	0.1	0.5	0.2	0.6	RI, MS
Germacrene D	1483	0.1	t	0.1	0.8	RI, MS
β-Selinene	1488	3.7	5.4	1.8	2.8	RI, MS
(*Z*)-β-guaiene	1494	–	–	–	0.2	RI, MS
α-Selinene	1498	2.8	4.0	1.6	2.0	RI, MS
Bicyclogermacrene	1500	t	0.1	–	0.1	RI, MS
(*E*,*E*)-α-farnesene	1503	–	–	0.1	0.1	RI, MS
β-Bisabolene	1505	2.5	3.0	1.7	2.9	RI, MS
Sesquicineole	1515	0.8	1.3	0.7	0.2	RI, MS
β-Sesquiphellandrene	1525	2.0	4.6	2.5	2.2	RI, MS
Unidentified ($C_{15}H_{24}$)[a]	1540	1.2	0.8	0.8	0.6	RI, MS
Germacrene B	1556	0.2	0.1	t	0.4	RI, MS
(*E*)-Nerolidol	1564	0.9	0.5	0.3	1.2	RI, MS
Spathulenol	1580	–	0.1	t	1.9	RI, MS
Caryophyllene oxide	1585	2.0	3.9	2.1	10.7	RI, MS
Viridiflorol	1595	0.2	0.3	0.2	0.2	RI, MS
Humulene epoxide II	1610	0.6	1.3	0.6	2.8	RI, MS
1-*epi*-Cubenol	1625	0.7	1.4	0.7	0.7	RI, MS
Unidentified ($C_{15}H_{24}O$)[b]	1632	0.3	0.6	0.2	0.4	RI, MS
Unidentified ($C_{15}H_{24}O$)[c]	1635	t	t	t	0.5	RI, MS

(*Contd*)

Table 1. (*Contd*)

Compound	RI	Content (%) VS April	FS May	FS October	FS November	Identification method
Class composition						
Monoterpene hydrocarbons		23.8	19.8	44.4	7.8	
Oxygenated monoterpenes		5.0	4.7	3.2	5.8	
Sesquiterpene hydrocarbons		56.7	56.8	40.9	58.4	
Oxygenated sesquiterpenes		5.2	8.8	4.6	17.7	
Benzenoids		0.1	0.2	t	1.1	
Others		0.2	0.2	t	0.2	
Total identified		91.0	90.5	93.1	91.0	
Essential oil (%)		0.10	0.10	0.13	0.12	

RI, Retention index determined with reference to homologous series of *n*-alkane (C_8–C_{30}) on DB-5 column; VS, Vegetative stage; FS, Flowering stage; t, Trace (<0.05%); MS, Mass spectrum; Std, Comparing the retention data and mass fragmentation pattern with known compound/standard.
[a]m/e: 41, 53, 55, 67, 77, 79, 80, 93 (100%), 105, 107, 109, 119, 121, 133, 161, 189, 204, 207.
[b]m/e: 41 (100%), 53, 55, 67, 69, 77, 79, 91, 105, 119, 131, 145, 159, 173, 187, 202, 207.
[c]m/e: 41 (100%), 44, 53, 55, 67, 69, 77, 79, 91, 105, 117, 119, 131, 145, 159, 173, 187, 202, 207.

Inoculum of the test bacteria [Gram-positive: *Staphylococcus aureus* (MTCC96), *Streptococcus mutans* (MTCC890), *Bacillus subtilis* (MTCC121), *Staphylococcus aureus* (MTCC2940) and Gram-negative: *Klebsiella pneumoniae* (MTCC109), *Escherichia coli* (MTCC723), *E. coli* (DH5α), *Pseudomonas aeruginosa* (MTCC741), *Salmonella typhimurium* (MTCC98)] was prepared equivalent to McFarland Standard 0.5. Uniform bacterial lawns were made using 100 µl inoculums on a nutrient agar plate. Filter paper (Whatman) discs (5.0 mm) soaked with test essential oil were placed over seeded plates. The plates were incubated at 37°C for 24 h. Activity was measured in terms of zone of growth inhibition (mm). The net zone of growth inhibition was determined by subtracting the disc diameter (i.e. 5.0 mm) from the total zone of inhibition shown by the test material in terms of clear zone around the disc. The tests were performed in triplicate. The bacterial strains were obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology Chandigarh, India.

To examine whether the essential oil constituents identified are useful in reflecting the chemical relationships between different compositions, their contents (%) were subjected to hierarchical cluster analysis using average method[22]. This software computes the hierarchical clustering of a multivariate dataset based on dissimilarities. The derived dendrogram depicts the grouping of chemical compositions according to their chemical constituents.

The *C. sativa* aerial parts collected at vegetative and reproductive stages gave 0.10% and 0.10–0.13% of essential oil on fresh weight basis respectively. The essential oil yields were observed to be higher in October (0.13%) and November (0.12%), when the plants were in flowering stage. The resulting essential oils were analysed by GC and GC/MS. A total of 57 constituents representing 90.5–93.1% of the total oil compositions were identified (Table 1). The *C. sativa* essential oils were dominated by sesquiterpene hydrocarbons (40.9–58.4%), followed by monoterpene hydrocarbons (7.8–44.4%), oxygenated sesquiterpenes (4.6–17.7%) and oxygenated monoterpenes (3.2–5.8%). Major constituents of the essential oils were (*E*)-caryophyllene (19.6–26.1%), limonene (4.1–15.8%), caryophyllene oxide (2.0–10.7%), (*E*)-β-farnesene (4.8–8.5%), α-humulene (5.4–7.8%), α-pinene (0.7–7.7%), myrcene (0.8–6.0%), terpinolene (0.2–6.0%), β-selinene (1.8–5.4%), β-sesquiphellandrene (2.0–4.6%), (*E*)-β-ocimene (0.4–4.1%), α-selinene (1.6–4.0%), β-bisabolene (1.7–3.0%), α-*trans*-bergamotene (2.1–3.9%), β-pinene (0.7–3.4%), humulene epoxide II (0.6–2.8%) and *cis*-sabinene hydrate (0.9–2.2%).

The essential oils obtained from different months/seasons showed significant variations in their terpenoid compositions. Oxygenated monoterpenes (5.8%), sesquiterpene hydrocarbons (58.4%) and oxygenated sesquiterpenes (17.7%) were found relatively higher in October. However, the amount of monoterpene hydrocarbons was higher in November (44.4%) compared to other months. To examine the similarity or differences among the essential oil compositions obtained from different months/seasons, 17 selected components (amount ≥ 2.0%) representing 74.8–86.4% of the oil compositions were subjected to the hierarchical cluster analysis. The similarity index among the different compositions is depicted in the derived dendrogram (Figure 1).

The chemical composition of the examined *C. sativa* essential oil was different from earlier reported compositions. According to an old study from India, major constituents of the *C. sativa* essential oil were caryophyllene (45.7%) and β-humulene (16.0%)[23]. Mediavilla and Steinemann[17] studied the essential oil composition of *C. sativa* from Switzerland and reported myrcene (29.4–65.8%) as the most abundant constituent, followed by (*E*)-caryophyllene (3.8–37.5%), α-pinene (2.1–31.0%), (*E*)-ocimene (0.3–10.2%) and α-terpinolene (trace-23.8%)[7].

Table 2. Antibacterial activity of *C. sativa* L. essential oil collected at flowering stage

Strain	Disease caused	Zone of growth inhibition (mm)
Gram-positive		
Staphylococcus aureus (MTCC2940)	Nasopharyngeal infection, fatal septicaemias, pneumonias, (secondary-endocarditis, meningitis, etc.)	10
S. aureus (MTCC96)	Nasopharyngeal infection, fatal septicaemias, pneumonias, (secondary-endocarditis, meningitis, etc.)	11
Streptococcus mutans (MTCC890)	Dental caries, endocarditis	9
Basillus subtilis (MTCC121)	Food poisoning	4
Gram-negative		
Klebsiella pneumonia (MTCC109)	Pneumonia, nosocomial infections, bronchitis, etc.	na
Escherichia coli (MTCC723)	Gastroenteritis	na
E. coli (DH5α)	Laboratory strain	na
Pseudomonas aerugenosa (MTCC741)	Nosocomial infection (hospital-acquired infections)	na
Salmonella typhimurium (MTCC98)	Gastroenteritis, septicaemia	7

na: not active.

Ross and ElSohly[16] analysed *C. sativa* essential oil from Mississippi and identified myrcene (58.7–67.1%) and limonene (16.4–17.2%) as the main constituents[16]. Moreover, Novak *et al*.[19] reported myrcene (21.1–35.0%), (*E*)-caryophyllene (12.2–18.9%), α-terpinolene (7.0–16.6%), α-pinene (7.2–14.6%), (*E*)-β-ocimene (7.3–9.0%) and α-humulene (6.1–8.7%) as the major constituents of Austrian *C. sativa* essential oil.

The essential oil of *C. sativa* was tested against four Gram-positive and five Gram-negative bacterial strains. The oil exhibited sensitivity against all tested Gram-positive strains and one Gram-negative strain (Table 2). The oil showed moderate to good activity against *S. aureus* strains (MTCC2940; MTCC96). However, it was found to be moderately active and low active against *Streptococcus mutans* and *B. subtilis* respectively. Further, the oil showed moderate activity towards *Salmonella typhimurium*; however, it was not active against the remaining Gram-negative bacteria.

In conclusion, the essential oil composition of *C. sativa* from the foothill region of northern India was rich primarily in (*E*)-caryophyllene, limonene, caryophyllene oxide, (*E*)-β-farnesene, α-humulene, α-pinene and myrcene. The oil showed moderate to good activity against most of the tested Gram-positive strains and one Gram-negative strain. Further, perusal of the essential oil profile of *C. sativa* revealed the presence of normal terpenoids, which are usually reported in various commercial essential oils. Consequently, the use of this hydro-distilled fraction (essential oil) of *C. sativa* is not expected to be used for psychoactive drug purposes. Similar conclusions have also been drawn earlier for this essential oil[17]. Therefore, the potential of *C. sativa* as a source of essential oil can be exploited.

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Isolation and characterization of salt-induced genes from *Rhizophora apiculata* Blume, a true mangrove by suppression subtractive hybridization

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Mangroves are plants that can tolerate salinity equivalent to that present in seawater. In the present study, we have attempted to isolate the genes upregulated at the early stage of salt stress response in *Rhizophora apiculata* Blume, a true mangrove, through suppression subtractive hybridization technique. Several genes were isolated, which were then classified to nine functional categories, viz. metabolism, protein degradation and folding, secondary metabolism, cell rescue and defence, transport facilitation, signal transduction, transcription and translation, photosynthesis and unclassified genes of unknown function. The expression patterns of 12 of the genes thus obtained were studied at 6, 12 and 24 h time points of salinity stress using real-time PCR. Most genes were found to be upregulated under salt stress and showed maximum upregulation at the 6 h time point. Two of the genes studied were downregulated after 6 h, implying that the pattern of gene expression varies with time of application of stress. The genes thus isolated may be used to confer the trait of salt tolerance to non-tolerant genotypes, which can ultimately prove beneficial for crop improvement programmes.

Keywords: Gene expression, *Rhizophora apiculata*, salt stress, suppression subtractive hybridization.

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SALT stress is one of the most serious factors limiting plant growth and productivity[1]. Several studies have focused upon the better utilization of saline soil in order to improve crop production. Mangrove plants may have acquired specific genes essential for salt tolerance during the course of their evolution. Identification and characterization of such genes can contribute towards better agricultural productivity in the future. *Rhizophora apiculata* Blume belongs to the category of Indo-West Pacific stilt mangroves which show tolerance to a wide range of salinities and is the dominant population in the southern coast of India[2]. It belongs to the category of non-secretor, true mangroves which accumulate the excess salt that enters through the transpiration stream within their leaves. This species exhibits several unique biological properties like antimicrobial, insecticidal, etc.

The tolerance of mangroves to a high saline environment is also tightly linked to the regulation of gene expression. The effect of stress upon a plant is best studied at the time of its application rather than at later stages after which the plant adapts to it. Some proteins like vacuolar Na^+/H^+ ATPase, that are upregulated at the initial point of stress, which usually lasts from 30 min to a few hours[3], are switched off once the adaptive stage is reached and their expression levels become comparable to those before stress treatment. Miyama and Tada[4] have reported that in Burma mangrove (*Bruguiera gymnorhiza*), which is also a non-secretor, true mangrove, the expression level of genes changed at 6 h after salt stress treatment, but recovered at 24 h and that the expression profile under salt stress was distinctly different from that under osmotic stress.

Understanding the mechanisms of salt tolerance in mangroves and identification of salt-tolerant genes from mangroves will lead to an effective means to breed or genetically engineer salt-tolerant crops. So the present study focuses on the identification and characterization of the genes involved in short-term response to salinity, especially the salt-tolerant genes in the mangrove, *R. apiculata* by means of suppression subtractive hybridization (SSH) technique, thus providing a new avenue for crop improvement programmes.

Seeds of *R. apiculata* Blume were collected from the Mangrove Research Station at Ayiramthengu, Kollam district, Kerala. The seeds were germinated and grown in potted soil in the greenhouse under freshwater conditions