

Production of monoterpenoids and aroma compounds from cell suspension cultures of *Camellia sinensis*

Abhinav Grover · Jayashankar S. Yadav ·
Ranjita Biswas · Choppakatla S. S. Pavan ·
Punita Mishra · Virendra S. Bisaria · Durai Sundar

Received: 22 December 2010 / Accepted: 20 August 2011 / Published online: 18 September 2011
© Springer Science+Business Media B.V. 2011

Abstract Cell suspension cultures of *Camellia sinensis* were established in 250 ml shake flasks. Flasks contained 50 ml liquid medium of either Murashige and Skoog (MS), N/5 MS or Heller medium containing different levels of 6-benzyladenine (BA) ($0.05\text{--}2\text{ mg l}^{-1}$), 2,4-dichlorophenoxyacetic acid (2,4-D) ($1\text{--}10\text{ mg l}^{-1}$), and sucrose ($10\text{--}50\text{ g l}^{-1}$). Moreover, the pH of the medium was varied from 5.2–6.2. In addition, cultures were subjected to light irradiation as well as to complete darkness. Following optimization of aroma and terpenoid extraction methods, cell cultures were analyzed for the volatile compounds using GC/MS. A total of 43 compounds were identified using the micro SDE apparatus. Among the major monoterpenoids obtained were α -terpineol and nerol. Moreover, other high aroma-value compounds, including 2-ethyl hexanol, benzyl alcohol, benzene acetaldehyde, nonanal and phenylethylalcohol were also detected. The highest levels of these compounds were obtained from cell suspension cultures grown in MS medium containing 5 mg l^{-1} 2,4-D, 1 mg l^{-1} BA and 30 g l^{-1} sucrose at pH of 5.8 with incubation in complete darkness.

Keywords *Camellia sinensis* cell suspension cultures · Monoterpenoids · Nerol · Tea · α -terpineol

Introduction

Camellia sinensis (tea) is a perennial evergreen shrub from Theaceae family. It is the most widely consumed non-alcoholic beverage in the world (Mondal et al. 2004). The quality or flavour (taste and aroma) of the beverage is completely dependent on the non-volatile and volatile constituents in the tea leaves (Ravichandran and Parthiban 1998). The non-volatile components give taste while the volatile components are responsible for aroma. Monoterpenes are the C_{10} class of isoprenoids encompassing nearly thousand colorless, lipophilic and volatile metabolites. These isoprenoids are derived from mevalonate pathway in plants but are not produced by mammals, fungi or other species (Supaibulwatana et al. 2010). Although found throughout nature, these compounds are best known as constituents of the essential oils, floral scents and defensive resins of aromatic plants, to which they impart their characteristic flavours (Bae et al. 2005). Monoterpenes like α -terpineol and nerol have been used either directly as pharmaceutical agents or as flavoring agents, topical medicines, perfumes and insecticides (Wise and Croteau 1999). The antimicrobial properties of monoterpenoids especially those of α -terpineol and nerol have also been widely reported (Aridogan et al. 2002; Jirovetz et al. 2007; Abbasi et al. 2010). There is also evidence that monoterpenes have potential use as herbicides, pesticides, and dietary anticarcinogens (Crowell 1999). Experimental studies using animal cancer models have demonstrated that some monoterpenes also possess antitumorigenic properties acting at different cellular and molecular levels (Loza-Tavera 1999).

A good amount of research has focused in studying the composition of these terpenoid-containing extracts from essential oils (Liu et al. 2007; Predieri and Rapparini

A. Grover · J. S. Yadav · R. Biswas · C. S. S. Pavan ·
P. Mishra · V. S. Bisaria · D. Sundar (✉)
Department of Biochemical Engineering and Biotechnology,
Indian Institute of Technology Delhi, Hauz Khas, New Delhi
110016, India
e-mail: sundar@dbec.iitd.ac.in

2007), fruits (Franco and Shibamoto 2000; Chen et al. 2008), plants (Saxena et al. 2007; Bitam et al. 2008; Zhu et al. 2008), in vitro cultures (Lopez et al. 1999), and flavoring agents (Oltramari et al. 2004). Advances in plant cell cultures have provided promising means for the production of valuable secondary metabolites like terpenoids. Production of monoterpenes (i.e., limonene, linalool among others) in callus tissues and cell suspensions of *Perilla frutescens* paved the way to their large scale production (Sahai 1994). Significant levels of mono- and sesqui-terpenoids have also been reported in callus cultures of *Lavandula angustifolia* (Banthorpe et al. 1995). Characterization of volatile flavour compounds has been reported in callus cultures of *Citrus sinensis* (Niedz et al. 1997) and in cell suspension cultures of Korean mint *Agastache rugosa* (Kim et al. 2001). Though the flavour profiles in *A. rugosa* cell cultures differed from those encountered in the parent plant, the cells produced a marked cucumber/wine-like aroma and some interesting flavour related alcohols (i.e. 2-phenylethanol).

Production of volatile and aroma components using biotechnological approaches finds a considerable market space and a significant scope for commercialization. Though conventional tea breeding is well established and contributed much for tea improvement over the past several decades, but the process is slow due to bottlenecks like perennial nature, long gestation periods and slow propagation rates (Mondal et al. 2004). Plant cell cultures represent a viable and attractive route for increased production of aroma compounds with high productivities and efficiencies with an additional advantage of invoking controls and regulations during the production stage. Callus cultures serve as useful systems for investigating factors influencing the biosynthesis of natural products (Pretto and Santarem 2000). Undifferentiated plant cell suspension cultures, though are known to produce less number of secondary metabolites owing to their lack of differentiation property, have an indispensable advantage of higher growth rates along with an ease in scale-up (Omidi et al. 2010). In this study, a bioprocess has been established for the production of monoterpenoids and aroma compounds from the cell suspension cultures of *Camellia sinensis*. Moreover, a methodology has also been developed to detect the presence of aroma compounds in the suspension cultures.

Materials and methods

Plant material and the explant

Young leaves of *Camellia sinensis* var. Assamica were obtained from Tocklai Experimental Station, Tea Research Association, Jorhat, Assam, India. The plant material was

washed in 1% Savlon and then treated with 0.1% Bavestin and rinsed five to six times with sterile double-distilled water. Surface sterilization was performed using 70% v/v ethanol treatment for 30 s and rinsed thrice with sterile double-distilled water. This was followed by treatment with 0.1% mercuric chloride for 5 min and rinsing with sterile double-distilled water four to five times. For culturing, sterilized leaves were dissected and placed on the callus initiation media under aseptic conditions.

Optimization of culture conditions

The explants were placed on three different media (MS, N/5 MS and Heller) containing varied composition of growth regulators and solidified with 8 g l⁻¹ agar. The pH of the media was adjusted to 5.8 before autoclaving. Explants were cultivated at 25°C under complete dark conditions. Once callus cells were established, they were subcultured every 3 weeks onto the original media from which they were derived. Suspension cultures were generated from 3 weeks old actively growing undifferentiated callus lines. Cell suspension cultures were initiated by transferring 500 mg fresh weight (FW) of friable callus into 250 ml Erlenmeyer flask containing 50 ml medium. The cultures were incubated on gyratory shaker (Scigenics Biotech, Chennai, India) rotating at 125 rpm and maintained at 25 ± 2°C under 16 h/8 h light/dark conditions. In order to optimize cell growth, cells were cultured in different compositions of media - Murashige and Skoog (MS), N/5 MS and Heller; and growth regulators (auxin and cytokinin). Experiments were also carried out with segregated concentration variations in auxin and cytokinin in order to investigate the effect of these growth regulators on cell growth which would eventually lead to optimization of growth regulators' concentrations. For auxin optimization, the cells were cultured on 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations of 1, 5 and 10 mg l⁻¹. Cytokinin concentration was optimized by using 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mg l⁻¹ of 6-benzyladenine (BA). The effect of different sucrose concentrations (1, 2, 3, 4 and 5%) and pH (5.2, 5.4, 5.6, 5.8, 6.0 and 6.2) was also investigated. For all the above experiments, the cells (500 mg fresh weight) were inoculated in 50 ml of the media in 250 ml Erlenmeyer flasks. The cells were incubated at 25°C in complete dark conditions. The effect of light irradiation on cell growth for three subsequent generations was also evaluated. The cells subcultured in dark were inoculated and then cultivated under continuous light irradiation for 3 weeks as a first generation. All treatments were replicated three times, except for sugar, pH, and light treatments, as these were replicated only twice. All data were subjected to ANOVA and the means were compared according to Fisher's Least Significant Difference using MSTAT-C.

Growth measurement

To study the growth of cells in suspension cultures, 1 g l^{-1} friable cells on DW basis were inoculated in 50 ml of medium supplemented with 30 g l^{-1} sucrose in 250 ml Erlenmeyer flasks. The cultures were incubated at 125 rpm and $25 \pm 2^\circ\text{C}$ under 16 h/8 h light/dark regime. Individual flasks (in duplicate) were harvested at a regular interval of 1 week and analyzed for DW. The suspension cultures were harvested by filtering the cells through $0.22\text{ }\mu\text{m}$ filter paper under vacuum. The dry cell weights were recorded after the harvested cells were dried at 60°C till constant weights were achieved. Growth ratio defined as the final dry weight divided by initial dry weight was also calculated.

Isolation and extraction of aroma compounds/terpenoids from suspension cultures

Since the sample preparation and extraction of metabolites from the tissue plays a critical role for an accurate analysis (Zhu et al. 2008), isolation and extraction of the aroma components was carried out using a number of volatile component extraction methods like direct solvent extraction, segregated distillation and extraction (Soxhlet and Clevenger) and simultaneous distillation extraction. The extraction solvent used in all cases was dichloromethane as it has been widely used in extractions from tea leaves (Kawakami et al. 1995; Rawat et al. 2007; Zhu et al. 2008). Simultaneous distillation extraction (SDE) was carried out by adopting a *micro* SDE apparatus (Godefroot et al. 1981). The particular apparatus chosen in fact reduced the amount of extraction solvent required to only 1–2 ml. For all the above-mentioned methods, extraction was carried out from 5 g dried cells. The cells were suspended in 30 ml of water and sonicated for 15 min. The first four conventional extraction procedures were employed in their regular fashion. The detailed description of the working of *micro* SDE apparatus has been described by (Godefroot et al. 1981). Briefly, the sonicated cell suspension was placed in a 100 ml flask. One milliliter dichloromethane (extraction solvent) containing 250 ppm hexadecane (internal standard) was introduced in another flask of 2 ml volume. Cleaned boiling chips were added to both the flasks. The dichloromethane reflux was started by heating the 2 ml flask in a water bath at 90°C when the two-phase system had settled in the demixing part of the apparatus. Shortly, steam was generated by applying heat to the 100 ml flask at 140°C . Chilled water was circulated in the vapour-mixing part by a cold finger to condense the vapours. The apparatus design drives the high-density layer (dichloromethane) and the low-density layer (water) to return to their respective flasks. The steam distillation was stopped after

1 h while the solvent extraction continued for some more time so as to capture even the last traces of the distilling vapours. In this way, all the volatile material was collected in $\sim 1\text{ ml}$ of dichloromethane. There might be some losses in dichloromethane because of its settling into different compartments other than its own or due to evaporation, but this is taken care of by the internal standard. The extract thus obtained was then passed through a $0.22\text{ }\mu\text{m}$ filter (Millipore, Ireland). Subsequently $1\text{ }\mu\text{l}$ of the solution was analysed using gas chromatography-mass spectrometry (GC–MS).

Characterization of aroma compounds/terpenoids

GC–MS analysis was carried out to identify the aroma compounds from the extract using an Agilent GC–MS apparatus. The Agilent (Agilent Technologies, USA) model 6890 GC and model 5973 N quadrupole mass selective detector that was used for characterization analysis was equipped with a $30\text{ m} \times 0.25\text{ mm ID} \times 0.25\text{ }\mu\text{m}$ fused silica capillary column (J & W Scientific USA) along with a 7,683 series autosampler. Helium served as the carrier gas. One microliter of the extract was injected in the chromatograph. In order to standardize characterization parameters, values of various parameters like split ratio (2:1, 1:1, 1:2), flow rate ($0.2\text{--}5\text{ ml min}^{-1}$), oven temperature ($50\text{--}280^\circ\text{C}$), temperature rate ($10\text{--}30^\circ\text{C min}^{-1}$), initial and final holding times (2–10 min) were optimized for identification of a large number of components in a single run. Injector and detector temperatures were set at 250°C . Mass range was recorded from m/z 45–450, with ionization energy of 70 eV.

Standards for various terpenoids and other major aroma compounds were purchased from Sigma Aldrich (USA). The identities of all compounds were determined by comparison of their retention times and mass spectra with those of authentic standards and with mass spectra reported by National Institute of Standards and Technology and Wiley library 05 (Agilent Technologies, Palo Alto, CA). Relative percentage amounts of the separated compounds were calculated from total ion chromatograms by the computerized integrator.

Results and discussion

Initiation of callus tissues

Though all of the selected media compositions were able to initiate callus from the explants, callus obtained from MS-based media were mostly friable. Callus obtained on MS media containing only 2,4-D was less friable while callus obtained on N/5 MS media containing 1-naphthaleneacetic

acid (NAA) and BA were friable. Further, the callus obtained on MS media containing 2,4-D and BA was friable as well as very soft in texture. Moreover, the size of callus lump obtained in this particular media was also large ranging from 11 to 20 mm. The calli obtained on Heller based media were compact in nature and thus were not compatible to be used for establishment of cell suspension cultures. The results also suggested the role of cytokinin in texture formation of callus as all media compositions containing BA resulted in the initiation of a friable callus. Callus growth was the highest in MS medium supplemented with 4.42 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA. Recently, the same combination of growth regulators, though at different concentrations, has been reported to be useful in initiation of callus tissues of Patchouli, an aromatic shrub of commercial interest (Santos et al. 2011).

Effect of different media, auxin concentration and cytokinin concentration on cell growth

Nutritional requirements for optimal growth of plant cells in vitro vary with the species. Even tissues/cells obtained from different explants of the same plant have different requirements for satisfactory growth (Murashige and Skoog 1962). As such, no single medium can be suggested as being entirely satisfactory for all types of plant cells. So in order to satisfy specific requirements of the tea cells, the cells were cultivated in varied combinations of media and growth hormones and their effect on growth was measured. Vigorous cell growth with a growth ratio of 4.08 was obtained in MS medium containing 2,4-D and BA as growth regulators as is evident from Fig. 1a.

On studying the effect of 2,4-D on cell growth, it was found to be maximum in MS basal medium supplemented with 5 mg l^{-1} 2,4-D (Fig. 1b). As a rule, 2,4-D promotes active proliferation of cells and steady growth of callus and suspension cultures with the rate of cell growth depending on 2,4-D concentration and cultivar characteristics of plants (Butenko 1999). It has been reported that in actively growing calli of wheat and barley, high concentrations of 2,4-D suppressed the elongation growth but stimulated cell division. Recently, a similar concentration of 2,4-D was found to be optimum for callus cultures of *C. sinensis* producing phenolic compounds (Nikolaeva et al. 2009). Thus a medium range concentration optimization of 2,4-D obtained for *Camellia* cells supports the previously reported results. Similarly, 1 mg l^{-1} concentration of BA was found to be optimum for highest cell growth as shown in Fig. 1c. At low concentrations of BA (0.05 , 0.1 and 0.5 mg l^{-1}) in the medium, the increment in biomass was nearly the same. The biomass growth became more appreciable only at a higher concentration of 1 mg l^{-1} . The same concentration of BA as a cytokinin in combination

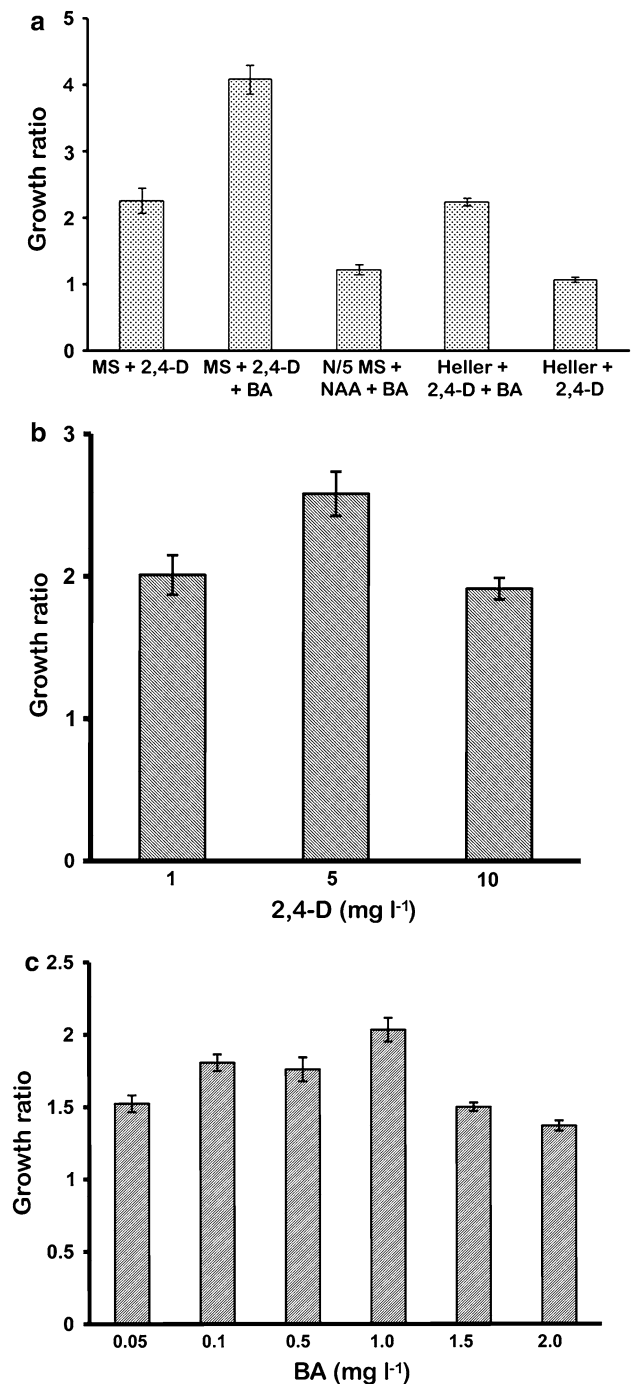


Fig. 1 Effect of **a** different media compositions, **b** different concentrations of 2,4-D and **c** different concentrations of BA on cell growth in suspension cultures of *Camellia sinensis*. Bars represent means \pm standard error of three replicates

with NAA as an auxin has also been reported for maximal optimum growth of *Cananga odorata* cells (Nurazah et al. 2009). Recently, production of pharmaceutically active constituents of *Catharanthus roseus* was established in liquid medium supplemented with a similar concentration of BA (Pati et al. 2011). However, for tea shoot

proliferation, a progressive increase in the multiplication rate has been reported when thidiazuron was used instead of BA (Sandal et al. 2001). The role of BA has also been reported in in vitro clonal propagation of tea plants (Agarwal et al. 1992); at BA concentrations higher than 1 mg l^{-1} , cell growth was suppressed. Thus significantly increased cell biomass could be achieved by making use of optimized concentrations of both 2,4-D (5 mg l^{-1}) and BA (1 mg l^{-1}) in cell suspension cultures of *C. sinensis*.

Growth kinetics and effect of sucrose concentration, initial medium pH and light irradiation on cell growth

Growth kinetics of *C. sinensis* cell suspension cultures was studied in MS media containing 5 mg l^{-1} 2,4-D and 1 mg l^{-1} BA. The maximum accumulation of biomass (10.1 g l^{-1} of DW) was observed after 3 weeks of cell growth (Fig. 2). Previously, the same growth period of tea cell suspension cultures has been reported for production of maximum cell biomass (Shibasaki-Kitakawa et al. 2003). So a growth period of 21 days was chosen as optimum in all the experiments that followed. Sucrose has been widely used as a principal carbon source for cultivation of plant tissue cultures. In general it has been observed that the rate of cell growth is in direct relation to sucrose concentration. Out of various sucrose concentrations used (1–5%), the highest biomass accumulation (10.22 g l^{-1}) was recorded in the medium supplemented with 3% sucrose (Fig. 3a). It has been reported that sucrose concentration affected the germination rate of tea somatic embryos with 3% being the optimum (Mondal et al. 2004). Generally, the pH of the culture medium is kept between 5 and 6 for plant cell growth. Therefore, the effect of pH (5.2–6.2) on cell growth was studied. The medium pH decreases during ammonia assimilation and increases during nitrate uptake (McDonald and Jackman 1989). It was observed that at pH

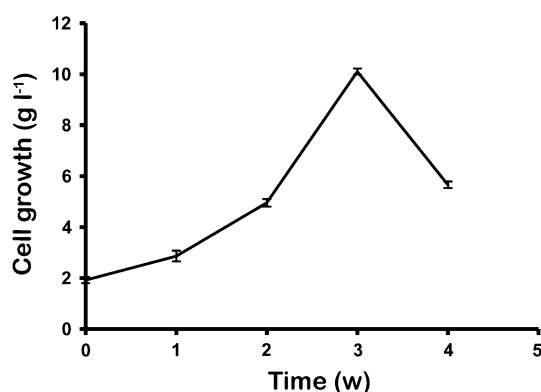


Fig. 2 Growth profile of *Camellia sinensis* in cell suspension cultures. Experiments were carried out in duplicate and the average values are reported. Bars represent means \pm standard error of two replicates

5.8 of the medium, the accumulation of the biomass was maximum (10.38 g l^{-1}) in *C. sinensis* suspension cultures (Fig. 3b).

The cell growth in the first generation of cells placed in light was very slow than that in the dark, as depicted in Fig. 3c. However, the cell concentration in the second

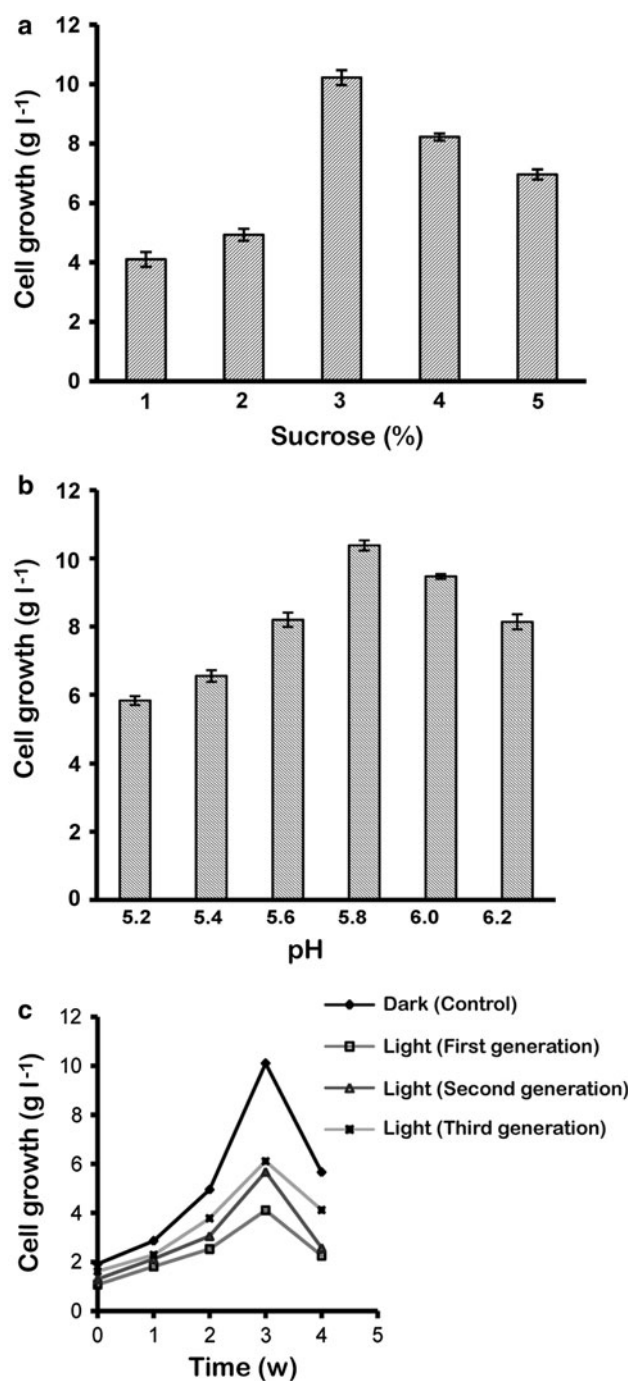


Fig. 3 Effect of **a** different concentrations of sucrose, **b** different pH and **c** light/dark conditions on cell growth in suspension cultures of *Camellia sinensis*. Bars represent means \pm standard error of two replicates

generation increased more rapidly than that in the first generation. There was little difference in the cell concentration between the second and third generations. The cell growth delay in the first generation seemed to arise from the photostress induced by transferring the cells from the dark to the bright place. But even after three generations, biomass accumulation in light-irradiated cultures seemed to be quite lower as compared to those incubated in complete dark conditions. A similar kind of behaviour was observed by Shibasaki-Kitakawa et al. (2003) where many chloroplasts were present in the cells under light irradiation, whereas the cells in the dark were colorless. The chloroplasts being the crucial sites of flavonoid synthesis, higher flavonoids contents were also observed in the suspension cultures. In the case of *C. sinensis* cell suspension cultures, it seems that the enzymes involved in the growth of tea cells get deregulated/inhibited in the presence of light thereby limiting the growth.

Extraction optimization of aroma compounds/terpenoids

Although all the five extraction methods employed were able to isolate aroma compounds but with varying degree of success. A majority of aroma compounds could not be isolated using direct solvent extraction. This is because most of the aroma compounds were found as bound glycosides in the cell wall thus making a heating/distillation step indispensable. In the volatile extracts obtained from Soxhlet and Clevenger type methods, both of which work as segregated distillation and solvent extraction procedures, an increased number of aroma compounds could be identified. However, majority of them were either off-shoot fatty acids or breakdown products of higher compounds, which give undesirable aroma. Since the extractions using distillation procedures require continued boiling of the crude sample for a considerable period of time in order to get noticeable amounts, the heating is also enough for generation of breakdown products. Even of the desired volatile compounds which were successfully isolated, the yields obtained were insignificant because of the low concentrations of volatiles in the aqueous distilled fraction. Typical concentrations of volatile aroma compounds in the aqueous solutions obtained from steam distillation are in parts per million range. Concentration of such highly diluted solutions remains a major limitation to the industrial development of techniques for the recovery and production of natural aroma compounds. However, the use of simultaneous steam distillation solvent extraction, as described in materials and methods section, overcame this limitation by employing extraction in the gaseous phase thus increasing the yields and extraction rates. This method had the advantage over other methods as it required dealing

with very low amount of organic solvents. In our case, a total of 43 volatile constituents were recovered in this small amount of solvent, thus excluding the necessity of time consuming solvent concentration steps.

Characterization of aroma compounds/terpenoids

The optimized values of various parameters for GC–MS were: A constant carrier gas flow rate of 1 ml min⁻¹ with a split ratio of 1:1, oven temperature programmed from 70 to 250°C at a rate of 10°C min⁻¹ with initial holding time of 2 min and final holding time of 5 min. A total of 43 volatile constituents could be identified from the cell extract in a single run as shown in Table 1. The compounds are listed in the order of elution from the HP-5MS column. The identified components accounted for 99.0952% of the total oil extracted. The major monoterpenoids obtained from the cell suspension cultures were α -terpineol and nerol (Fig. 4). It has been reported that α -terpineol can arise from non-enzymatic rearrangements of acyclic monoterpenes such as geraniol, nerol, and linalool (Girard et al. 2002). Under acidic conditions, terpenes such as d-limonene and linalool can transform to other terpenes, such as α -terpineol via the hydration of double bonds, dehydration, cyclation and hydrolysis of esters (Clark and Chamblee 1992). Apart from monoterpenoids, various other aroma compounds having high flavour value like 2-ethyl hexanol, benzyl alcohol, benzene acetaldehyde, nonanal and phenylethylalcohol were also produced.

Monoterpene production and accumulation are restricted to highly specialized anatomical structures in plants. For example, monoterpene biosynthesis in mints originates in the secretory cells of peltate glandular trichomes (Mccaskill et al. 1992) while various types of resin ducts or blisters have been reported as sites of terpenoid biosynthesis in other plants (Katoh and Croteau 1998). Plants that lack such specialized structures typically accumulate only trace quantities of monoterpenes. Research over the past decade has yielded substantial insight into the metabolism of monoterpenes in plants (Davis and Croteau 2000). In a recent study on production of the catechins in tea, it was shown that the distribution of catechins was concentrated in the vascular bundle and palisade tissue (Liu et al. 2009), suggesting the possibility of site specific accumulation in tea. But tea leaves are only available on a seasonal basis limiting the large scale production of the key metabolites. Thus plant cell cultures remain a favored methodology for biotechnological production of these natural compounds. Characterization of volatile flavour compounds and their large scale production using callus and suspension cultures of several plants including *Perilla frutescens*, *Lavandula angustifolia*, *Oryza sativa* and *Agastache rugosa* have been reported (Nabeta et al. 1983; Sahai 1994; Suvarnalatha et al. 1994; Banthorpe et al. 1995).

Table 1 Volatile compounds produced from the cell suspension cultures of *Camellia sinensis*

S. no.	Retention time	Peak area %	Library/ID	Odor/quality	CAS number
1	4.7035	0.9602	Benzaldehyde	Almond, nutty	000100-52-7
2	5.1205	1.5121	2-pentyl-Furan	Weak, grape, sweet	003777-69-3
3	5.6749	1.2626	2-ethyl-1-Hexanol	Apple, green	000104-76-7
4	5.808	4.1059	Benzyl Alcohol	Floral, rose	000100-51-6
5	5.9854	7.9126	Benzeneacetaldehyde	Floral and sweet	000122-78-1
6	6.083	0.6856	2-methyl-Phenol	Burnt, smoky	000095-48-7
7	6.1451	1.0471	Dodecane		000112-40-3
8	6.2338	0.4995	2-Propenamide		000079-06-1
9	6.7439	0.4895	2-methyl-5-propyl-Nonane		031081-17-1
10	6.8459	1.6895	3,7-dimethyl-Decane		017312-54-8
11	6.8903	0.5248	Nonanal	Oily-floral, citrus-like sweet	000124-19-6
12	6.9346	1.0016	3,7-dimethyl-Decane		017312-54-8
13	7.081	8.4074	Phenylethyl Alcohol	Rose-honey-like	000060-12-8
14	7.4048	0.6476	2,3-dimethyl-Phenol		000526-75-0
15	7.5689	0.8076	1-methoxy-2-methyl Benzene	Floral, rosy, green	000578-58-5
16	7.6665	1.0398	1,1-dimethyl-2-(2-methyl-2-propenyl) Cyclopropane		069147-03-1
17	7.7774	0.7267	2-Dimethylamino-3-methylpyridine	Weak, earthy	061713-46-0
18	7.8395	0.4726	3-methyl 1,5-Cyclooctadiene	Sweet, floral	056564-88-6
19	8.2875	0.7939	α -terpineol	Deal, clove	1000157-89-9
20	8.4383	0.4345	Decanal		000112-31-2
21	9.1569	1.6985	Nerol	Floral, herbal	000624-15-7
22	9.5117	0.7734	Hexadecane		000544-76-3
23	9.7911	2.1528	Indole	Floral, green,	000120-72-9
24	10.066	1.1185	2-Methoxy-4-vinylphenol	Medicinal, vegetative	007786-61-0
25	10.155	1.212	Dodecane		000112-40-3
26	11.135	0.6	Tetradecane		000629-59-4
27	12.599	1.5712	2,4-bis(1,1-dimethylethyl)-Phenol	Antioxidant/Prodox 146	000096-76-4
28	12.674	4.2082	Butylated Hydroxytoluene	Antioxidant	000128-37-0
29	12.803	1.6926	4-ethoxy-Benzoic acid ethyl ester		023676-09-7
30	13.788	1.1569	Heptadecyl-Oxirane		067860-04-2
31	14.511	0.8137	Cyclotetradecane		000295-17-0
32	14.945	11.0074	Hexadecanal	Fatty, herbal	000629-80-1
33	15.668	0.7538	3,5-di-tert-Butyl-4-hydroxybenzaldehyde		001620-98-0
34	16.174	0.6802	Acetate, (Z)-7-Hexadecen-1-ol		023192-42-9
35	16.773	4.2697	1H-Purine-2,6-dione, 3,7-dihydro-1,3,7-trimethyl-	Weak, earthy	000058-08-2
36	16.995	7.5911	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester		000084-69-5
37	17.261	0.8153	7-Pentadecyne		022089-89-0
38	17.358	1.219	(Z,Z,Z)-9,12,15-Octadecatrienoic acid		000463-40-1
39	17.722	1.4478	Pentadecanal		002765-11-9
40	18.534	3.5191	n-Hexadecanoic acid		000057-10-3
41	18.622	4.1724	Dibutyl phthalate		000084-74-2
42	19.549	10.1901	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester		004376-20-9
43	23.12	1.4104	Isoproterenol tri-TMS derivative		029522-12-1

The volatiles were extracted using micro simultaneous steam distillation solvent extraction (micro SDE) and analyzed using GC–MS

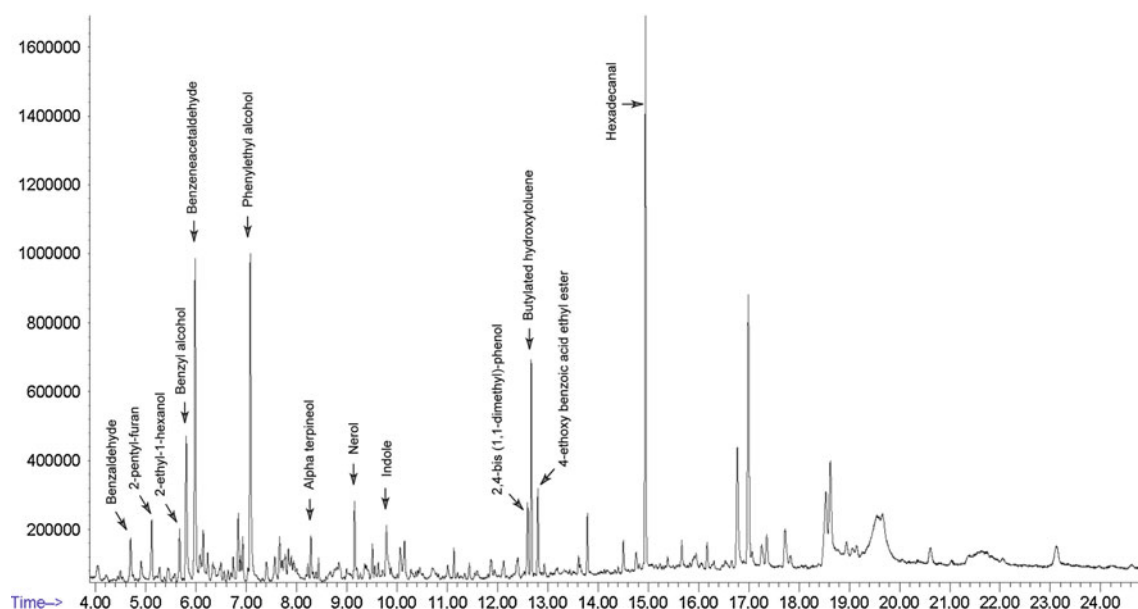


Fig. 4 Total ion chromatogram of *Camellia sinensis* cell suspension cultures: the volatiles were extracted using micro simultaneous steam distillation solvent extraction (micro SDE) and analyzed using GC–MS

Conclusion

Cell suspension cultures of *Camellia sinensis* were successfully established in MS medium containing 5 mg l^{-1} 2,4-D and 1 mg l^{-1} BA. Maximum cell growth was obtained under optimized concentrations of sucrose, pH of the medium and under complete darkness. Production of 43 monoterpenoids and aroma compounds was found to take place in the cell suspension cultures when micro SDE was employed for their detection and subsequent characterization by GC–MS. Since tissue cultures have a higher metabolic rate than the plant, the findings would be particularly useful for designing bioprocesses for the production of these aroma/monoterpenoids at a large scale and for further research on their biosynthesis.

Acknowledgments We thank Dr. Sudipta Das (Tea Research Association, Tocklai Experimental Station, Jorhat, Assam, India) for providing the tea plant material. This study was made possible in part through the support of a grant from Department of Biotechnology (DBT), Govt. of India to VSB and DS.

References

- Abbasi BH, Ahmad N, Fazal H, Rashid M, Mahmood T, Fatima N (2010) Efficient regeneration and antioxidant potential in regenerated tissues of *Piper nigrum* L. *Plant Cell Tiss Org Cult* 102(1):129–134. doi:10.1007/s11240-010-9712-x
- Agarwal B, Singh U, Banerjee M (1992) In vitro clonal propagation of tea (*Camellia sinensis* (L.) O. Kuntze). In vitro clonal propagation of tea (*Camellia sinensis* (L.) O. Kuntze). *Plant Cell Tiss Org Cult* 30:1–5
- Aridogan BC, Baydar H, Kaya S, Demirci M, Ozbasar D, Mumcu E (2002) Antimicrobial activity and chemical composition of some essential oils. *Arch Pharm Res* 25(6):860–864
- Bae TW, Park HR, Kwak YS, Lee HY, Ryu SB (2005) Agrobacterium tumefaciens-mediated transformation of a medicinal plant *Taraxacum platycarpum*. *Plant Cell Tiss Org Cult* 80(1):51–57
- Banthorpe DV, Bates MJ, Ireland MJ (1995) Stimulation of accumulation of terpenoids by cell suspensions of *Lavandula angustifolia* following pretreatment of parent callus. *Phytochemistry* 40(1):83–87
- Bitam F, Gavatta ML, Manzo E, Dibi A, Gavagnin M (2008) Chemical characterisation of the terpenoid constituents of the Algerian plant *Launaea arborescens*. *Phytochemistry* 69(17):2984–2992
- Butenko RG (1999) Biology of higher plant cells in vitro as the basis for biotechnology. FBK-PRESS, Moscow
- Chen QC, Youn U, Min BS, Bae K (2008) Pyronane monoterpenoids from the fruit of *Gardenia jasminoides*. *J Nat Prod* 71(6):995–999
- Clark JB, Chamblee TS (1992) Acid-catalyzed reactions of citrus oils and other terpene-containing flavors. In: Charalambous G (ed) Off-flavors in food beverages. Elsevier Science, New York, pp 229–285
- Crowell PL (1999) Prevention and therapy of cancer by dietary monoterpenes. *J Nutr* 129(3):775–778
- Davis EM, Croteau R (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. *Top Curr Chem* 209:53–95
- Franco MRB, Shibamoto T (2000) Volatile composition of some Brazilian fruits: Umbu-caja (*Spondias citrherea*), camu-camu (*Myrciaria dubia*), arapa-boi (*Eugenia stipitata*), and cupuacu (*Theobroma grandiflorum*). *J Agr Food Chem* 48(4):1263–1265
- Girard B, Fukumoto L, Mazza G, Delaquis P, Ewert B (2002) Volatile terpene constituents in maturing Gewurztraminer grapes from British Columbia. *Am J Enol Viticult* 53(2):99–109
- Godefroot M, Sandra P, Verzele M (1981) New method for quantitative essential oil analysis. *J Chromatogr* 203:325–335
- Jirovetz L, Buchbauer G, Schmidt E, Stoyanova AS, Denkova Z, Nikolova R, Geissler M (2007) Purity, antimicrobial activities

- and olfactive evaluations of geraniol/nerol and various of their derivatives. *J Essent Oil Res* 19(3):288–291
- Katoh S, Croteau R (1998) Individual variation in constitutive and induced monoterpene biosynthesis in grand fir. *Phytochemistry* 47(4):577–582
- Kawakami M, Ganguly SN, Banerjee J, Kobayashi A (1995) Aroma composition of oolong tea and black tea by brewed extraction method and characterizing compounds of darjeeling tea aroma. *J Agr Food Chem* 43(1):200–207
- Kim TH, Shin JH, Baek HH, Lee HJ (2001) Volatile flavour compounds in suspension culture of *Agastache rugosa* Kuntze (Korean mint). *J Sci Food Agr* 81(6):569–575
- Liu K, Rossi PG, Ferrari B, Berti L, Casanova J, Tomi F (2007) Composition, irregular terpenoids, chemical variability and antibacterial activity of the essential oil from *Santolina corsica* Jordan et Fourr. *Phytochemistry* 68(12):1698–1705
- Liu YJ, Gao LP, Xia T, Zhao L (2009) Investigation of the site specific accumulation of catechins in the tea plant (*Camellia sinensis* (L.) O. Kuntze) via vanillin-HCl staining. *J Agr Food Chem* 57(21):10371–10376
- Lopez MG, Sanchez-Mendoza IR, Ochoa-Alejo N (1999) Comparative study of volatile components and fatty acids of plants and in vitro cultures of parsley (*Petroselinum crispum* (Mill) Nym ex hill). *J Agr Food Chem* 47(8):3292–3296
- Loza-Tavera H (1999) Monoterpenes in essential oils: biosynthesis and properties. *Adv Exp Med Biol* 464:49–62
- Mccaskill D, Gershenzon J, Croteau R (1992) Morphology and monoterpene biosynthetic capabilities of secretory-cell clusters isolated from glandular trichomes of peppermint (*Mentha piperita* L.). *Planta* 187(4):445–454
- Mcdonald KA, Jackman AP (1989) Bioreactor studies of growth and nutrient utilization in alfalfa suspension-cultures. *Plant Cell Rep* 8(8):455–458
- Mondal TK, Bhattacharya A, Laxmikumaran M, Ahuja PS (2004) Recent advances of tea (*Camellia sinensis*) biotechnology. *Plant Cell Tiss Org Cult* 76(3):195–254
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15:473–497
- Nabeta K, Ohnishi Y, Hirose T, Sugisawa H (1983) Monoterpene biosynthesis by callus tissues and suspension cells from *Perilla* species. *Phytochemistry* 22(2):423–425
- Niedz RP, Moshonas MG, Peterson B, Shapiro JP, Shaw PE (1997) Analysis of sweet orange (*Citrus sinensis* (L.) Osbeck) callus cultures for volatile compounds by gas chromatography with mass selective detector. *Plant Cell Tiss Org* 51(3):181–185
- Nikolaeva TN, Zagoskina NV, Zaprometov MN (2009) Production of phenolic compounds in callus cultures of tea plant under the effect of 2, 4-D and NAA. *Russ J Plant Physl* 56(1):45–49. doi:10.1134/S1021443709010075
- Nurazah Z, Radzali M, Syahida A, Maziah M (2009) Effects of plant growth regulators on callus induction from *Cananga odorata* flower petal explant. *Afr J Biotechnol* 8(12):2740–2743
- Oltamari AC, Wood KV, Bonham C, Verpoorte R, Caro MSB, Viana AM, Pedrotti EL, Maraschin RP, Maraschin M (2004) Safrole analysis by GC-MS of prototrophic (*Ocotea odorifera* (Vell.) Rohwer) cell cultures. *Plant Cell Tiss Org* 78(3):231–235
- Omidi Y, Zare K, Nazemiyeh H, Movafeghi A, Khosrowshahli M, Motalebi-Azar A, Dadpour M (2010) Bioprocess engineering of *Echium italicum* (L.): induction of shikonin and alkannin derivatives by two-liquid-phase suspension cultures. *Plant Cell Tiss Org Cult* 100(2):157–164. doi:10.1007/s11240-009-9631-x
- Pati PK, Kaur J, Singh P (2011) A liquid culture system for shoot proliferation and analysis of pharmaceutically active constituents of *Catharanthus roseus* (L.) G. Don. *Plant Cell Tiss Org* 105(3):299–307. doi:10.1007/s11240-010-9868-4
- Predieri S, Rapparini F (2007) Terpene emission in tissue culture. *Plant Cell Tiss Org Cult* 91(2):87–95. doi:10.1007/s11240-007-9250-3
- Pretto FR, Santarem ER (2000) Callus formation and plant regeneration from *Hypericum perforatum* leaves. *Plant Cell Tiss Org* 62(2):107–113
- Ravichandran R, Parthiban R (1998) Occurrence and distribution of lipoxygenase in *Camellia sinensis* (L.) O Kuntze and their changes during CTC black tea manufacture under southern Indian conditions. *J Sci Food Agr* 78(1):67–72
- Rawat R, Gulati A, Babu GDK, Acharya R, Kaul VK, Singh B (2007) Characterization of volatile components of Kangra orthodox black tea by gas chromatography-mass spectrometry. *Food Chem* 105(1):229–235
- Sahai OM (1994) Plant tissue culture. In: Gabelman A (ed) *Bioprocess production of flavour, fragrances and color ingredients*. Wiley, New York
- Sandal I, Bhattacharya A, Ahuja PS (2001) An efficient liquid culture system for tea shoot proliferation. *Plant Cell Tiss Org Cult* 65(1):75–80
- Santos AV, Arrigoni-Blank MF, Blank AF, Diniz LEC, Fernandes RMP (2011) Biochemical profile of callus cultures of *Pogostemon cablin* (Blanco) Benth. *Plant Cell Tiss Org* 107(1):35–43. doi:10.1007/s11240-011-9953-3
- Saxena G, Banerjee S, Laiq-ur-Rahman VermaPC, Mallavarapu GR, Kumar S (2007) Rose-scented geranium (*Pelargonium* sp.) generated by *Agrobacterium rhizogenes* mediated Ri-insertion for improved essential oil quality. *Plant Cell Tiss Org Cult* 90(2):215–223. doi:10.1007/s11240-007-9261-0
- Shibasaki-Kitakawa N, Takeishi J, Yonemoto T (2003) Improvement of catechin productivity in suspension cultures of tea callus cells. *Biotechnol Progr* 19(2):655–658. doi:10.1021/bp025539a
- Supaibulwatana K, Banyai W, Kirdmanee C, Mii M (2010) Overexpression of farnesyl pyrophosphate synthase (FPS) gene affected artemisinin content and growth of *Artemisia annua* L. *Plant Cell Tiss Org Cult* 103(2):255–265. doi:10.1007/s11240-010-9775-8
- Suvarnalatha G, Narayan MS, Ravishankar GA, Venkataraman LV (1994) Flavour production in plant cell cultures of basmati rice (*Oryza sativa* L.). *J Sci Food Agr* 66(4):439–442
- Wise ML, Croteau R (1999) Monoterpene biosynthesis. In: Cane DE (ed) *Comprehensive natural products chemistry: Isoprenoids including carotenoids and steroids*, vol 2. Elsevier Science, London, pp 97–153
- Zhu M, Li E, He H (2008) Determination of volatile chemical constituents in tea by simultaneous distillation extraction, vacuum hydrodistillation and thermal desorption. *Chromatographia* 68(7–8):603–610