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Metabolic profiling of in vitro micropropagated and conventionally greenhouse grown ginger (*Zingiber officinale*)

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Abstract

Ginger is an important medicinal and culinary herb, known worldwide for its health promoting properties. Because ginger does not reproduce by seed, but is clonally propagated via rhizome division and replanting, it is susceptible to accumulation and transmittance of pathogens from generation to generation. In addition, such propagation techniques lead to slow multiplication of particularly useful stocks. We have developed an in vitro propagation method to alleviate these problems. Metabolic profiling, using GC/MS and LC-ESI-MS, was used to determine if chemical differences existed between greenhouse grown or in vitro micropropagation derived plants. Three different ginger lines were analyzed. The constituent gingerols and gingerol-related compounds, other diarylheptanoids, and methyl ether derivatives of these compounds, as well as major mono- and sesquiterpenoids were identified. Principal component analysis and hierarchical cluster analysis revealed chemical differences between lines (yellow ginger vs. white ginger and blue ring ginger) and tissues (rhizome, root, leaf and shoot). However, this analysis indicated that no significant differences existed between growth treatments (conventional greenhouse grown vs. in vitro propagation derived plants). Further statistical analyses (ANOVA) confirmed these results. These findings suggest that the biochemical mechanisms used to produce the large array of compounds found in ginger are not affected by in vitro propagation.

Keywords: *Zingiber officinale*; Zingerbraceae; Ginger; Metabolic profiling; Terpenoids; Gingerols; Diarylheptanoids

1. Introduction

Ginger, *Zingiber officinale* Rosc. (Zingiberaceae), and particularly its rhizome, which is also known as "ginger root", has been used in Asia for thousands of years for relief from arthritis, rheumatism, muscular aches and pains, coughs, sinusitis, sore throats, diarrhea, colic, cramps, indigestion, loss of appetite, motion sickness, fever, flu, chills, and infectious disease, and also as a popular culinary spice (College, 1985). The medicinal value of ginger is due to pharmacologically active compounds, such as the anti-inflammatory gingerols, that the plant produces and stores in its rhizomes. To date, more than 100 chemical constituents from ginger have been isolated or detected (Jolad et al., 2004; Jiang et al., 2005a,b). One such compound, [6]-gingerol, has attracted the most attention because of its potential use in treating chronic inflammation, such as asthma and rheumatoid arthritis (Jolad et al., 2004; Jiang et al., 2005b).

In our efforts to elucidate the biochemical pathways to important compounds, such as the gingerols, in these plants, we found it necessary to develop a method to rapidly produce large numbers of ginger plants from small amounts of starting material. In vitro micropropagation approaches provide alternatives to the whole plant for molecule hunters and may guarantee constant and stable supplies of active constituents of ginger. In the present communication, we report a highly efficient in vitro micropropagation procedure for ginger that does not rely on toxic treatments and that is easy to perform (several undergraduate students with no tissue culture experience were

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able to quickly learn the technique with great success). One concern with such methods is that the resulting plants may not possess the same properties (such as presence or concentration of specific metabolites) as the parents.

We used a metabolic profiling approach to address this concern, by comparing ginger plants that were propagated in vitro to plants propagated by traditional agronomic means in the greenhouse. These experiments demonstrated that the micropropagated plants produce the same metabolites at approximately the same concentration as corresponding non-in vitro propagated plants. The targets of these investigations were the medicinally important gingerols, mono- and diacetylgingerols, gingerdiols, 1-dehydrogingerdiones, paradols, shogaols, 3-dihydroshagaols, other diarylheptanoids, and methyl ether derivatives of these compounds, as well as major mono- and sesquiterpenoids.

2. Results and discussion

The aim of this study was to develop an efficient, economical, and safe in vitro propagation procedure for ginger (*Z. officinale*), and to determine whether ginger plants derived from in vitro micropropagated plantlets produced the same chemical profiles as traditionally propagated ginger plants. Because in vitro propagation procedures are often only successful with a particular cultivar or breeding line, we also evaluated our procedure on the three ginger lines that are most commonly used for cultivation and available in the market in the USA.

2.1. In vitro micropropagation

A highly efficient in vitro micropropagation procedure was developed that yielded high rates of multiplication, low costs and genetic stability. In ginger, traditional multiplication produces 10–25 lateral buds in a season of 8–10 months, with only 3–5 buds actively producing plantlets in the wild (College, 1985). The reproducing part (the rhizome) is also the spice-yielding part of the ginger plant, which restricts the amount of seed ginger available to the grower. This report indicates the potential of a single explant (bud) to produce millions of plantlets within a year.

2.1.1. An efficient and safe decontamination pretreatment for ginger explants

The explant pretreatment procedure described in Section 3, especially the rhizome bud treatment, showed significant improvement for ginger organogenesis when compared to other methods previously described. The rate of bud germination and differentiation increased from 4% to 50% after the 50 °C hot water and plant preservative mixture (PPM) treatments were introduced. This pretreatment regime also eliminated the need for $HgCl_2$ treatment, which was used in the other reports for ginger explant sterilization (Hosoki and Sagawa, 1977; Bhagyalakshmi and Singh, 1988; Rout et al., 2001). One goal of our investigation was to develop a ginger sterilization technique that did not require the use of $HgCl_2$ because it is toxic to humans, it is a significant environmental pollutant, and it can cause problems for plant growth and development. In addition, our procedure showed very low levels of contamination from endogenous bacteria and fungi, with less than 10% of explants becoming contaminated, even when inexperienced undergraduate lab assistants performed the experiments. All three ginger lines that were tested showed essentially the same results in terms of germination efficiency and response to growth regulator concentrations, therefore, only results for ginger line GBR are described below unless otherwise indicated.

2.1.2. Callus induction

Callus (Figs. 1a and b) could be produced when rhizome buds, shoot tips, leaf bases, and inflorescence parts (petals, anthers and ovaries) of ginger were placed on M2 medium. However, the rate of callus formation varied dramatically, depending on the tissue used. Rhizome buds, shoot tips and leaf bases were poor tissues for callus formation, with only 5% of explants producing callus. Developing inflorescence tissues, on the other hand, were much more efficient at producing callus, with 90% of explants from dissected anthers, petals, or ovaries from unopened ginger inflorescences yielding callus. The presence of 2,4-dichlorophenoxyacetic acid (2,4-D) at 1.5–5.0 mg l^{-1} in the culture medium resulted in callus growth for ginger explants. Organogenesis and plantlet formation occurred when the concentration of 2,4-D was reduced to 0.25 mg l^{-1} accompanied by addition of 1 mg l^{-1} 6-benzylaminopurine (BA) to the medium, with growth in the dark for 4–6 weeks. The explants were then transferred to fresh medium without growth regulators, where leaf development, shoot elongation and rooting occurred within 4–6 weeks, under a 16 h photoperiod. The rate of plant regeneration increased when the growth regulators were completely removed from the culture medium in subsequent subcultures. However, these growth regulators were required to initiate organogenesis and plantlet formation. Regeneration of plantlets obtained thusly from callus was most successful on Gamborg's B-5 basal medium with minimal organics (B_5) medium without regulators at 27 ± 2 °C under a 16 h photoperiod. After this, the resulting plantlets were used to obtain sufficient material for further studies.

Transfer of callus to M1 medium containing different regulators was also evaluated. Callus transferred to M1 medium containing BA (1.0 mg l^{-1}) promoted root primordia production. The use of α-naphthaleneacetic acid (NAA) (0.5–1.0 mg l^{-1}) in combination with BA (0.5 mg l^{-1}) produced abnormal shoots with callus. The incorporation of kinetin (Kn) (0.01–0.2 mg l^{-1}) into the medium did not improve the differentiation of callus into plantlets. Indole-3-acetic acid (IAA) (0.5–5.0 mg l^{-1} were tested) at higher concentration (5 mg l^{-1}) produced some abnormal shoots with swollen bases.

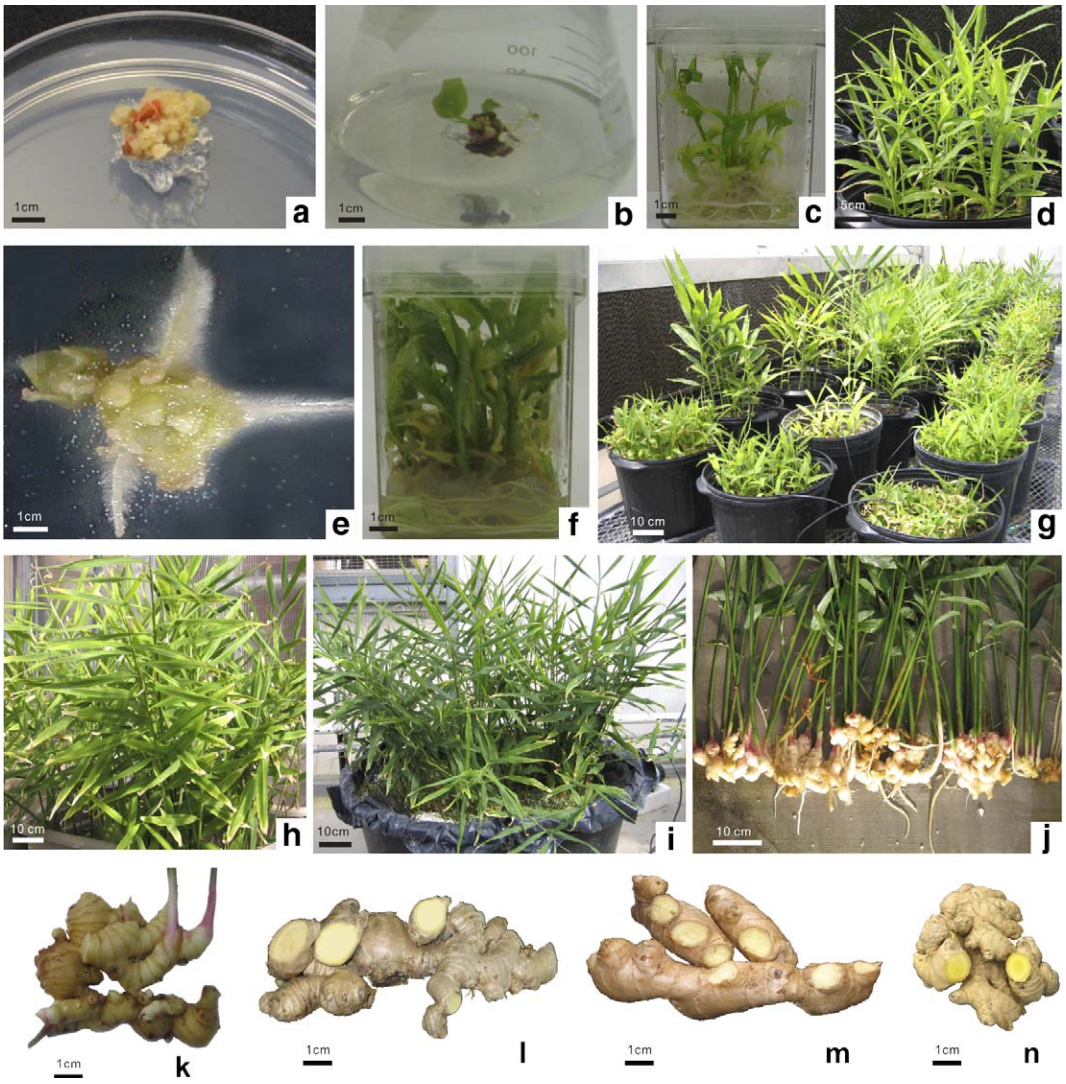

Fig. 1. Callus cultures, multiple shoot formation, maintenance and plant regeneration in in vitro propagated GBR ginger: (a) callus induced from ginger petals after 6 weeks on B_5 medium containing sucrose (3%), 2,4-D (5 mg l^{-1}), and phytagel (0.8%); (b) ginger plantlet induced from callus after 8 weeks on B_5 medium containing sucrose (2%), AA (100 mg l^{-1}), BA (0.5 mg l^{-1}), TDZ 0.1 mg l^{-1}, and phytagel (0.8%); (c) multiple shoot plantlets of ginger, 6 weeks after callus induced plantlet transferred on B_5 medium containing sucrose (2%), Kn (2 mg l^{-1}), NAA (0.5 mg l^{-1}), and phytagel (0.8%); (d) two month old ginger plants (derived from multiple shoot plantlets obtained from callus) growing in 20 l pots in the greenhouse; (e) cotyledonary nodes from ginger rhizome buds 10 days after germination on B_5 medium containing sucrose (2%), AA (100 mg l^{-1}), BA (0.5 mg l^{-1}), and phytagel (0.8%); (f) multiple shoot plantlets of ginger 4-6 weeks after bud germination on B_5 medium containing the same components as if (c); (g) ginger plants (derived from multiple shoot plantlets obtained from bud explants) growing in 20 l pots in the greenhouse; (h) greenhouse grown ginger plants (from rhizomes) in 80 l totes in the greenhouse; (i) ginger hydroponic culture (four month old plants) derived from multiple shoot plantlets obtained from bud explants; (j) in vitro propagated ginger with rhizomes after 4 months growing in greenhouse; (k) in vitro propagated ginger rhizomes followed by hydroponic growth in the greenhouse for 6 months prior to harvest;(l) GBR rhizomes from in vitro propagated ginger plants; (m) GW rhizomes from in vitro propagated ginger plants; (n) GY rhizomes from in vitro propagated ginger plants.

2.1.3. Shoot induction from rhizome buds

Because of the low rate of callus induction from easily obtained tissues (buds, leaves) and the difficulty of obtaining tissues that were more efficient at producing callus (inflorescences are produced very rarely on ginger plants), we developed a method to propagate ginger plantlets directly without the need to go through callus. Several tissues were tested for their ability to produce shoots from explants in vitro, including leaves (leaf bases, veins, leaf margins, and leaf tips), rhizome internodes, rhizome buds, and roots. Only rhizome bud explants were found to be productive sources of new shoots under the in vitro conditions tested (data not shown). M4 medium was then optimized using Kn (1–2 mg l^{-1}), BA (1–3 mg l^{-1}), IAA (1 mg l^{-1}), and NAA (0.5–1 mg l^{-1}) as growth regulators for rapid in vitro micropropagation of ginger from bud explants. Ten different combinations of growth regulators were used for method optimization. Shoot and root number and length data were collected for each combination (see Fig. 2). The results of these experiments indicated that

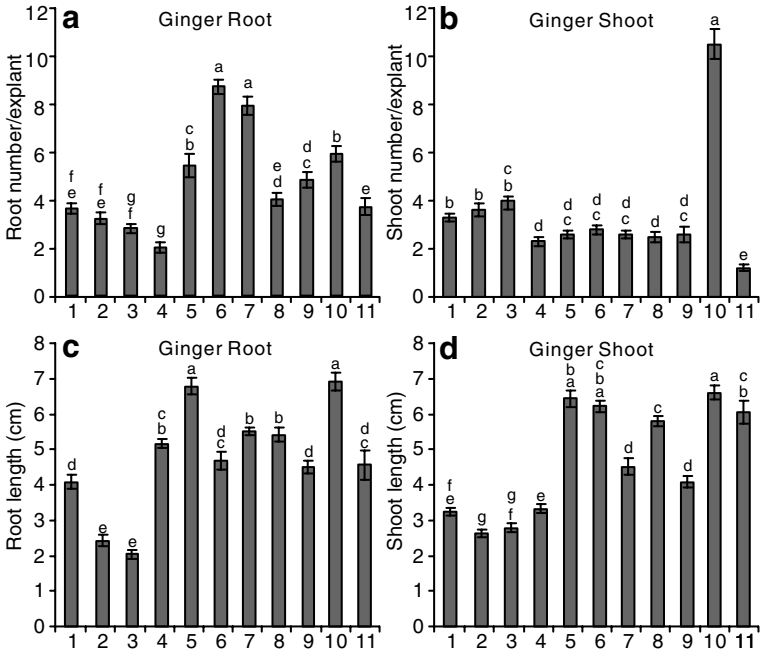

Fig. 2. Effect of Kn, BA, IAA, and NAA combinations in M4 medium on number (a,b) or length (c,d) of shoots and roots of GY ginger (a–d) produced in culture. The following combinations were tested: 1, BA 1 mg l^{-1} + IAA 1 mg l^{-1}; 2, BA 2 mg l^{-1} + IAA 1 mg l^{-1}; 3, BA 1 mg l^{-1} + NAA 1 mg l^{-1}; 4, BA 2 mg l^{-1} + NAA 1 mg l^{-1}; 5, BA 2.5 mg l^{-1} + NAA 0.5 mg l^{-1}; 6, BA 3 mg l^{-1} + NAA 0.5 mg l^{-1}; 7, Kn 1 mg l^{-1} + IAA 1 mg l^{-1}; 8, Kn 2 mg l^{-1} + IAA 1 mg l^{-1}; 9, Kn 1 mg l^{-1} + NAA 1 mg l^{-1}; 10, Kn 2 mg l^{-1} + NAA 0.5 mg l^{-1}; 11, Basal (M4) medium. $n = 10$ for all samples. Error bars are ±SE. Treatments that are not significantly different at the $P < 0.05$ level (determined with one way ANOVA) are indicated by the same letters.

the combination of 2 mg l^{-1} Kn with 1 mg l^{-1} NAA (treatment 10) was the most efficient for obtaining a maximum number of ginger shoots (average 11.5 shoots per bud). Plantlet production of white ginger (GW) and yellow ginger (GY) with best root growth was observed with 2 mg l^{-1} Kn with 1 mg l^{-1} of IAA.

2.1.4. Effect of subculture

Shoot multiplication efficiency was tested for a period of 32 months (33 subcultures). Single plantlets separated from multiple shoot cultures were subcultured and the shoot multiplication rate (over 10 new shoots per culture) persisted in B_5 medium with 2.0 mg l^{-1} Kn and 0.5 mg l^{-1} NAA. The amplification rate did not decrease in continuous subcultures on the same medium. About 5% of the shoots, which had no roots, were used for further root formation studies. Cultures without roots were transferred to M3 medium and grown under 16 h day light conditions as described above. Root formation was observed after two weeks of cultivation.

2.1.5. Ex vitro establishment

Initial acclimatization studies indicated that no special treatment was needed when moving in vitro propagated plantlets to the greenhouse. Rooted plantlets were taken from in vitro cultures and placed directly into soil in pots in the greenhouse. After one month in soil, the yield of plants produced by tissue culture and then transferred to the greenhouse were compared with that of conventionally propagated plants. On average, 98% of in vitro plants survived transfer to soil and maintenance under greenhouse conditions. This was comparable to traditionally propagated rhizomes. The only difference observed in morphological characters between the in vitro propagated plants and their traditionally propagated clonal siblings (both groups of plants were derived from the same parental stock) was that in vitro derived ginger plants grew better (were larger and greener) and produced more rhizomes than plants derived from rhizomes that had not undergone the in vitro propagation procedure (see Table 1). Elimination of endogenous pathogens because of the sterilization regime or residual affects of the exogenously applied growth regulators could explain this improvement. Further experiments are underway to determine whether these improvements are transient. In addition, metabolite profiling of these propagated plants was performed to determine whether other, non-morphologically active somatic mutations may have occurred during the in vitro propagation regime. Results of these experiments are described below.

Table 1
Comparison of rhizome weight (g per plant, fresh weight) of in vitro micropropagated (bud explants) and rhizome-derived ginger plants grown in the greenhouse for one year

Ginger line	In vitro micropropagated	Rhizome-derived
GBR (blue ring ginger)	268.5 ± 2.2	235.6 ± 2.5
GY (yellow ginger)	254.3 ± 2.5	216.5 ± 2.4
GW (white ginger)	214.1 ± 1.8	172.3 ± 1.6

Note: Mean (±SE) of an average of 10 replicates. SE: standard error.

Using the in vitro propagation procedure with harvested buds, where about 10 new plants are produced every four weeks from each bud explant, it is possible to obtain 1000 plants in 3 months from a single bud (see Supplementary Table 1). Compared to the 4–6 ginger plants produced per rhizome per year under standard cultivation practices, this is a dramatic improvement indeed. Furthermore, the tissue used for reproduction is also the tissue harvested for human use in these plants, which restricts the amount of seed ginger available if reasonable yields are to be obtained from the crop each year. In contrast, the findings of this report suggest the potential of a single bud explant to produce tens of thousands to millions of individual plants within a single year. Because ginger is already propagated exclusively clonally under traditional propagation techniques, concerns of planting a genetically uniform crop are not an issue with these plants. Growth space instead of propagation efficiency is now the limiting factor in our ability to use ginger as a model plant to study rhizome development and metabolism.

2.2. Metabolic profiling analysis

A problem that has often been observed with plants reproduced via in vitro propagation regimes is a high rate of somatic mutation (Meins, 1983; Mangolin et al., 1994; Rani et al., 1995; Wang et al., 2000). Because the major goal of our research was to develop an in vitro propagation method for rapid propagation of plants for use in genomics-based metabolism investigations, we wanted to be certain that the method did not introduce mutations that could lead to alterations in metabolism, which would then make such investigations impossible to perform. To evaluate whether such mutation occurred, we utilized a metabolic profiling scheme to evaluate the content and composition of both non-polar volatile and polar non-volatile compounds in the plants resulting from in vitro propagation compared to plants produced through traditional propagation methods. In these experiments, we took plantlets derived from in vitro propagation and plants resulting from traditional propagation methods and grew them in pots on the same bench in the same greenhouse for a whole growing season prior to chemical composition evaluation. This was done to make sure that the plants were grown under as close to identical conditions as possible prior to chemical analysis. The results of these experiments are described in detail below.

In addition, we grew some of the in vitro propagated plantlets (from line GBR) in a hydroponics system to see if plants grown in this way differed in metabolic capability from plants grown in traditional soil conditions. Interestingly, no apparent differences were observed in chemical content or composition (see Table 2) for the rhizomes of in vitro propagated GBR plantlets that were subsequently grown in soil or in the hydroponics system, even though these two growth regimes differed dramatically. This observation suggested some important conclusions. First, the composition of the compounds produced in ginger rhizomes appears to be controlled more by genetic than by environmental factors, provided that growth conditions are favorable. This has important implications for future investigations that seek to use this tissue in genomics-based investigations of rhizome metabolism. Second, future studies that utilize the capabilities of the hydroponics system, e.g., the ability to provide a controlled environment or the ability to apply specific elicitors, will be able to be performed with confidence that the observed effects of such treatments on metabolism (if any effects are indeed observed) will not be due to the use of the hydroponics system but rather would be caused by the applied treatment. Such treatments should be directly transferable to the field.

2.2.1. GC/MS based comparison of three ginger lines and ginger tissues

Non-polar compounds in ginger tissues were extracted with methyl *t*-butyl ether (MTBE) and thereafter analyzed by GC/MS (see Supplementary Figs. 1 and 2, and Supplementary Table 2). Compound identifications indicated by the library search program as being >80% probable were viewed as being likely hits. Spectra for each eluting compound were then compared (by hand) to the standard spectrum for the best hit to determine if the molecular ion peaks and the fragmentation patterns did in fact match. For each of the three samples per ginger line, two injections were made and the mean peak area was calculated. Many compounds present in small quantities in ginger tissues were not included in our analysis because they could not be readily identified due to insufficient mass spectrum quality or because their relative concentrations could not be adequately evaluated. However, 131 compounds, mainly monoterpenoids and sesquiterpenoids, were readily identified from extracts of rhizomes from three ginger lines (GBR, GW and GY) and different tissues (rhizome, root, leaf, and shoot) of in vitro propagated GW (obtained from in vitro propagated and greenhouse grown plantlets that were subsequently grown in the greenhouse for one growing season). Several compounds found at high levels could not be unambiguously assigned, even though they could be tentatively assigned to compound classes. These compounds, identified as DRG-GM1-N1-11.1-150-95-69, DRG-GM1-N1-12.49-154-71-95 and DRG-GM1-N1-16.8-168-100-69 (see Table 2), were named following the nomenclature rules outlined by Bino et al. (2004) for the naming of unknown compounds in metabolic profiling investigations. All of the compounds identified could be classified into five groups: monoterpenoids, sesquiterpenoids, diterpenoids, gingerol-related compounds, and others (alcohols, ketones, aldehydes, and long chain acids, etc.) based on their chemical structures, mass spectra and retention times.

2.2.1.1. GC/MS-based comparison of rhizomes from three ginger lines. One hundred and two compounds were identified from rhizomes of the three lines (GBR, GW and GY).

Table 2
Relative content of volatile compounds identified by GC/MS based metabolic profiling of one year old plant rhizomes (Rh) that were produced from in vitro micropropagated plantlets (IV), hydroponically grown from in vitro micropropagated plantlets (IVH), or produced from greenhouse grown plants (GH) of three ginger lines (GBR, GW and GY), and different tissues (included root (R), leaf (L) and shoot (S)) of in vitro propagated GW plantlets

RT	Name	Formula	MW	GBR (Rh)			GY (Rh)		GW (different tissues)				
				IV	IVH	GH	IV	GH	IV				GH (Rh)
									Rh	R	L	S	
5.75	3-Hexen-1-ol	$C_6H_{12}O$	100	0	0	0	0	0	0	0	2	0	0
6.65	2-Heptanol	$C_7H_{16}O$	116	0	0	0	0	0	0	0	2	0	0
7.10	Tricyclene	$C_{10}H_{16}$	136	1	1	1	1	1	1	1	0	0	1
7.35	(1*R*)-(+)-α-Pinene	$C_{10}H_{16}$	136	2	2	2	1	2	2	3	3	3	2
7.60	*R*-Citronellene	$C_{10}H_{18}$	138	1	1	1	1	1	1	1	0	0	1
7.65	Camphene	$C_{10}H_{16}$	136	3	3	3	1	3	3	3	0	0	3
8.16	4(10)-Thujene	$C_{10}H_{16}$	136	1	1	1	1	1	1	0	0	0	1
8.24	2(10)-Pinene	$C_{10}H_{16}$	136	1	1	2	2	2	1	1	2	2	2
8.50	α-Pinene	$C_{10}H_{16}$	136	2	2	2	1	2	2	0	2	2	2
8.78	α-Phellandrene	$C_{10}H_{16}$	136	1	1	1	1	1	1	0	0	0	1
8.89	3-Carene	$C_{10}H_{16}$	136	1	1	1	1	1	1	0	0	0	1
9.17	*p*-Cymene	$C_{10}H_{16}$	134	1	1	1	1	1	1	0	1	0	1
9.25	*m*-Mentha-6,8-diene	$C_{10}H_{16}$	136	1	1	1	1	1	1	1	0	0	1
9.28	β-Phellandrene	$C_{10}H_{16}$	136	2	2	2	2	2	2	0	3	3	2
9.35	Cineole	$C_{10}H_{18}O$	154	2	2	2	2	2	2	2	2	2	2
9.61	(*Z*)-Alloocimene	$C_{10}H_{16}$	136	0	0	0	0	0	0	0	1	0	0
10.55	*p*-Mentha-1,4(8)-diene	$C_{10}H_{16}$	136	1	1	1	1	1	1	0	0	0	1
10.77	Linalool	$C_{10}H_{16}$	136	1	1	1	1	1	1	0	2	1	1
10.85	Nonanal	$C_9H_{18}O$	142	0	0	0	0	0	0	0	2	2	0
11.10	DRG-GM1-N1-11.1-150-95-69	$C_{10}H_{14}O$	150	1	1	1	1	1	1	0	0	0	1
11.30	(*E*)-2-Pinanol	$C_{10}H_{18}O$	154	1	1	1	1	1	1	0	0	0	1
11.70	Photocitral A	$C_{10}H_{16}O$	152	0	0	0	0	0	0	0	1	0	0
11.86	(−)-Alcanfor	$C_{10}H_{16}O$	152	1	1	1	1	1	1	0	0	0	1
12.03	Citronellal	$C_{10}H_{18}O$	154	1	1	1	1	1	1	0	0	0	1
12.42	(−)-Borneol	$C_{10}H_{18}O$	154	2	2	2	2	1	2	0	0	0	1
12.70	DRG-GM1-N1-12.49-154-71-95	$C_{10}H_{18}O$	154	1	1	1	1	1	1	0	0	0	1
13.06	*p*-Menth-1-en-8-ol	$C_{10}H_{18}O$	154	1	1	1	1	1	1	0	1	0	1
13.23	2-Pinen-4-one	$C_{10}H_{14}O$	150	0	0	0	0	0	0	1	0	0	0
13.40	*n*-Decaldehyde	$C_{10}H_{20}O$	156	1	1	1	1	1	1	0	0	0	1
14.44	Neral	$C_{10}H_{16}O$	152	2	2	2	2	2	2	0	2	2	2
14.89	Geraniol	$C_{10}H_{18}O$	154	2	2	2	2	2	2	0	3	2	2
15.39	(*E*)-Citral	$C_{10}H_{16}O$	152	3	3	3	2	3	3	0	3	3	2
15.84	Borneyl acetate	$C_{12}H_{20}O_2$	196	1	1	1	1	1	1	2	0	0	1
16.04	2-Undecanone	$C_{11}H_{22}O$	170	1	1	1	1	2	2	0	2	0	1
16.40	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150	0	0	0	0	0	0	0	1	0	0
16.80	DRG-GM1-N1-16.8-168-100-69	$C_{10}H_{16}O_2$	168	0	0	0	0	0	0	0	1	0	0
17.10	Myrtenyl acetate	$C_{12}H_{18}O_2$	194	1	1	1	1	1	1	0	0	0	1
17.55	δ-Elemene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	0	0	1
18.00	Citronellyl butyrate	$C_{14}H_{26}O_2$	226	1	1	1	1	1	1	0	0	0	1
18.20	Geranic acid	$C_{10}H_{16}O_2$	168	1	1	1	1	1	1	0	2	0	1
18.39	Neryl acetate	$C_{12}H_{20}O_2$	196	1	1	1	1	1	1	0	0	0	1
18.50	(+)-Cyclosativene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	0	0	1
18.84	α-Copaene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	1	1	1
19.12	Geraniol acetate	$C_{12}H_{20}O_2$	196	3	3	3	3	1	3	0	1	0	1
19.40	β-Elemene	$C_{15}H_{24}$	204	1	1	1	2	2	1	1	0	0	2
19.85	7-epi-Sesquithujene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	0	0	1
20.35	(*E*)-Caryophyllene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	3	3	1
20.69	β-Cubebene	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	0	0	1
20.83	γ-Elemene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	1	0	1
20.91	(*E*)-α-Bergamotene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	0	0	1
21.16	DRG-GM1-N1-21.16-204-93-69	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	0	0	1
21.20	α-Caryophyllene	$C_{15}H_{24}$	204	0	0	0	0	0	0	2	1	2	0
21.39	α-Guaiene	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	0	0	1
21.53	(*Z*)-α-Bisabolene	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	0	0	1
21.64	(*Z*)-β-Farnesene	$C_{15}H_{24}$	204	1	1	1	2	2	1	1	0	0	1
21.81	Aromadendrene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	1	1	1
22.05	2-Dodecena	$C_{12}H_{22}O$	180	0	0	0	0	0	0	0	1	0	0
22.31	β-Patchoulene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	0	0	1
22.54	Germacrene D	$C_{15}H_{24}$	204	2	2	2	2	2	2	1	0	1	2

Table 2 (*continued*)

RT	Name	Formula	MW	GBR (Rh)			GY (Rh)		GW (different tissues)				
				IV	IVH	GH	IV	GH	IV				GH (Rh)
									Rh	R	L	S	
22.59	*ar*-Curcumene	$C_{15}H_{22}$	202	2	2	2	2	2	2	1	0	0	2
22.70	γ-Cadinene	$C_{15}H_{24}$	204	0	0	0	0	0	0	0	2	0	0
22.96	γ-Maaliene	$C_{15}H_{24}$	204	2	2	2	2	2	2	2	0	0	2
23.18	α-Zingiberene	$C_{15}H_{24}$	204	3	3	3	3	3	3	3	0	0	3
23.30	α-Muurolene	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	2	1	1
23.58	(*E*,*E*)-α-Farnesene	$C_{15}H_{24}$	204	3	3	2	3	3	3	3	2	1	3
23.69	Bicyclosesquiphellandrene	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	0	0	1
23.86	Selina-3,7(11)-diene	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	0	0	1
24.14	β-Sesquiphellandrene	$C_{15}H_{24}$	204	3	3	3	3	3	3	3	0	0	3
24.36	(*E*)-γ-Bisabolene	$C_{15}H_{24}$	204	1	1	1	1	1	1	1	0	0	1
24.98	Elemol	$C_{15}H_{26}O$	222	1	1	1	1	1	1	1	0	0	1
25.10	Nerolidyl acetate	$C_{17}H_{28}O_2$	264	0	0	0	0	0	0	0	1	0	0
25.23	Germacrene B	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	0	0	1
25.48	(*E*)-Nerolidol	$C_{15}H_{26}O$	222	1	1	1	2	2	1	0	2	0	1
25.70	Caryophyllene oxide	$C_{15}H_{24}O$	220	0	0	0	0	0	0	0	2	3	0
25.90	Coniferyl alcohol	$C_{10}H_{12}O_3$	180	1	1	1	1	1	1	0	0	0	1
26.43	DRG-GM1-N1-26.43-222-82-69	$C_{15}H_{26}O$	222	1	1	1	1	1	1	0	0	0	1
26.66	Globulol	$C_{15}H_{26}O$	222	1	1	1	1	1	1	0	0	0	1
26.97	8-Cedren-13-ol	$C_{15}H_{24}O$	220	1	1	1	1	1	1	0	0	0	1
27.05	Humulene-epoxide-II	$C_{15}H_{24}O$	220	0	0	0	0	0	0	2	0	0	0
27.25	(*E*)-Sesquisabinene hydrate	$C_{15}H_{26}O$	222	1	1	1	1	1	1	1	0	0	1
27.47	Germacrene-D-4-ol	$C_{15}H_{26}O$	222	1	1	1	1	1	1	1	0	0	1
27.60	Caryophylla-4(14),8(15)-dien-5-α-ol	$C_{15}H_{24}O$	220	0	0	0	0	0	0	0	1	0	0
27.67	Isoaromadendrene epoxide	$C_{15}H_{26}O$	222	1	1	1	1	1	1	1	0	0	1
27.86	(*Z*)-Sesquisabinene hydrate	$C_{15}H_{26}O$	222	1	1	1	1	1	1	1	0	0	1
28.42	Zingerone	$C_{11}H_{14}O_3$	194	2	2	2	2	2	2	0	1	0	2
29.32	DRG-GM1-N1-29.32-220-93-137	$C_{15}H_{24}O$	220	1	1	1	1	1	1	0	0	0	1
29.99	DRG-GM1-N1-29.99-222-119-137	$C_{15}H_{26}O$	222	1	1	1	2	2	1	0	0	0	2
30.19	DRG-GM1-N1-30.19-220-91-69	$C_{15}H_{24}O$	220	1	1	1	1	1	1	0	0	0	1
30.30	Indan, 1,1,3-trimethyl-3-phenyl-	$C_{18}H_{20}$	236	0	0	0	0	0	0	0	1	0	0
31.25	(*Z*,*E*)-Farnesol	$C_{15}H_{24}O$	220	1	1	1	1	1	1	0	0	0	1
31.73	Farnesal	$C_{15}H_{24}O$	220	1	1	1	1	1	1	0	0	0	1
31.96	(*Z*)-α-(*E*)-Bergamotol	$C_{15}H_{24}O$	220	1	1	1	1	1	1	0	0	0	1
32.10	3,7,11-Trimethyl-dodeca-2,4,6,10-tetraenal	$C_{15}H_{22}O$	218	1	1	1	1	1	1	0	0	0	1
32.93	Myristic acid	$C_{14}H_{28}O_2$	228	0	0	0	0	0	0	0	2	2	0
33.90	1-Heptatriacontanol	$C_{37}H_{76}O$	536	0	0	0	0	0	0	0	1	1	0
34.88	2-(*Z*)-9-Octadecenyloxyethanol	$C_{20}H_{40}O_2$	312	0	0	0	0	0	0	0	1	2	0
35.09	All-(*E*)-Farnesyl acetate	$C_{17}H_{28}O_2$	264	1	1	1	1	1	1	0	0	0	1
36.13	DRG-GM1-N1-36.13-220-119-93	$C_{15}H_{24}O$	220	1	1	1	1	1	1	0	0	0	1
38.71	(±)-(*E*)-Nuciferol	$C_{15}H_{22}O$	218	1	1	1	1	1	1	1	0	0	1
39.07	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1	1	1	1	1	1	2	3	3	1
39.58	Helifolenol B	$C_{15}H_{24}O$	220	1	1	1	1	1	1	2	0	0	1
41.06	Geranyl linalool	$C_{20}H_{34}O$	290	1	1	1	1	1	1	0	0	0	1
43.20	Phytol	$C_{20}H_{40}O$	296	0	0	0	0	0	0	0	2	0	0
44.23	Linoleic acid	$C_{18}H_{32}O_2$	280	1	1	1	1	1	1	2	2	0	1
44.27	DRG-GM1-N1-44.27-280-55-69	$C_{18}H_{32}O_2$	280	0	0	0	0	0	0	2	0	0	0
44.30	Linolenic acid	$C_{18}H_{30}O_2$	278	0	0	0	0	0	0	0	2	3	0
44.37	Oleic acid	$C_{18}H_{34}O_2$	282	1	1	1	1	1	1	2	1	0	1
44.60	α-Springene	$C_{20}H_{32}$	272	1	1	1	1	1	1	0	0	0	1
45.07	DRG-GM1-N1-45.07-284-55-69	$C_{20}H_{28}O$	284	1	1	1	1	1	1	0	0	0	1
45.21	Stearic acid	$C_{18}H_{36}O_2$	284	0	0	0	0	0	0	0	2	0	0
45.40	Palmitamide	$C_{16}H_{33}NO$	255	0	0	0	0	0	0	0	1	0	0
45.52	[4]-Gingerol	$C_{15}H_{22}O_4$	266	1	1	1	1	1	1	0	0	0	1
46.90	[6]-Paradol	$C_{17}H_{26}O_3$	278	1	1	1	1	1	1	0	0	0	1
48.60	[6]-Shogaol	$C_{17}H_{24}O_3$	276	2	2	2	2	2	2	0	0	0	2
49.61	[7]-Paradol	$C_{18}H_{28}O_3$	292	1	1	1	1	1	1	0	0	0	1
50.30	Oleamide	$C_{18}H_{35}NO$	281	0	0	0	0	0	0	0	2	2	0
50.34	DRG-GM1-N1-50.34-302-81-137	$C_{20}H_{30}O_2$	302	2	2	2	2	2	2	3	0	0	2
51.30	[6]-Gingerol	$C_{17}H_{26}O_4$	294	3	3	3	2	2	3	0	0	0	2
53.17	Acetoxy-[6]-gingerol	$C_{19}H_{28}O_5$	336	2	2	2	2	2	2	0	0	0	2
54.13	[8]-Shogaol	$C_{19}H_{28}O_3$	304	1	1	1	1	1	1	0	0	0	1

(*continued on next page*)

Table 2 (*continued*)

RT	Name	Formula	MW	GBR (Rh)			GY (Rh)		GW (different tissues)				
				IV	IVH	GH	IV	GH	IV				GH (Rh)
									Rh	R	L	S	
54.52	DRG-GM1-N1-54.52-368-175-191	$C_{24}H_{32}O_3$	368	1	1	1	1	1	1	0	0	0	1
54.88	Diacetoxy-[6]-gingerdiol	$C_{21}H_{32}O_6$	380	1	1	1	1	1	1	1	0	0	1
55.07	DRG-GM1-N1-55.05-274-164-151	$C_{18}H_{26}O_2$	274	0	0	0	0	0	0	0	1	1	0
55.11	[9]-Paradol	$C_{20}H_{32}O_3$	320	1	1	1	1	2	1	0	0	0	1
55.50	Methyl diacetoxy-[6]-gingerdiol	$C_{22}H_{34}O_6$	394	1	1	1	1	1	1	0	0	0	1
56.87	[8]-Gingerol	$C_{19}H_{30}O_4$	322	2	2	1	1	1	1	0	0	0	1
58.68	2-Hexadecanol	$C_{16}H_{34}O$	242	0	0	0	0	0	0	0	1	0	0
59.48	[10]-Shogaol	$C_{21}H_{32}O_3$	332	1	1	1	1	1	1	0	0	0	1
60.35	[11]-Paradol	$C_{22}H_{36}O_3$	348	1	1	1	1	1	1	0	0	0	1
68.17	Vitamin E	$C_{29}H_{50}O_2$	430	0	0	0	0	0	0	0	2	1	0
71.36	β-Sitosterol	$C_{29}H_{50}O$	414	0	0	0	0	0	0	1	2	2	0

Note: 1 indicates <0.5%, 2 indicates 0.5–5%, and 3 indicates >5% of total integrated peak area of total ion chromatogram (TIC) of a particular sample.

Of these, 79 are terpenoids and 16 are gingerol-related compounds (gingerols, shogaols and paradols) as listed in Tables 2 and 3. There was no apparent qualitative difference in volatile compound composition: (a) between the three lines; (b) between plants derived from in vitro propagated or greenhouse grown plantlets for each of the three lines; or (c) between GBR plants propagated in the soil or in the hydroponics system. There were, however, some quantitative differences for some compounds when these different treatments were considered. As shown in Table 2, for example, the contents of (*E*,*E*)-α-farnesene and 8-gingerol in GBR plants derived from in vitro propagated plantlets were higher than in traditionally propagated GBR plants. (*E*)-citral, 2-undecanone, and geraniol acetate from GW plants derived from in vitro propagated plantlets showed higher concentrations than from traditionally grown GW. In contrast, traditionally propagated GY possessed higher levels of (*E*)-citral and 2-undecanone than the corresponding plants derived from in vitro propagated GY. In general, however, the composition and content matched very closely between growth treatments, and even between the three lines. They were not significantly different at the $P < 0.05$ level (determined with 2-factorial ANOVA).

It is difficult to distinguish the three ginger cultivars (GBR, GW, and GY) from their aerial parts, such as leaves and shoots. However, they can be easily identified from the appearance of their rhizomes (see Fig. 1). Rhizomes of yellow ginger (GY) are smaller in size than those of GBR (blue ring ginger) and GW (white ginger), and the color of the rhizome cross-section is yellow. GBR and GW rhizome cross-sections are whitish in color, but GBR has a light blue ring close to the epidermal layer. These three lines are the most widespread and popular ginger lines cultivated in and sold on the United States market for culinary use. Their volatile metabolite profiles, based on this investigation of these lines, are almost identical qualitatively. Any quantitative differences for specific compounds did not appear at first glance to be significant.

Table 3
Molecule classification of the metabolites of the ginger rhizome samples (rhizomes of 3 lines from in vitro micropropagation derived and greenhouse grown plants) and the total ginger samples (including all tissues and treatments from all three lines)

	Ginger rhizome samples		Total ginger samples	
	Compounds	% of Total	Compounds	% of Total
Monoterpenoid	29	28.4	33	25.2
Sesquiterpenoid	47	46.1	54	41.2
Diterpenoid	3	2.9	4	3.1
Gingerol-related	16	15.7	18	13.7
Others	7	6.9	22	16.8
Total	102	100	131	100

2.2.1.2. Comparison of ginger tissues using GC/MS-based metabolic profiling. In addition to the comparison of the rhizomes of three ginger lines, we also used metabolic profiling to evaluate the chemical composition of different tissues of one of these lines, GW. One hundred thirty-one compounds were identified in extracts of different tissues (rhizomes, roots, leaves, and shoots) from GW plants derived from in vitro propagated plantlets. These included 91 terpenoids and 18 gingerol-related compounds (gingerols, shogaols and paradols) as listed in Tables 2 and 3. The major compounds in the various tissues are: rhizome: α-zingiberene (19%), (*E*,*E*)-α-farnesene (15%), and geranyl acetate (7%); root: α-zingiberene (24%), camphene (17%), and β-sesquiphellandrene (14%); leaf: (*E*)-caryophyllene (14%), linolenic acid (8%), and *n*-hexadecanoic acid (7%); and shoot: (*E*)-caryophyllene (40%), *n*-hexadecanoic acid (12%), and β-phellandrene (7%). The rhizome possesses 78% of the compounds identified from all tissues, and 48% of these were only found in the rhizome. The root had 33% of all compounds, with only 7% of these being root-specific. The root shared 88% of its compounds with the rhizome. The leaf contained 39% of the total compounds, with 29% of these being leaf-specific. And the shoot possessed 21% of the total compounds, with none being shoot-specific. The shoot shared 96% of its compounds with the leaf. Table 4 lists the number of

Table 4
Number of GC/MS identified compounds shared by different tissue types

	Rhizome	Root	Leaf	Shoot
Rhizome	102:49	38:26	26:6	17:0
Root		43:3	13:0	10:0
Leaf			51:15	26:9
Shoot				27:0

Note: For 38:26; "38" is the total number of compounds shared by both rhizome and root, "26" indicates the number of compounds found only in the rhizome and root and not in other tissues.

compounds shared between tissues and specific to each tissue pair when compared to other tissue types.

As can readily be seen in Tables 2 and 3, the different tissues presented very different metabolic profiles, with most gingerol-related compounds (89% of those identified) being restricted to the rhizome, whereas most of the mono- and sesquiterpenoids were found in multiple tissues. Nevertheless, there were a number of terpenoids that were found in only one tissue. These included, among others, compounds such as citronellal found only in the rhizome, β-sesquiphellandrene found in the rhizome and root, *p*-menth-1-en-8-ol in the rhizome and leaf, and α-caryophyllene found in the root and leaf and but not in the rhizome. These results suggested that differing mechanisms must be in play that lead to differential production and/or accumulation of specific metabolites in these different ginger tissues.

2.2.1.3. Principal component analysis (PCA) of GC/MS data. Principal component analysis (PCA) was employed in an effort to distinguish the metabolic profiles of the three ginger lines (GBR, GW and GY), and of different growth treatments (conventional greenhouse grown vs. in vitro propagation derived plants) and different tissues (rhizome, root, leaf and shoot) of in vitro propagated GW. Two general comparisons were made. In the first (rhizome samples only), we compared only the GC/MS profiles from the rhizome samples for the three lines. This included the samples grown under different conditions. In the second comparison (all tissues), we included all tissues in the analysis, leaf, root, shoot and rhizome, from all three lines under the different growth conditions. From the first analysis (rhizome samples only), three principle components were generated, which represented >99% of the observed variance in the sample set (see Table 4). From the second analysis (all tissues), four separate principle components were generated, representing 98.6% of the variance (Table 5). Loading variables of >0.45 were selected as high-loading, as defined by Comrey and Lee (1992), and used to generate clusters of compounds that appeared to be distinguished by principal components (see Table 6, Supplementary Table 3). In the dataset from the first analysis (comparing only rhizome samples), none of the principal components (PC-1, PC-2 or PC-3) could be clustered or separated between all three lines or between all growth treatments (see Figs. 3a–c). However, in the second comparison (including all tissues from all lines), PC-2 and PC-3 could be effectively clustered and separated between the four different tissues (see Figs. 3d–i), supporting the conclusions discussed above (Section 2.2.1.2) regarding the differences in metabolic profiles that exist between ginger tissues. Furthermore, when rhizome samples from line GY were compared in this analysis to rhizome samples from lines GW and GBR, both PCA and HCA results (see below) suggested that the rhizomes of line GY differed significantly from the other two, but that the other two lines were indistinguishable.

Table 5
Percentage of total variance accounted for by combinations of extracted principal components of the ginger rhizome samples (rhizomes of 3 lines from in vitro micropropagation derived and greenhouse grown plants) and the total ginger samples (including all tissues and treatments from all three lines)

Component	Initial eigenvalues		
	Total	% of Variance	Cumulative %
1. The first three principal components of ginger rhizome samples			
1	98.033	96.111	96.111
2	2.102	2.061	98.172
3	1.038	1.018	99.190
2. The first four principal components of total ginger samples			
1	89.947	68.662	68.662
2	28.598	21.830	90.492
3	8.739	6.671	97.164
4	1.943	1.483	98.647

Table 6
Compound classification of the metabolites loading highly in factors of PCA component matrices for the extracted principal components of 1. the ginger rhizome samples (rhizomes of 3 lines from in vitro micropropagation derived and greenhouse grown plants) and 2. the total ginger samples (including all tissues and treatments from all three lines)

	Monoterpenoid	Sesquiterpenoid	Diterpenoid	Gingerol-related	Others	Total
1. The first three principal components of ginger rhizome samples						
PC-1	28	47	3	15	7	100
PC-2	1	0	0	0	0	1
PC-3	0	0	1	0	0	1
2. The first four principal components of total ginger samples						
PC-1	21	43	3	15	1	83
PC-2	13	8	1	2	16	40
PC-3	5	5	0	0	4	14
PC-4	0	1	0	0	1	2

Note: Numbers represent the number of molecules from the stated class that load high in specific principal components.

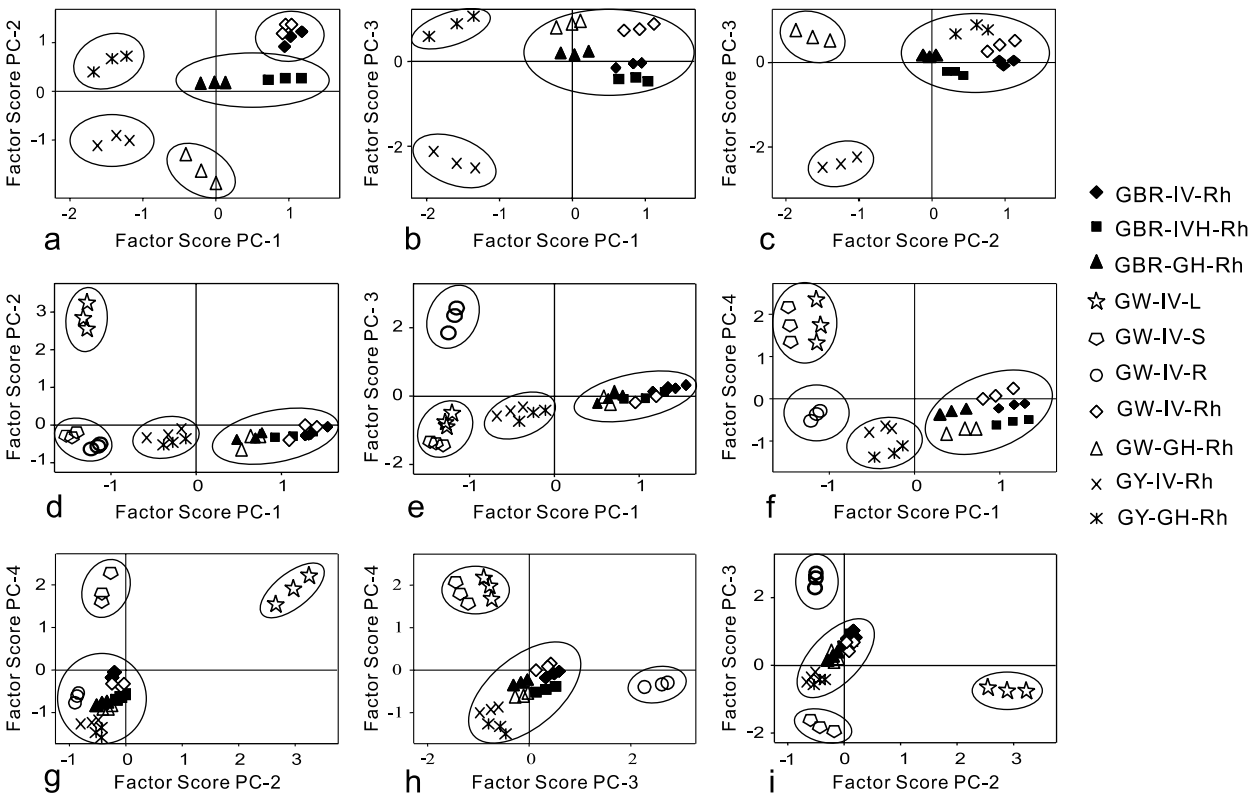

Fig. 3. Scatter plots of PCA factor scores for ginger rhizome samples (rhizomes of 3 lines from in vitro micropropagation derived and greenhouse grown plants, a–c, PC-1-3) and all ginger samples (including all tissues and treatments from all three lines, d–i, PC-1-4). Axes of two-dimensional plots are derived from (a) PC-1 and PC-2, (b) PC-1 and PC-3, and (c) PC-2 and PC-3 of rhizome samples; (d) PC-1 and PC-2, (e) PC-1 and PC-3, (f) PC-1 and PC-4, (g) PC-2 and PC-4, (h) PC-3 and PC-4, and (i) PC-2 and PC-3 of all ginger samples. Plotted points represent individual samples, while arbitrary ellipses or circles have been included to assist interpretation. This PCA analysis represents the differentiation of 21 individual rhizome samples (biological triplicates of GBR-IV, GBR-IVH, GBR-GH, GW-IV, GW-GH, GY-IV and GY-GH rhizomes) and 30 individual samples for all ginger samples (biological triplicates of GW-IV-Rh, GW-IV-R, GW-IV-L and GW-IV-S, with GBR-IV, GBR-IVH, GBR-GH, GW-GH, GY-IV and GY-GH rhizomes). *Note*: abbreviations used to define samples are as follows: IV = plants derived from in vitro propagated plantlets; IVH = hydroponically grown plants derived from in vitro propagated plantlets; GH = plants from traditionally greenhouse propagation. Rh = rhizome; R = root; L = leaf; S = shoot.

Interestingly, ginger rhizome samples from different growth treatments were grouped together for each ginger line in this second analysis, indicating that the in vitro propagation procedure had little or no significant effect on metabolism in ginger rhizomes.

2.2.1.4. Hierarchical cluster analysis (HCA). To further explore the relationships between different lines, treatments and tissues, hierarchical cluster analysis (HCA) of the GC/MS data was performed. A heatmap was developed based on the HCA results comparing the different samples to the peak areas of the identified compounds (see Fig. 4). Three major clusters among the sample treatments could be identified in this analysis: cluster **A** contained all of the rhizome samples, with the samples from line GY forming a subcluster separated from the GBR and GW rhizome samples; cluster **B** contained all root samples; and cluster **C** contained the leaf and shoot samples. In addition, the rhizome samples derived from in vitro propagated plantlets formed closely aligned, but distinct groups from the rhizome samples from traditionally greenhouse grown plants.

In addition, five major clusters among the 131 compounds could be identified in this analysis. Clusters **1**, **2**, **3**, **4** and **5** have 6, 39, 6, 10, and 70 compounds, respectively (see Fig. 4 and Table 7, and Supplementary Table 4). Notably, rhizome samples in cluster **C** possess the highest levels of compounds in cluster **5,** including 78% of total gingerol-related compounds, 75% of total diterpenoids, 70% of total sesquiterpenoids, and 42% of total monoterpenoids. However, compounds belonging to the "others" class were only 4% of the total in this cluster. In contrast, leaf and shoot samples in cluster **A** contained high levels of compounds belonging to cluster **2**, including large numbers of other compounds (86% of total others), 27% of total monoterpenoids, 25% of total diterpenoids, 15% of total sesquiterpenoids and 11% of total gingerol-related compounds. Cluster **1** contained mostly compounds that were found to be highly abundant in the root (cluster **B**), including 9% of total others, 6% of total gingerol-related compounds, 6% of total monoterpenoids and 2% of total sesquiterpenoids. No diterpenoids were found in this cluster. In addition, the compound [9]-paradol (peak no. 124) was assigned to this cluster, but unlike the rest of the compounds in cluster 1, it was found at high levels only in the rhizome of GY ginger. Clusters **3** and **4** contained compounds that were

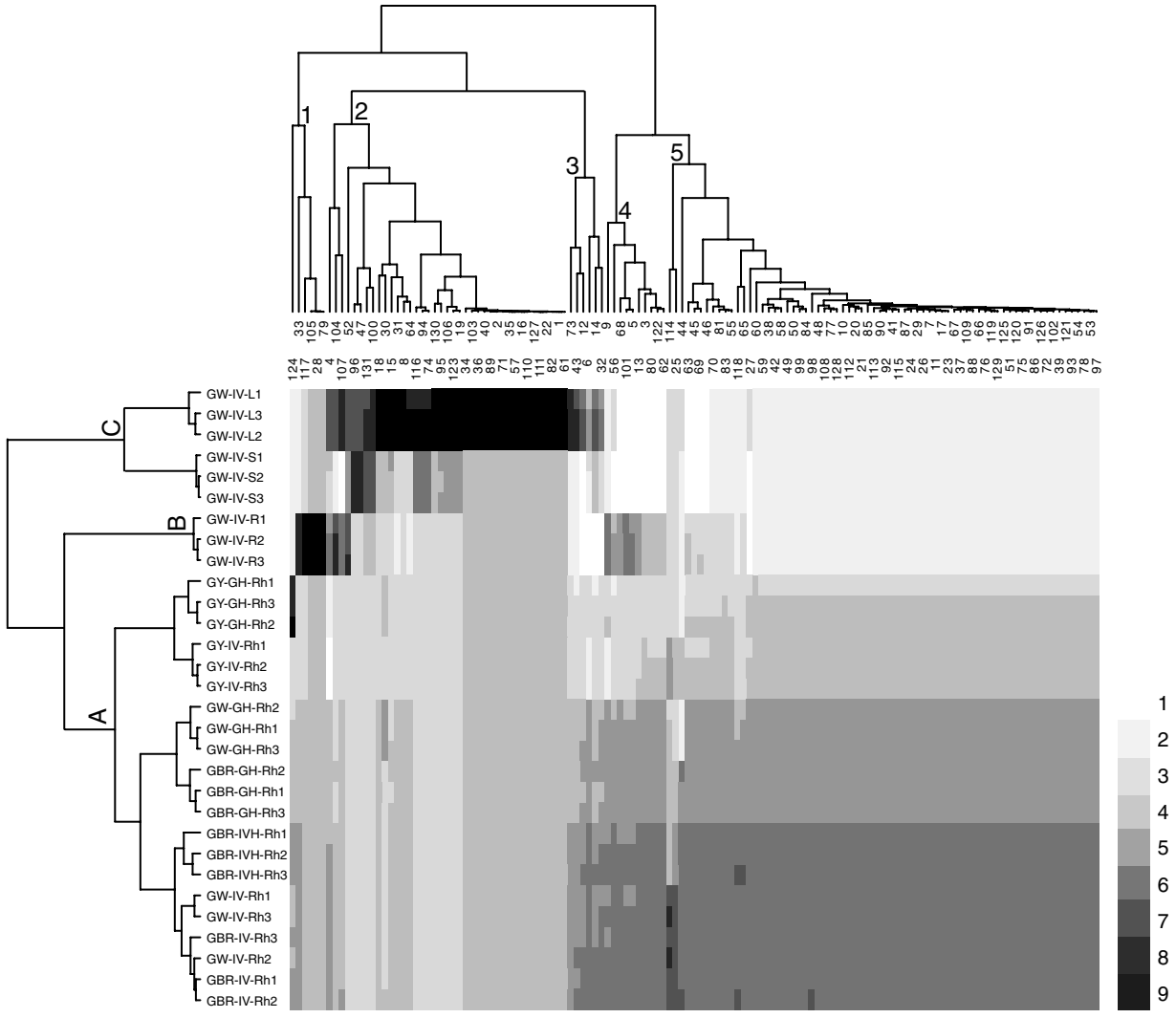

Fig. 4. Hierarchical clustering results comparing metabolic profiles of ginger samples to growth and tissue treatments. 131 compounds from different tissues and growth treatments of 3 ginger lines (GBR, GW and GY) were compared. Clusters **1**, **2**, **3**, **4**, and **5** represent groupings of compounds. Clusters **A**, **B**, and **C** show the cluster groups of 30 individual plant samples (biological triplicates of GBR-IV-Rh, GBR-IVH-Rh, GBR-GH-Rh, GW-GH-Rh, GY-IV-Rh, GY-GH-Rh, GW-IV-Rh, GW-IV-R, GW-IV-L and GW-IV-S). *Note*: abbreviations to define samples as for Fig. 5.

Table 7
Number of compounds from each compound class belonging to each HCA cluster

Cluster	Compound class					Total
	Monoterpenoid	Sesquiterpenoid	Diterpenoid	Gingerol-related	Others	
1	2	1	0	1	2	6
2	9	8	1	2	19	39
3	4	2	0	0	0	6
4	4	5	0	1	0	10
5	14	38	3	14	1	70
Total	33	54	4	18	22	131

found in multiple tissues and that belonged to multiple classes of compounds.

2.2.2. LC-ESI-MS based comparison of three ginger lines

The pungency of fresh ginger is due to a series of homologous phenolic ketones, of which [6]-gingerol is the most abundant. The gingerols (see Fig. 5) are thermally unstable and can be readily converted by exposure to air to their corresponding shogaols, which are abundant in dried ginger. Fresh rhizomes of the three ginger lines (GBR, GW and GY) were assayed in triplicate by LC-ESI-MS/MS to determine the composition and relative content of gingerol-related compounds (see Supplementary Fig. 3). In total, 31 gingerol-related compounds

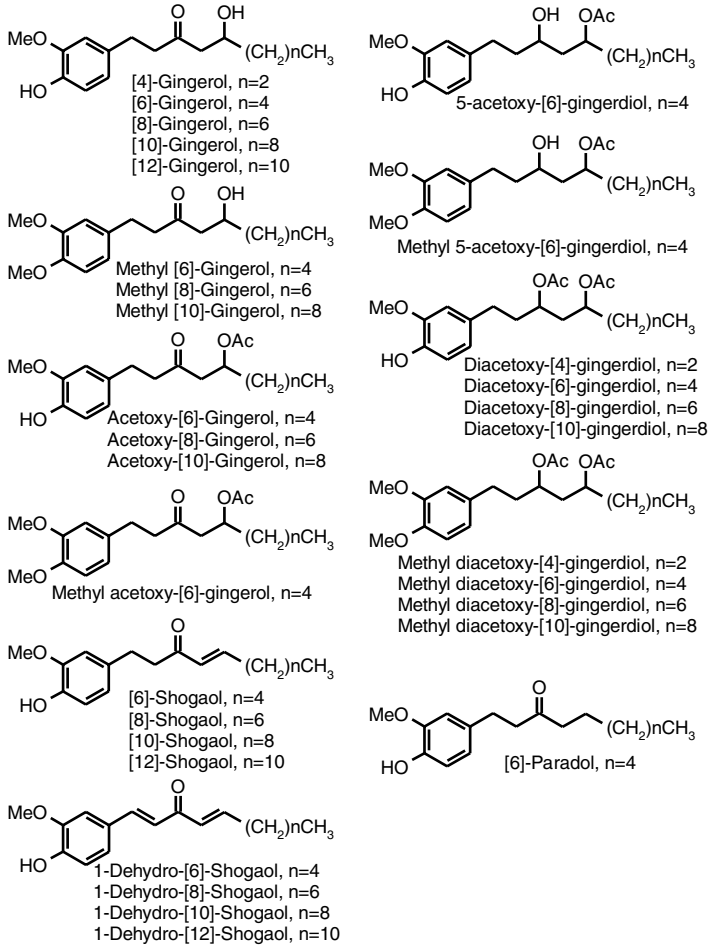

Fig. 5. Structures of gingerol-related compounds identified by LC-ESI-MS analysis.

were identified in extracts from plants derived from in vitro propagated plantlets or from traditionally propagated rhizomes, as well as in extracts from hydroponically grown plants derived from in vitro propagated plantlets (see Fig. 5 and Table 8). All of these compounds were present in all samples. Moreover, [6]-gingerol was identified as the major pungent phenolic compound in all samples, whereas [8]- and [10]-gingerol were found at lower concentrations. Although other reports have claimed that the shogaols do not naturally occur in live ginger (Wohlmuth et al., 2005), and have been presumed to the by products of dehydration of the gingerols when ginger rhizomes are dried, we found significant levels of the shogaols in freshly prepared samples, suggesting that these plants do in fact accumulate these compounds, although the means by which they are formed from the gingerols in fresh tissue is yet to be determined.

Interestingly, unlike most of the terpenoid constituents of the ginger rhizomes (see Table 2), which were pretty constant in concentration between the different lines and between growth regimes, the levels of some of the gingerol-related compounds differed greatly from line to line (see Table 8). In addition to these metabolic profiling based results, we also evaluated the concentrations of the three major gingerols ([6]-, [8]-, and [10]-gingerol) in a more quantitative manner using the LC-MS. Calibration curves were first derived from three independent injections of five concentrations of [6], [8]-, and [10]-gingerol. Linearity in peak area versus injection amount for all three compounds was found in the concentration range between 10 and 250 μg ml^{-1}, with high reproducibility and accuracy. Regression analysis of the calibration curve data points showed a linear relationship with excellent correlation coefficients (r^2) for [6]-, [8]-, and [10]-gingerol, being 0.9979, 0.9984, and 0.9968, respectively, suggesting high precision in this analysis. The linear regression equations for the curves of [6]-, [8]-, and [10]-gingerol concentrations were $y = 577126x - 371280$, $y = 169728x - 22343$, and $y = 189936x - 24348$, respectively, where x was the concentration of standard gingerol (μg ml^{-1}) and y was the total peak area. The content of gingerols in in vitro propagated and greenhouse grown samples were then determined by LC-MS. The gingerol content varied from 1.39 to 2.25 mg/g for [6]-gingerol, and 0.32 to 1.15 mg/g for both [8]-, and [10]-gingerol, respectively (see Table 9). These results support the contention that ginger from different sources is in fact likely

Table 8
Relative content of gingerol-related compounds identified by LC-ESI-MS based metabolic profiling of one year old plant rhizomes that were produced from in vitro micropropagated plantlets (IV), hydroponically grown from in vitro micropropagated plantlets (IVH), or produced from greenhouse grown plants (GH) of three ginger lines (GBR, GW and GY)

RT	Name	Formula	MW	GBR			GW		GY	
				IV	IVH	GH	IV	GH	IV	GH
25.92	[4]-Gingerol	$C_{15}H_{22}O_4$	266	3	3	3	3	3	3	3
32.89	[6]-Gingerol	$C_{17}H_{26}O_4$	294	3	3	3	3	3	3	3
36.02	Methyl [6]-gingerol	$C_{18}H_{28}O_4$	308	1	1	1	1	1	1	1
36.44	3- or 5-Acetoxy-[6]-gingerdiol	$C_{19}H_{30}O_5$	338	1	1	1	1	1	1	1
37.06	Diacetoxy-[4]-gingerdiol	$C_{19}H_{26}O_6$	350	3	2	3	3	3	1	1
38.91	Acetoxy-[6]-gingerdiol	$C_{19}H_{28}O_5$	336	3	3	3	3	2	1	1
39.03	[8]-Gingerol	$C_{19}H_{30}O_4$	322	2	2	2	2	1	1	1
39.48	Methyl 3- or 5- acetoxy-[6]-gingerdiol	$C_{20}H_{32}O_5$	352	3	3	3	2	2	1	1
39.91	[6]-Shogaol	$C_{17}H_{24}O_3$	276	1	1	1	1	1	1	1
40.35	Methyl diacetoxy-[4]-gingerdiol	$C_{20}H_{28}O_6$	364	2	2	2	2	2	1	1
41.80	Methyl [8]-gingerol	$C_{20}H_{32}O_4$	336	3	3	3	3	3	2	2
42.24	Methyl acetoxy-[6]-gingerol	$C_{20}H_{30}O_5$	350	2	2	2	2	2	1	1
42.57	Diacetoxy-[6]-gingerdiol	$C_{21}H_{32}O_6$	380	3	3	3	3	3	3	3
42.78	[6]-Paradol	$C_{17}H_{26}O_3$	278	1	1	1	1	1	1	1
43.31	1-Dehydro-[6]-gingerdione	$C_{17}H_{22}O_3$	274	3	3	3	3	2	2	2
44.44	Acetoxy-[8]-gingerol	$C_{21}H_{32}O_5$	364	1	1	1	1	1	1	1
44.59	[10]-Gingerol	$C_{20}H_{32}O_4$	336	2	2	2	2	2	1	1
45.17	Methyl diacetoxy-[6]-gingerdiol	$C_{22}H_{34}O_6$	394	1	1	1	1	1	1	1
45.88	[8]-Shogaol	$C_{19}H_{28}O_3$	304	1	1	1	1	1	1	1
47.41	Methyl [10]-gingerol	$C_{21}H_{34}O_4$	350	1	1	1	1	1	1	1
47.56	Diacetoxy-[8]-gingerdiol	$C_{23}H_{36}O_6$	398	1	1	1	1	1	1	1
48.78	1-Dehydro-[8]-gingerdione	$C_{19}H_{26}O_3$	302	1	1	1	1	1	1	1
49.82	Acetoxy-[10]-gingerol	$C_{22}H_{34}O_5$	378	3	3	2	2	2	1	1
50.24	[12]-gingerol	$C_{23}H_{38}O_4$	378	2	2	2	2	2	3	1
50.47	Methyl diacetoxyl-[8]-gingerdiol	$C_{24}H_{38}O_6$	412	2	2	2	1	1	2	2
51.43	[10]-Shogaol	$C_{21}H_{32}O_3$	332	1	1	1	2	2	3	1
52.75	Diacetoxy-[10]-gingerdiol	$C_{24}H_{38}O_6$	412	2	2	2	1	1	3	3
53.92	1-Dehydro-[10]-gingerdione	$C_{21}H_{28}O_3$	328	3	3	3	1	1	3	3
55.18	Methyl diacetoxyl-[10]-gingerdiol	$C_{25}H_{40}O_6$	426	3	3	3	3	2	2	2
56.34	[12]-Shogaol	$C_{23}H_{36}O_3$	360	3	3	3	3	3	3	3
58.03	1-Dehydro-12-gingerdione	$C_{23}H_{34}O_3$	358	2	2	1	3	2	1	1

Note: 1 indicates <0.5%, 2 indicates 0.5–5%, and 3 indicates >5% of mass peak area of a particular sample.

Table 9
Quantitative comparison of gingerol content in rhizomes of one year old plants that were produced from in vitro micropropagated plantlets (IV), hydroponically grown from in vitro micropropagated plantlets (IVH), or produced from greenhouse grown plants (GH) of three ginger lines (GBR, GW and GY)

	GBR			GW		GY	
	Greenhouse grown	In vitro propagated		Greenhouse grown	In vitro propagated	Greenhouse grown	In vitro propagated
		Pots	Hydroponic				
[6]-Gingerol	1.823 ± 0.010	2.247 ± 0.02	2.081 ± 0.014	1.553 ± 0.011	1.840 ± 0.018	1.385 ± 0.004	1.740 ± 0.036
[8]-Gingerol	0.542 ± 0.024	0.924 ± 0.011	0.846 ± 0.007	0.484 ± 0.005	0.712 ± 0.006	0.319 ± 0.002	0.482 ± 0.004
[10]-Gingerol	0.781 ± 0.004	1.154 ± 0.009	1.058 ± 0.002	0.757 ± 0.003	0.954 ± 0.024	0.492 ± 0.005	0.832 ± 0.012

Note: All values are given in mg/g fresh weight, average of triplicate biological samples ± SE.

to possess different properties, both in flavor and pungency, but also in potential bioactivity and health benefits.

In addition to the gingerols, several members of the biogenetically related diarylheptanoid class were also identified in these plants using LC-MS based metabolic profiling. Diarylheptanoids belong to a class of natural products with a 1,7-diarylheptane skeleton (Roughley and Whiting, 1971) that include the curcuminoids, characteristic medicinal compounds of turmeric, a relative of ginger in the Zingiberaceae. Diarylheptanoids have been found to possess a variety of biological and pharmacological activities including antioxidant, antihepatotoxic, antiinflammatory, antiproliferative, antiemetic, chemopreventive, and antitumor activities (Hikino et al., 1985; Flynn et al., 1986; Ishida et al., 2000; Ali et al., 2001; Ishida et al., 2002; Shin et al., 2002; Egan et al., 2004; Masuda et al., 2004; Sharma et al., 2004), leading to increased interest in recent years in this group of compounds. LC-ESI-MS/MS analysis of the rhizomes of the three ginger lines and performed for this investigation identified eight

diarylheptanoids as being highly abundant (see Table 10 and Fig. 6 and Supplementary Fig. 3). Interestingly, several of these compounds differed in concentration between the different lines (see Table 10) and between growth treatments.

2.3. Conclusions

The in vitro propagation procedure described in this report allows for conservation and rapid amplification of highly productive ginger stocks, such as stocks high in particular bioactive constituents like [6]-gingerol, or of new varieties of ginger. In addition, a unique, safe, and effective method for explant decontamination was presented. This method will allow for the serious problem of microbial contamination in ginger to be easily overcome. Because the rhizomes can be produced in vitro, a highly controlled environment can be utilized for metabolism investigations in ginger. Metabolic profiling of plants derived from in vitro propagated plantlets showed no qualitative differences in major volatile or non-volatile compound composition when compared to traditionally propagated plants. PCA and HCA supported the conclusion that no significant differences existed in the metabolic

Table 10
Relative content of diarylheptanoids identified by LC-ESI-MS based metabolic profiling of one year old plant rhizomes that were produced from in vitro micropropagated plantlets (IV), hydroponically grown from in vitro micropropagated plantlets (IVH), or produced from greenhouse grown plants (GH) of three ginger lines (GBR, GW and GY)

RT	Name	Formula	MW	GBR			GW		GY	
				IV	IVH	GH	IV	GH	IV	GH
19.9	5-Hydroxy-1-(3,4-dihydroxy-5-methoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone (diarylheptanoid 1)	$C_{21}H_{26}O_7$	390	1	1	1	1	1	1	1
22.3	5-Hydroxy-l-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone (diarylheptanoid 2)	$C_{22}H_{28}O_7$	404	1	1	1	1	1	1	1
22.7	5-Hydroxy-l,7-bis(4-hydroxy-3-methoxyphenyl)-3-heptanone (diarylheptanoid 3)	$C_{21}H_{26}O_6$	374	1	1	1	1	1	1	1
25.1	3,5-Diacetoxy-1,7-bis(3,4-dihydroxyphenyl)heptane (diarylheptanoid 4)	$C_{23}H_{28}O_8$	432	2	2	2	2	2	2	2
28.3	3,5-Diacetoxy-7-(3,4-dihydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)heptane (diarylheptanoid 5)	$C_{24}H_{30}O_8$	446	2	2	2	2	1	1	1
30.7	3,5-Diacetoxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)heptane (diarylheptanoid 6)	$C_{26}H_{34}O_9$	490	3	3	2	2	2	3	3
31.3	3,5-Diacetoxy-1,7-bis(4-hydroxy-3-methoxyphenyl)heptane (diarylheptanoid 7)	$C_{25}H_{32}O_8$	460	2	2	2	2	1	1	1
31.9	Dihydrocurcumin	$C_{21}H_{22}O_6$	370	1	1	1	1	1	1	1

Note: 1 indicates <0.5%, 2 indicates 0.5–5%, and 3 indicates >5% of mass peak area of a particular sample.

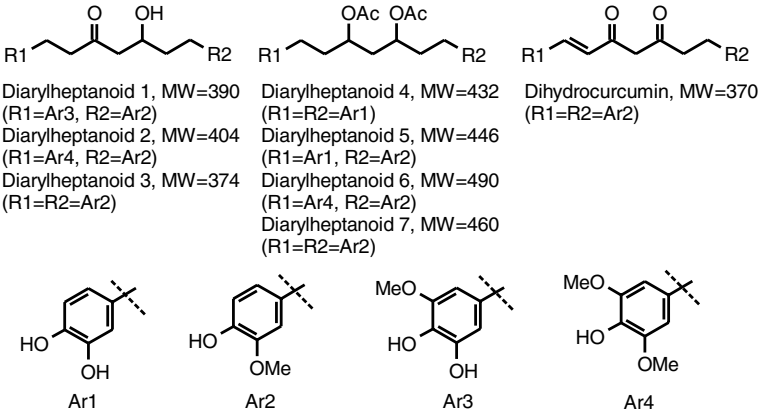

Fig. 6. Structures of diarylheptanoids identified by LC-ESI-MS analysis. Trivial names indicated correspond to those given in Table 4 to aid in identification of the correct structures, due to the long standard names of the diarylheptanoids.

profiles between in vitro propagated and traditionally greenhouse grown plants, at least for the compounds that we were able to detect in our analysis. Targeted metabolite analysis, however, indicated that some significant quantitative differences did exist between these ginger lines and even between growth treatments, suggesting that although the tendency to produce specific compounds was under genetic control, the level of production may also be affected by environmental factors. For example, media conditions used for micropropagation, including differences in concentrations of specific growth regulators, appear to have led to quantitative differences in accumulation of compounds such as [6]-, [8]-, and [10]-gingerols in the resulting plants. Modulation of these media conditions could potentially produce ginger plants with desired complements of metabolites, with potential medicinal application. Nevertheless, when specific conditions were applied during in vitro micropropagation, ginger plants with essentially the same metabolic profiles as the parental lines could be produced. Finally, callus production and successful induction of plantlets from callus establishes an ideal platform for future transgenic research in Zingiberaceae plants.

3. Experimental

3.1. Chemicals and reagents

Gamborg's B-5 basal medium with minimal organics, Phytagel, naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid were purchased from Sigma (St Louis, MO, USA). Acetonitrile and methanol were from Burdick & Jackson (Muskegon, MI). Methyl *t*-butyl ether (MTBE, High Purity Solvent) was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Authentic standards of [6]-gingerol, [8]-gingerol, and [10]-gingerol were purchased from ChromaDex, Inc. (Santa Ana, CA).

3.2. Plant material

The rhizomes of ginger (*Z. officinale*) were obtained from different sources. Blue ring ginger (GBR) was purchased from a supermarket in Tucson, Arizona; white ginger (GW) and yellow ginger (GY) were obtained from Dean Pinner at Pinner Creek Organics, Hilo, Hawaii. Plant identification was performed by Dr. Steven P. McLaughlin at the University of Arizona Herbarium. Voucher specimens were deposited in the University of Arizona Herbarium.

The plants used for this analysis were grown in the same greenhouse at the University of Arizona in five gallon pots, in Scott's Metromix soil, and watered by drip irrigation. Fresh young rhizomes, leaves, shoots and roots were collected on the same day in the middle of October (one month prior to dormancy onset) for chemical extraction. The collected plant material was immediately frozen in liquid nitrogen, and kept at −80 °C until analyzed.

Each sample consisted of three replicates and each replicate was a bulk of three individual plants from three pots. 20 g tissue from three individual plants were mixed and ground under N_2(l). Two separate 1 g aliquots were taken from each ground bulked sample and processed for GC/MS and LC-MS analysis, respectively, according to a previously published method (Jiang et al., 2005a).

3.3. In vitro micropropagation

No differences were observed in propagation efficiency from the individual plant within each line. Therefore, except for Table 1, all data are shown for ginger line GBR.

3.3.1. Explants and pretreatment

Different plant parts, such as rhizome buds, shoot tips, leaf bases, and inflorescence parts were selected as potential explants. Prior to placement on culture media, rhizomes were pretreated in the following manner: washed briefly with Tween 20 (0.06%), rinsed with tap water for 5 min, soaked in hot water (50 °C) for 10 min, transferred to laminar flow hood, washed 3–4 times with sterile double distilled H_2O (ddH_2O), soaked for 30 min in bleach (10%), rinsed once with sterile ddH_2O, buds (5–20 mm) dissected using a sterile scalpel, dissected buds treated with 0.5% PPM (plant preservative mixture, Plant Cell Technology, Inc., Washington, DC) for 5–10 min, rinsed again in sterile ddH_2O, submerged in EtOH–H_2O (7:3) for 1–2 min, and sterilized buds cultured on selected media. Shoot tips and leaf bases were treated as just described from the bleach step to the end of the protocol. Anthers, petals, and ovaries from unopened ginger inflorescences were directly dissected and put on media after the bracts were carefully removed, without further sterilization or treatment.

3.3.2. Culture medium and conditions

Medium for development (M1): B_5 medium containing sucrose (3%), ascorbic acid (AA, 100 mg l^{-1}), BA (0.5 mg l^{-1}), thidiazuron (TDZ, 0.1 mg l^{-1}), and phytagel (0.8%); Medium for callus induction from rhizome buds, shoot tips, leaf bases, and inflorescences (petals, anthers and ovaries) (M2): B_5 medium containing sucrose (3%), 2,4-D (1.5–5 mg l^{-1}), and phytagel (0.8%); Medium for root induction (M3): Three-quarter strength B_5 medium containing sucrose (1.5%), AA (100 mg $\cdot$ l^{-1}), and phytagel (0.8%) without growth regulators; Medium for micropropagation (M4): B_5 medium containing sucrose (2%) and phytagel (0.8%) with different concentrations of BA, IAA, NAA, and Kn. This medium without growth regulators (basal M4 medium) served as control.

In all the cases the pH of the medium was adjusted to 5.8 prior to autoclaving. The plantlet cultures were maintained at 27 ± 2 °C under a 16 h photoperiod (fluorescent cool white light, 100 μmol m^{-2} s^{-1}). Callus cultures were maintained at 25 ± 2 °C under darkness.

3.3.3. Shoot multiplication, root induction, and ex vitro establishment

Shoots obtained from cultured buds were subcultured once on M1 medium to allow further development and then transferred to M4 medium for multiplication. Six weeks later, the first subculture on M4 medium was performed and the expended leaves and roots were trimmed away. This procedure was applied to eliminate synergistic effects of growth regulators used during earlier culture. The numbers and lengths of shoots and roots were recorded. In most combinations of media, some shoots in a cluster were produced without roots. Such shoots were transferred to basal M3 medium to induce root formation.

Plantlets with well developed roots were thoroughly washed in running tap water to remove adhering medium, and transplanted to hydroponic culture or to pots containing soil and placed directly in the greenhouse without acclimatization. The potted plants (Figs. 1d and g) were maintained under greenhouse conditions (Jiang et al., 2005a) and regularly irrigated with tap water. Hydroponics culture (Figs. 1i) was performed by placing plantlets in perlite under which hydroponic solution was circulated. The hydroponic solution consisted of tap water supplemented with micro and macro organic/inorganic elements. Stock solution A (100×) was produced by dissolving 12.3 kg $Ca(NO_3)_2$ and 800 g chelated iron in hot H_2O (2 l) and adding this to tap water (160 l). Stock solution B (100×) was produced by adding 6.344 kg KH_2PO_4, 3.570 kg KNO_3, 8.080 kg Epsom salts, 56.4 g $MnCl_2\,4H_2O$, 1.76 g $CuSO_4\,5H_2O$, 7.05 g $ZnSO_4\,7H_2O$, 1.03 g Na_2MoO_4 or 1.21 g $Na_2MoO_4\,2H_2O$ to about 150 l tap water, adding to this 27.43 g H_3BO_3 dissolved in hot water (2 l), and bringing to with tap water (160 l). Stock solutions A and B were added in equal parts to tap water (1 l each to 98 l water) to produce the working hydroponics solution. This recipe was based on the method of Dr. Howard Resh (Resh and Barbara, 1989).

3.4. Metabolic profiling analysis

Frozen fresh ginger rhizome, root, leaf or shoot tissue was ground into a fine powder in the presence of N_2(l) with a mortar and pestle and divided into 1 g aliquots for extraction with methyl *t*-butyl ether (MTBE) or MeOH, transferred to a 4 ml glass vial, covered with 2 ml of solvent (MTBE or MeOH) and capped with PTFE lined cap. After sonication for 30 min, each sample was centrifuged in the same glass vial in a SORVALL RC-5 Superspeed Refrigerated Centrifuge, GSA Rotor (Du Pont instruments, Norwalk, CT, USA) at 1500 rpm for 25 min to pellet the ground plant material and then filtered through an Acrodisc® CR 13 mm syringe filter with 0.20 μm PTFE membrane. The MTBE filtrate was used directly for GC/MS analysis. The MeOH extracts were used for gingerol quantitation by LC-ESI-MS analysis. Triplicate extracts were used for quantitation analysis. The samples were never dried prior to analysis.

For GC/MS analysis, a Thermo Electron Trace GC ultra coupled to a DSQ mass spectrometer was equipped with an Alltech EOONO-CAP™-EC™-5 capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness). UHP helium was used as the carrier gas at a flow rate of 1.2 ml/min. Oven temperature was set initially at 40 °C, for 2 min, then raised to 100 °C at 8 °C min^{-1} and held at 100 °C for 3.5 min, then increased to 280 °C at 3 °C min^{-1}, and then raised to 300 °C at 10 °C min^{-1} and held at 300 °C for 3.5 min prior to column reequilibration at 40 °C. The injector/transfer line/trap temperatures were 220/250/200 °C, respectively. Electron impact ionization was carried out at 70 eV. Eluted compounds were identified using the NIST Mass Spectral library Version 2.0 (NIST/EPA/NIH, USA) and essential oil library of HP CHEMSTATION from Dr. Robert P. Adams, and by referring to publications from Jolad et al. (2004) and Jiang et al. (2005b, 2006a,b,c).

For LC-ESI-MS/MS analysis, a Thermo Electron Surveyor HPLC system coupled to an LCQ Advantage mass spectrometer was used. The HPLC was equipped with a Discovery® HS C18 column, 150 × 2.1 mm, i.d., 3 μm, with guard column (Supelco, Bellefonte, PA, USA), a photodiode array (PDA) detector. The mass spectrometer was equipped with an electrospray ion source. The HPLC elution conditions were as follows: flow rate, 0.25 ml min^{-1}; column temperature, 40 °C; injection volume, 5 μl; gradient of solvent A (5 mM ammonium formate, 0.1% formic acid) and solvent B (CH_3CN): 0–2 min, 5% B; 2–57 min, 5–100% B; 57–60 min, 100% B; 60–65 min. 100–5% B; 65–75 min, 5% B. The acquisition parameters for MS were: negative mode; drying N_2 temperature, 350 °C, 10 l min^{-1}; nebulizer pressure 60 psi; HV capillary 4500 V; HV end plate offset −500 V; current capillary 62.3 nA; current end plate 1378.7; RF amplitude capillary exit −99.3 V; skimmer −40.0 V; mass range measured: 50–900 m/z. These were the optimized parameters for the maximum transmission of the gingerol-derived ions.

3.5. Data processing and statistical analysis

Xcalibur™ (version 1.4) system was used as instrument control and data processing platform for both GC/MS and LC-MS data collection, peak identification and measurement. Data reductions by principal components analysis (PCA) and classifications by hierarchical cluster analysis (HCA) were carried out using the Statistical Package for the Social Sciences (SPSS v12.0). The heatmap was developed using the R project for statistical computing (v2.2.1). Analysis of variance (ANOVA) were performed using the SAS system. All other intermediate data manipulation was carried out using Microsoft Excel 2000.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.07.012.

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