JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 9573−9583 **9573**

Metabolic Profiling of Turmeric (*Curcuma longa* L.) Plants Derived from in Vitro Micropropagation and Conventional Greenhouse Cultivation

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Turmeric (*Curcuma longa*) was considered only a culinary spice in many parts of the world until the notable anti-inflammation curcuminoids were discovered from this herb. Because it is a sterile triploid and is propagated vegetatively by rhizome division, turmeric is susceptible to pathogens that accumulate and are transmitted from generation to generation, and amplification of particularly useful stocks is a slow process. An in vitro propagation method has been developed to alleviate these problems. Metabolic profiling, using GC-MS and LC-ESI-MS, was used to determine if chemical differences existed between greenhouse-grown and in vitro micropropagation derived plants. The major chemical constituent curcuminoids, a group of diarylheptanoid compounds, as well as major mono- and sesquiterpenoids were identified and quantified. Principal component analysis and hierarchical cluster analysis revealed chemical differences between lines (T3C turmeric vs Hawaiian red turmeric) 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).

KEYWORDS: Diarylheptanoids; curcuminoids; curcumin; turmeric; *Curcuma longa*; GC-MS; LC-ESI-MS

INTRODUCTION

The rhizome of turmeric (*Curcuma longa* L., Zingiberaceae, see **Figure 1**) has been used for centuries in Asia to treat indigestion and a host of other ailments (*1*). However, it was considered only a culinary spice in many other parts of the world until the early 1970s (*2*, *3*), when the notable anti-inflammation curcuminoids, including curcumin, demethoxycurcumin, and bisdemethoxycurcumin that belong to the class of compounds known as diarylheptanoids, were discovered from this herb. Diarylheptanoids and sesquiterpenoids, the two major groups of natural products known from turmeric, have been shown to possess antioxidant, anticarcinogenic, and anti-inflammatory activities (*4*). The most important of these compounds and the most intensively studied by far is curcumin, which has been shown to also possess the remarkable activities of preventing or treating Alzheimer's disease, immunomodulation, and correcting cystic fibrosis defects, among others (*5*−*13*). Turmeric accumulates these important pharmacologically active metabolites at high levels in its rhizomes, >3% of the dry weight of the tissue for some of the above constituents (*14*, *15*).

Because of the high levels of these compounds in the rhizome, turmeric is an excellent model species to study rhizome metabolism. Investigations into the biosynthesis and function of such compounds in these plants, however, are currently limited by the ability to rapidly reproduce valuable stock lines. Turmeric, which is a sterile triploid, is clonally propagated via rhizome division and replanting. Because of this, it is susceptible to accumulation and transmittance of pathogens from generation to generation, and amplification of particularly useful stocks is a slow process. Turmeric and other members of the Zingiberaceae reproduce almost exclusively by asexual clonal propagation of the rhizomes. The risk of transmittance of diseases from one generation to the next is great, and *Pseudomonas solancearum* (bacterial wilt disease), *Fusarium oxysporum* f. sp. *zingiberi* (fusarium yellows disease), and *Pythium* species (soft/root rot) are transmitted in this manner (they remain in the tissue used as seed) and lead to significant losses to growers. It is estimated that a 3-fold increase in the production of rhizomes could be possible by the control of these diseases (*16*). Other investigators have reported in vitro propagation based methods that were developed in part to deal with this problem (*17*, *18*). Traditional tissue culture sterilization methods (such as bleach solutions) could not eliminate endogenous bacteria and fungi growing within turmeric explants. For this reason, these other reports for turmeric explant sterilization required the use of $HgCl_2$ treatment to eliminate microbial contamination (*17*, *18*). Nevertheless, >40% of explants were contaminated in these other studies. We have developed an in vitro propagation method (described herein) to alleviate these problems.

One concern with in vitro propagation methods is that the resulting plants may not possess the same properties (such as

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10.1021/jf061658k CCC: $33.50
Published on Web 11/10/2006

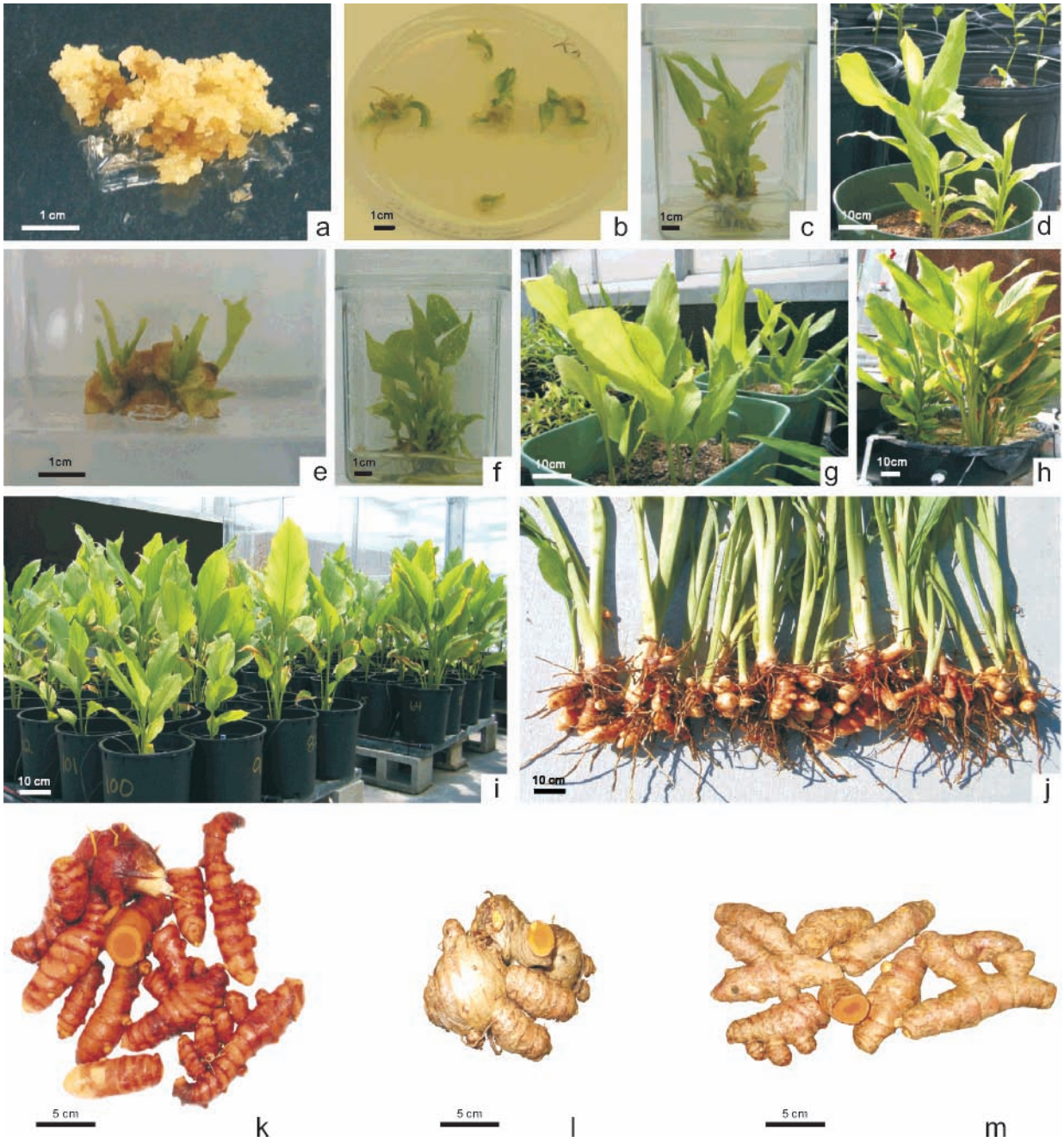

Figure 1. Callus cultures, multiple shoot formation, maintenance, and plant regeneration in in vitro propagated T3C turmeric: (**a**) callus induced from turmeric cotyledonary nodes after 4 weeks on B_5 medium containing sucrose (3%), 2,4-D (5 mg L^{-1}), and phytagel (0.8%); (**b**) turmeric plantlet induced from callus after 4 weeks on B_5 medium containing sucrose (3%), 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 turmeric, 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**) 2-month-old turmeric plants (derived from multiple shoot plantlets obtained from callus) growing in 20 L pots in the greenhouse; (**e**) shoot primordia from turmeric rhizome buds 3 weeks 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 turmeric 3 weeks after bud germination on B_5 medium containing sucrose (2%), Kn (2 mg L^{-1}), BA (1.0 mg L^{-1}), and phytagel (0.8%); (**g**) greenhouse-grown tumeric plants (from rhizomes) in 80 L totes in the greenhouse; (**h**) turmeric hydroponic culture (4-month-old plants) derived from multiple shoot plantlets obtained from bud explants; (**i**) turmeric plants from in vitro propagated and conventional rhizome derived randomly arranged growing in the same greenhouse; (**j**) in vitro propagated turmeric with rhizomes after 4 months of growth in the greenhouse; (**k**) in vitro propagated turmeric rhizomes followed by hydroponic growth in the greenhouse for 6 months prior to harvest; (**l**) rhizomes from in vitro propagated T3C turmeric plants; (**m**) rhizomes from in vitro propagated HRT turmeric plants.

the presence or concentration of specific metabolites) as the parents, due to somatic mutagenesis. Somatic mutation has often been observed with in vitro propagated plants (*19*−*22*). Because we wanted to develop an in vitro propagation method that allowed for rapid propagation of plants, to be used in our genomics-based metabolism investigations, we had to be certain that mutations that could lead to alterations in metabolism, which would then make such investigations impossible to perform, would not be introduced by the in vitro propagation method.

The aim of this study was to evaluate the effect that an efficient, economical, and safe in vitro propagation procedure for turmeric (*C. longa*) had on the chemical composition of the resulting plants. A metabolic profiling strategy was employed to determine whether there were differences between plants that

were derived from the in vitro propagation procedure and plants that had been propagated through traditional vegetative means. To our knowledge, no investigations have been reported that compare the metabolic profiles of turmeric plants derived from in vitro micropropagation with those produced by conventional cultivation techniques.

MATERIALS AND METHODS

Chemicals and Reagents. Gamborg's B-5 basal medium with minimal organics (B5), phytagel, naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) were purchased from Sigma (St. Louis, MO). Methanol and acetonitrile were purchased from Burdick & Jackson (Muskegon, MI). Methyl *tert*-butyl ether (MTBE; high-purity solvent) was from EMD Chemicals, Inc. (Gibbstown, NJ). Authentic standards of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were purchased from ChromaDex, Inc. (Santa Ana, CA). Plant Preservative Mixture (PPM) was purchased from Plant Cell Technology, Inc. (Washington, DC).

Plant Material. Turmeric (*C. longa*) rhizomes were obtained from two different sources. T3C turmeric (T3C) was from plants grown in a greenhouse at the University of Arizona; Hawaiian red turmeric (HRT) was obtained from Dean Pinner at Pinner Creek Organics, Hilo, HI. Voucher specimens of plants identified by Dr. Steven P. McLaughlin at the University of Arizona were deposited in the University of Arizona Herbarium.

Greenhouse Growth Conditions and Sample Collection. The plants used for this analysis were all grown as previously described (*23*) in Scott's Metromix soil in 20 L pots in the same greenhouse at the University of Arizona and were watered by drip irrigation. Fresh young rhizomes, leaves, shoots, and roots were collected 1 month prior to dormancy onset (on the same day in the middle of October) for chemical extraction. The plant samples were immediately frozen in liquid nitrogen and kept at −80 °C until analyzed.

Sample Preparation. All samples used for analysis were obtained in triplicate, and each replicate was a bulk of three individual plants from three pots. Twenty grams of tissue from three individual plants was mixed and ground in the presence of liquid nitrogen. Two separate 1 g aliquots were taken from each ground bulked sample and processed for gas chromatography−mass spectrometry (GC-MS) and liquid chromatography−electrospray ionization mass spectrometry (LC-ESI-MS) analysis, respectively, according to previously published methods (*24, 25*).

In Vitro Micropropagation. Except when specifically indicated, all data are shown for turmeric line T3C, because no differences were observed in propagation efficiency from the individual plants within each line and comparable results were obtained from both lines.

Explant production and pretreatment, culture medium and conditions, shoot multiplication, root induction, and ex vitro establishment were performed using a method that we developed for ginger (*26*).

Plantlets derived from the in vitro propagation procedure and that possessed 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 (**Figure 1d**,**i**) were maintained under greenhouse conditions (*24*). Hydroponics culture (**Figure 1h**) was performed by placing plantlets in perlite under which hydroponic solution was circulated. The hydroponic solution recipe has been previously described (*26*) and was based on the method of Dr. Howard Resh (*27*).

Metabolic Profiling Analysis. Frozen fresh turmeric rhizome, root, leaf, or shoot tissue was extracted with MTBE or MeOH as previously described (*26*) and used directly for GC-MS and LC-ESI-MS analysis, respectively. Triplicate extracts were used for quantitation analysis. The samples were never dried or concentrated prior to analysis.

A Thermo Electron Trace GC Ultra coupled to a DSQ mass spectrometer and equipped with an Alltech ECONO-CAP-EC-5 capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness) was used for GC-MS analysis. The instrument method was the same as previously described (*24*). Eluted compounds were identified using the NIST Mass Spectral Library version 2.0 (NIST/EPA/NIH) and the essential oil GC-MS mass spectra library from Dr. Robert P. Adams and by referring to publications from Jolad et al. (*28*) and Jiang et al. (*24, 25, 29, 30*).

A Thermo Electron Surveyor HPLC system equipped with a Discovery HS C18 column, 150 × 2.1 mm, i.d., 3 μm, with a guard column (Supelco, Bellefonte, PA) and a photodiode array (PDA) detector was coupled to an LCQ Advantage mass spectrometer equipped with an electrospray ion source for LC-ESI-MS analysis. The HPLC elution conditions and the acquisition parameters for MS were the same as previously described (*24, 29*).

Quantitative Analysis of the Three Major Curcuminoids. LC-MS was used to evaluate the concentrations of the three major curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) in turmeric samples. Calibration curves were derived from three independent injections of five concentrations of the three major curcuminoids (see Supporting Information).

Data Processing and Statistical Analysis. Thermo Electron's Xcalibur (version 1.4) was used as instrument control and data processing platform for both LC-MS and GC-MS data collection, peak identification, and measurement. Classifications by hierarchical cluster analysis (HCA) and data reductions by principal component analysis (PCA) were carried out using the Statistical Package for the Social Sciences (SPSS v. 12.0). The heatmap was developed using the R project for statistical computing (v. 2.2.1). Analyses of variance (ANOVA) were performed using the SAS system. All other intermediate data manipulation was carried out using Microsoft Excel 2000.

RESULTS AND DISCUSSION

We have developed an efficient, economical, and safe in vitro propagation procedure for turmeric (*C. longa*). This procedure utilizes a special sterilization method for reducing the rate of contamination, which uses PPM to sterilize the tissue and eliminate endogenous fungi and bacteria from turmeric explants (see Supporting Information) while allowing for a much higher in vitro propagation rate. We then performed metabolic profiling experiments to evaluate the composition and content of both polar nonvolatile and nonpolar volatile compounds in plants produced via the in vitro propagation procedure compared to plants derived from traditional propagation. We evaluated two turmeric lines that are most commonly found at the market in the United States, because we wanted to make sure that our in vitro propagation procedure would be useful with more than one cultivar or breeding line.

In Vitro Micropropagation. We developed a highly efficient in vitro micropropagation procedure that yielded high rates of multiplication while maintaining genetic stability and low costs. Traditional multiplication of turmeric in the wild or under field conditions produces 10−25 lateral buds per plant in a season of 8−10 months, with only 4−6 of the buds actively producing plantlets (*1*). The rhizome (the reproducing part) is also the spice-yielding part of the turmeric plant. This limits the amount of seed turmeric available to the grower each year. This paper demonstrates that a single explant (bud) can produce millions of plantlets within a year.

Efficient and Safe Decontamination Pretreatment for Turmeric Explants. The method employed to pretreat the explants, especially the rhizome bud treatment, greatly improved turmeric organogenesis compared to previously described methods. Use of the 50 °C hot water and PPM treatments increased the bud germination and differentiation rate from 4 to 50%. Other methods for turmeric explant sterilization (*17, 18*) required the use of $HgCl_2$ treatment to prevent microbial contamination. Our method does not require the use of $HgCl_2$, a well-known toxin and significant environmental pollutant, which can cause problems for plant growth and development. Our procedure showed very low levels of contamination from endogenous bacteria and fungi, with <10% of explants becoming contami-

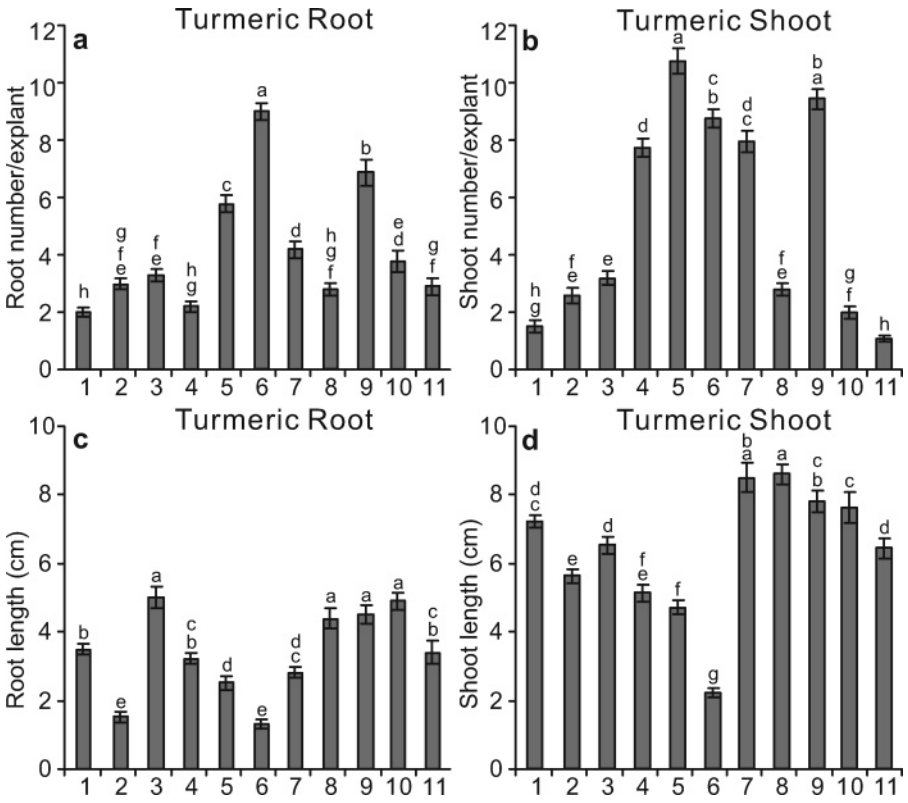

Figure 2. Effect of Kn, BA, IAA, and NAA combinations in M4 medium on number (**a**, **b**) or length (**c**, **d**) of shoots and roots of T3C turmeric (**a**–**d**) produced in culture. The following combinations were tested: 1, BA 1 mg L^{-1} + IAA 1 mg L^{-1}; 2, BA 2 mg L^{-1} + IAA 1 mg L^{-1}; 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 ± standard error. Treatments that are not significantly different at the $P < 0.05$ level (determined with one-way ANOVA) are indicated by the same letters.

nated, even when inexperienced undergraduate laboratory assistants performed the experiments.

Callus Induction. When rhizome buds, leaf bases, shoot tips, and cotyledonary nodes from in vitro propagated turmeric plantlets were placed on M2 medium (see Supporting Information), callus was produced (**Figure 1a**,**b**). The rate of callus formation varied dramatically, however, and depended on the tissue used. Leaf bases, rhizome buds, and shoot tips were poor tissues for callus induction, with only 5% of explants producing callus. With 95% of explants from cotyledonary nodes of in vitro propagated plantlets yielding callus, this tissue, in contrast, was much more efficient at producing callus. Culture medium containing 2,4-D at 1.5−5.0 mg L^{-1} produced callus growth from turmeric explants. When the concentration of 2,4-D in the medium was reduced to 0.25 mg L^{-1} and accompanied by the addition of 1 mg L^{-1} BA, organogenesis and plantlet formation occurred after growth in the dark for 4−6 weeks.

Shoot Induction from Rhizome Buds. We developed a method to propagate turmeric plantlets directly without the need to go through callus, because callus was not easily induced from easily obtained tissues (buds, leaves) and it was difficult to obtain tissues that were more efficient at producing callus. M4 medium (see Supporting Information) was optimized using differing levels of growth regulators, BA (1−3 mg L^{-1}), Kn (1−2 mg L^{-1}), IAA (1 mg L^{-1}), and NAA (0.5−1 mg L^{-1}), to yield rapid in vitro micropropagation of turmeric from bud explants. Ten different combinations of growth regulators were tested for this optimization. Data for shoot and root number and length were collected for each combination (see **Figure 2**). The results of these experiments indicated that the combination of 2.5 mg L^{-1} BA and 0.5 mg L^{-1} NAA (treatment 5) was the most efficient at producing a maximum number of turmeric shoots (average 10.85 shoots per bud).

Effect of Subculture. The efficiency of shoot multiplication was tested for 32 consecutive months (33 subcultures). Single plantlets obtained from multiple shoot cultures were subcultured, and the shoot multiplication rate of over 10 new shoots per culture persisted in B_5 medium containing 22.5 mg L^{-1} BA 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.

Ex Vitro Establishment. Initial acclimatization studies indicated that no special treatment was needed when in vitro propagated turmeric plantlets were moved to the greenhouse. Rooted plantlets obtained from in vitro cultures were placed directly into soil in pots in the greenhouse. After 1 month in the soil, the yield of plants produced by tissue culture and then transferred to the greenhouse was compared with that of conventionally propagated plants. On average, 98% of in vitro propagated turmeric plants survived transfer to soil and maintenance under greenhouse conditions. This was similar 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 came from the same parental stock) was that in vitro derived turmeric plants were larger and greener (they grew better) and produced more rhizomes than plants derived from rhizomes that had pass through the in vitro propagation procedure (see **Table 1**). An explanation for this increased vigor could be elimination of endogenous pathogens because of the sterilization regime or residual affects of the exogenously applied

Table 1. Comparison of Rhizome Weight (Grams per Plant, Fresh Weight) of in Vitro Micropropagated (Bud Explants) and Rhizome-Derived Turmeric Plants Grown in the Greenhouse for 1 Year

turmeric line	in vitro micropropagated[a]	rhizome derived[a]
T3C	290.4 ± 2.0	254.5 ± 2.2
Hawaii red	288.6 ± 2.6	257.8 ± 2.5

[a] Mean ± standard error of an average of 10 replicates.

growth regulators. Further experiments are underway to determine whether these improvements are permanent. In addition, we performed metabolite profiling of these propagated plants to determine whether other, nonmorphologically active somatic mutations may have occurred during the in vitro propagation regime. Results from these experiments are described below.

About 10 new plants are produced every 4 weeks from each bud explant using this in vitro propagation procedure, and it is thereby possible to obtain 1000 plants in 3 months from a single bud. This is a dramatic improvement over the four to six

Table 2. Relative Content[a] of Volatile Compounds Identified by GC-MS-Based Metabolic Profiling of 1-Year-old Turmeric 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 Two Turmeric Lines (T3C and HRT), and Different Tissues, Including Root (R), Leaf (L), and Shoot (S), of in Vitro Propagated HRT Plantlets

					T3C (Rh)			HRT (different tissues)				
								IV				
peak	RT	name	formula	MW	IV	IVH	GH	Rh	R	L	S	GH (Rh)
1	7.23	(1*R*)-(+)-α-pinene	$C_{10}H_{16}$	136	1	1	1	1	1	2	2	1
2	7.44	*R*-citronellene	$C_{10}H_{18}$	138	1	1	1	1	0	0	0	1
3	7.51	camphene	$C_{10}H_{16}$	136	0	0	0	0	2	0	0	0
4	8.06	β-pinene	$C_{10}H_{16}$	136	1	1	1	1	1	2	2	1
5	8.63	α-phellandrene	$C_{10}H_{16}$	136	2	2	2	2	3	3	3	2
6	8.72	3-carene	$C_{10}H_{16}$	136	1	1	1	1	0	2	0	1
7	8.86	α-terpinene	$C_{10}H_{16}$	136	1	1	1	1	0	2	0	1
8	9.01	*o*-cymene	$C_{10}H_{14}$	134	1	1	1	1	2	2	2	1
9	9.08	sylvestrene	$C_{10}H_{16}$	136	1	1	1	1	1	2	2	1
10	9.13	cineole	$C_{10}H_{18}O$	154	2	2	2	2	1	2	3	2
11	9.66	γ-terpinene	$C_{10}H_{16}$	136	1	1	1	1	2	2	2	1
12	10.29	*p*-mentha–1,4(8)-diene	$C_{10}H_{16}$	136	2	2	2	2	3	3	3	2
13	10.51	linalool	$C_{10}H_{18}O$	154	0	0	0	0	0	1	0	0
14	10.6	(*Z*)-cinerone	$C_{10}H_{14}O$	150	1	1	1	1	1	1	2	1
15	10.77	(*E*)-carveol	$C_{10}H_{16}O$	152	0	0	0	0	0	1	1	0
16	10.82	2-norpinanone, 3,6,6-trimethyl-	$C_{10}H_{16}O$	152	1	1	1	1	1	0	0	1
17	11.28	3,5-heptadienal, 2-ethylidene-6-methyl-	$C_{10}H_{14}O$	150	1	1	1	1	1	1	1	1
18	11.39	*p*-menth-8-en-2-one	$C_{10}H_{16}O$	152	1	1	1	1	1	1	1	1
19	12.08	δ-terpineol	$C_{10}H_{18}O$	152	1	1	1	1	0	1	0	1
20	12.34	terpinen-4-ol	$C_{10}H_{18}O$	154	1	1	1	1	1	1	2	1
21	12.55	*p*-cymen-8-ol	$C_{10}H_{14}O$	150	1	1	1	1	0	1	1	1
22	12.73	α-terpineol	$C_{10}H_{18}O$	154	1	1	1	1	0	2	1	1
23	13.05	(*Z*)-sabinol	$C_{10}H_{16}O$	152	1	1	1	1	1	1	1	1
24	13.57	(*E*)-chrysanthenyl acetate	$C_{12}H_{18}O_2$	194	1	1	1	1	0	1	2	1
25	15.46	bornyl acetate	$C_{12}H_{20}O_2$	196	0	0	0	0	1	0	0	0
26	16.65	piperitone epoxide	$C_{10}H_{16}O_2$	168	1	1	1	1	1	1	2	1
27	17.58	ascaridole	$C_{10}H_{16}O_2$	168	1	1	1	1	1	2	2	1
28	18.95	β-elemene	$C_{15}H_{24}$	204	0	0	0	0	1	0	0	0
29	19.43	7-epi-sesquithujene	$C_{15}H_{24}$	204	1	1	1	1	1	0	0	1
30	19.9	(*E*)-caryophyllene	$C_{15}H_{24}$	204	1	1	1	1	1	1	2	1
31	20.47	(*Z*)-α-bergamotene	$C_{15}H_{24}$	204	1	1	1	1	1	0	0	1
32	21.06	α-humulene	$C_{15}H_{24}$	204	1	1	1	1	1	1	0	1
33	21.17	(*Z*)-β-farnesene	$C_{15}H_{24}$	204	1	1	1	1	1	2	0	1
34	22.18	*ar*-curcumene	$C_{15}H_{22}$	202	1	1	1	1	2	0	0	1
35	23.05	(*E,E*)-α-farnesene	$C_{15}H_{24}$	204	0	0	0	0	0	2	0	0
36	23.26	α-zingiberene	$C_{15}H_{24}$	204	3	3	3	3	3	0	0	3
37	23.48	β-bisabolene	$C_{15}H_{24}$	204	3	3	3	3	2	0	0	3
38	24.21	β-sesquiphellandrene	$C_{15}H_{24}$	204	3	3	3	3	3	0	0	3
39	24.27	(*E*)-γ-bisabolene	$C_{15}H_{24}$	204	1	1	1	1	0	0	0	1
40	24.8	(*Z*)-sesquisabinene hydrate	$C_{15}H_{26}O$	222	1	1	1	1	0	0	0	1
41	26.02	(*E*)-sesquisabinene hydrate	$C_{15}H_{26}O$	222	1	1	1	1	0	0	0	1
42	29.26	tumerone	$C_{15}H_{22}O$	218	3	3	3	3	3	0	0	3
43	29.66	γ-curcumene	$C_{15}H_{24}$	204	2	2	2	2	2	0	0	2
44	30.18	curlone	$C_{15}H_{22}O$	218	3	3	3	3	3	0	0	3
45	30.5	curcuphenol	$C_{15}H_{22}O$	218	1	1	1	1	0	0	0	1
46	31.54	α-oxobisabolene	$C_{15}H_{24}O$	220	2	2	2	2	2	0	0	2
47	32.3	corymbolone	$C_{15}H_{24}O_2$	236	1	1	1	1	0	0	0	1
48	32.59	DRG-GM1-N1-23.59-250-149-83	$C_{16}H_{26}O_2$	250	1	1	1	1	0	0	0	1
49	33.95	DRG-GM1-N1-33.95-236-109-69	$C_{15}H_{22}O_2$	234	2	2	2	2	1	0	0	2
50	35.03	DRG-GM1-N1-35.03-236-82-69	$C_{15}H_{24}O_2$	236	1	1	1	1	1	0	0	1
51	35.6	DRG-GM1-N1-35.60-238-135-109	$C_{15}H_{26}O_2$	238	1	1	1	1	1	0	0	1
52	36.5	DRG-GM1-N1-36.43-238-95-109	$C_{15}H_{26}O_2$	238	1	1	1	1	1	0	0	1
53	38.85	*n*-hexadecanoic acid	$C_{16}H_{32}O_2$	256	1	1	1	1	1	2	2	1
54	41.53	hexadecane-1,2-diol	$C_{16}H_{34}O_2$	258	1	1	1	1	0	0	0	1
55	42.5	methyl 8,11-octadecadienoate	$C_{19}H_{34}O_2$	294	1	1	1	1	0	0	0	1
56	43.09	phytol	$C_{20}H_{40}O$	296	0	0	0	0	0	1	0	0
57	43.82	linoleic acid	$C_{18}H_{32}O_2$	280	1	1	1	1	1	0	2	1
58	43.93	oleic acid	$C_{18}H_{34}O_2$	282	1	1	1	1	1	0	2	1
59	44.23	α-linolenic acid	$C_{18}H_{30}O_2$	278	0	0	0	0	0	1	0	0
60	44.6	stearic acid	$C_{18}H_{36}O_2$	284	1	1	1	1	1	1	1	1
61	49.97	DRG-GM1-N1-50.00-302-81-137	$C_{20}H_{30}O_2$	302	1	1	1	1	3	3	2	1

[a] 1 indicates <0.5%, 2 indicates 0.5%–5%, and 3 indicates >5% of total integrated peak area of TIC of a particular sample.

turmeric plants produced per rhizome per year under standard cultivation practices. Furthermore, the amount of tissue available for seed through traditional cultivation practices is restricted if reasonable yields are to be realized each year, because the seed tissue is also the tissue used for human consumption. In contrast, the findings of this study suggest that a single bud explant can be used to produce tens of thousands to millions of individual plants, even within a single year. Because turmeric is a sterile triploid that must be propagated clonally, even via traditional propagation techniques, there are no concerns about planting a genetically uniform crop; it already is uniform. Propagation efficiency is no longer the limiting factor in our ability to use turmeric as a model plant to study rhizome development and metabolism.

Metabolic Profiling Analysis. To ensure that somatic mutations affecting metabolism were not introduced during the in vitro propagation process, we used a metabolic profiling approach to compare plants derived from in vitro propagated plantlets to those derived from traditionally greenhouse grown plants. We took plantlets derived from both methods and grew them in soil in pots on the same bench in the same greenhouse for an entire growing season and then performed the metabolic profiling experiments. The plants were grown in this manner to make sure that they were grown under as close to identical conditions as possible prior to chemical analysis.

We also grew some in vitro propagated plantlets (from line T3C) in a hydroponics system to test whether plants grown under such conditions differed in metabolic capability as compared to plants grown in soil. No such differences were observed in either chemical composition or specific compound gross quantitation (see **Table 2**) for the rhizomes of in vitro propagated T3C plantlets that were then grown in the hydroponics system or in soil, even though these growth regimens were very different. This observation led to some important conclusions. First, as long as the growth conditions are favorable, genetic rather than environmental factors appeared to play a larger role in controlling the composition of the compounds produced in turmeric rhizomes. This has significant implications for future investigations that will utilize this tissue to investigate metabolism in turmeric rhizomes. Second, future studies utilizing the capabilities of a hydroponics system, such as the ability to precisely control the environment or the concentration of specific elicitors, may be performed with assurance that any observed effects of such treatments on metabolism (if any effects are observed) will not be caused by the hydroponics system but rather would be due to the applied treatment. Such treatments should be directly applicable to the field.

GC-MS-Based Comparison of Two Turmeric Lines and Turmeric Tissues. Turmeric tissues were extracted with MTBE and analyzed by GC-MS to detect and quantify nonpolar compounds (see **Table 2**, Supporting Information Figures 1 and 2, and Supporting Information Table 1). Compound identifications indicated as being >80% probable by the library search program were considered to be likely hits. Spectra for each eluting compound were then compared to standard spectra for the best hits to determine whether the molecular ion peaks and the fragmentation patterns matched. Two injections were made for each of the three replicates per turmeric line, and the mean peak areas were calculated. Many compounds present at low levels in turmeric tissues were not included in our analysis because they could not be clearly identified due to low mass spectrum quality for low-abundance peaks or because their relative concentrations could not be adequately determined. Sixty-one compounds, mainly monoterpenoids and sesquiterpenoids, were easily identified from extracts of rhizomes from the two turmeric lines (T3C and HRT) and the different tissues (rhizome, root, leaf, and shoot) obtained from in vitro propagated HRT. Several highly abundant compounds could not be unambiguously assigned, even though they could be tentatively assigned to compound classes. These compounds, identified as DRG-GM1-N1-23.59-250-149-83, DRG-GM1-N1-33.95-236-109-69, and DRG-GM1-N1-35.03-236-82-69 (see **Table 2**), were named following the nomenclature rules outlined by Bino et al. (*31*) for the naming of unknown compounds in metabolic profiling investigations. All of the compounds that were identified, on the basis of their chemical structures, mass spectra, and retention times, belonged to four major compound classes: monoterpenoids, sesquiterpenoids, diterpenoids, and the others (alcohols, aldehydes, ketones, and long-chain acids, etc.).

Table 3. Molecule Classification of the Metabolites of the Turmeric Rhizome Samples (Rhizomes of Two Lines from in Vitro Micropropagation Derived and Greenhouse-Grown Plants) and the Total Turmeric Samples (Including All Tissues and Treatments from the Two Lines)

	turmeric rhizome samples		total turmeric samples	
	compounds	% of total	compounds	% of total
monoterpenoid	23	43.4	27	44.3
sesquiterpenoid	23	43.4	25	41.0
diterpenoid	0	0	1	1.6
others	7	13.2	8	13.1
total	53	100	61	100

GC-MS-Based Comparison of Rhizomes from Two Turmeric Lines. Fifty-three compounds were identified in rhizome samples of the two turmeric lines. Forty-six of these were terpenoids, as listed in **Tables 2** and **3**. There were no apparent qualitative differences in volatile compound composition: (a) between plants derived from in vitro propagated or greenhouse-grown plantlets for each of the two lines; (b) between the two lines; or (c) between T3C plants propagated in the hydroponics system or in the soil. The composition and content were very similar between growth treatments and even between the two lines. These were not significantly different at the $P < 0.05$ level.

It is difficult to distinguish the two turmeric cultivars (T3C and HRT) from their aerial parts, such as leaves and shoots (see **Figure 1**). Moreover, their rhizomes are difficult to tell apart at first glance (see **Figure 1k−m**). The main difference is that the color of the T3C rhizome cross section is orange, and the HRT rhizome cross section is reddish in color. Their volatile metabolite profiles are almost identical qualitatively, on the basis of this investigation. Moreover, any quantitative differences for specific compounds did not appear at first glance to be very significant.

Comparison of Turmeric Tissues Using GC-MS-Based Metabolic Profiling. In addition to comparing the rhizomes of the two turmeric lines, we also used metabolic profiling to evaluate the chemical composition of extracts from different tissues of one of these lines, HRT. Sixty-one compounds were identified in the extracts from the four tissues analyzed (rhizomes, roots, leaves, and shoots) from HRT plants derived from in vitro propagated plantlets. These included 53 terpenoids, as shown in **Tables 2** and **3**. The major compounds in the various tissues were as follows: rhizome, α-zingiberene (38% of total peak area), β-sesquiphellandrene (20%), and tumerone (17%); root, α-zingiberene (27%), β-sesquiphellandrene (20%), and tumerone (17%); leaf, DRG-GM1-N1-50.00-302-81-137 (38%), α-phellandrene (33%), and *p*-mentha-1,4(8)-diene (10%); and shoot, α-phellandrene (31%), *p*-mentha-1,4(8)-diene (30%), and cin-

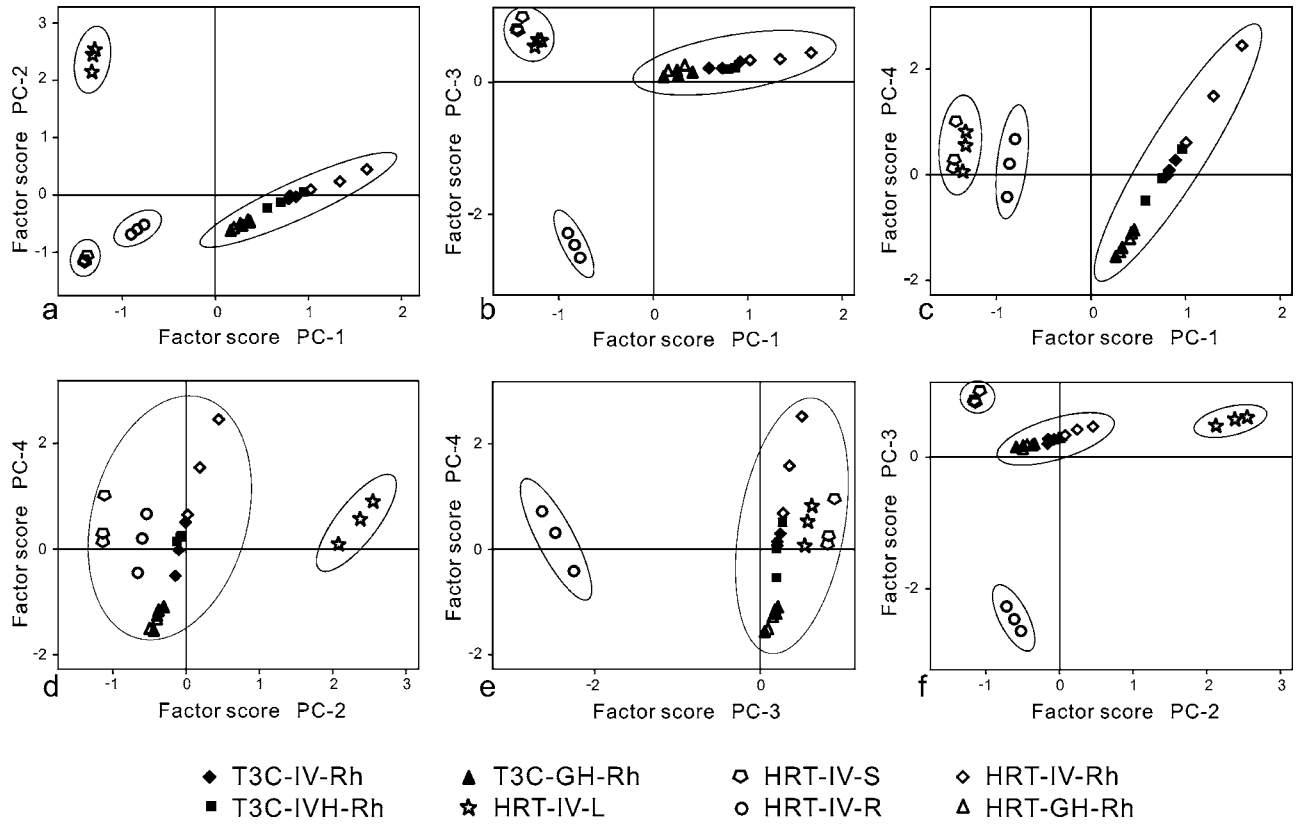

Figure 3. Scatter plots of PCA factor scores for all turmeric samples (including all tissues and treatments from the two lines). Axes of two-dimensional plots are derived from (**a**) PC-1 and PC-2, (**b**) PC-1 and PC-3, (**c**) PC-1 and PC-4, (**d**) PC-2 and PC-4, (**e**) PC-3 and PC-4, and (**f**) PC-2 and PC-3 of all turmeric samples. Plotted points represent individual samples, whereas arbitrary ellipses have been included to assist interpretation. This PCA represents the differentiation of 24 individual turmeric samples (biological triplicates of T3C-IV-Rh, T3C-IVH-Rh, T3C-GH-Rh, HRT-IV-Rh, HRT-IV-R, HRT-IV-L, HRT-IV-S, and HRT-GH-Rh). 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 traditional greenhouse propagation; Rh, rhizome; R, root; L, leaf; S, shoot.

Table 4. Number[a] of GC-MS-Identified Compounds Shared by Different Tissue Types of HRT Line

	rhizome	root	leaf	shoot
rhizome	53:9	34:14	27:3	24:0
root		41:3	19:0	21:0
leaf			32:4	23:1
shoot				25:0

[a] For, e.g., 34:14, "34" is the total number of compounds shared by both rhizome and root and "14" indicates the number of compounds found only in the rhizome and root and not in other tissues.

eole (6%). The rhizome possesses 87% of the compounds identified from all tissues, with 17% of these being found only in the rhizome. The root contained 67% of all compounds identified, with only 7% of these being root-specific. The root shared 83% of its compounds with the rhizome. The leaf possessed 52% of all of the compounds identified, and 13% of these were leaf-specific. Only 41% of the compounds identified were found in the shoot, with none being shoot-specific. The shoot shared 92% of its compounds with the leaf. **Table 4** lists the number of 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 possessed very different metabolic profiles, with most of the mono- and sesquiterpenoids being found in multiple tissues. Terpenoids that were found in only one tissue included, among others, compounds such as *R*-citronellene, (*E*)-γ-bisabolene, (*Z*)-sesquisabinene hydrate, (*E*)-sesquisabinene hydrate, curcuphenol, corymbolone, and DRG-GM1-N1-23.59-250-149-83, found only in the rhizome; camphene, bornyl acetate, and β-elemene, found only in the root; and linalool, (*E*,*E*)-α-farnesene, phytol, and α-linolenic acid, found only in the leaf. A number of compounds were found in only two tissues, such as 2-norpinanone, 3,6,6-trimethyl-, 7-epi-sesquithujene, (*Z*)-α-bergamotene, *ar*-curcumene, α-zingiberene, β-bisabolene, β-sesquiphellandrene, tumerone, γ-curcumene, and curlone in the rhizome and root; 3-carene, α-terpinene, and δ-terpineol in the rhizome and leaf; and (*E*)-carveol in the leaf and shoot but not in the rhizome and root. These results suggest that specific mechanisms must be involved in differential production and/or accumulation of specific metabolites in these tissues.

PCA of GC-MS Data. We used PCA (see **Figure 3**) in an attempt to distinguish metabolic profiles of (1) plants from the two turmeric lines, (2) plants propagated by different means (conventional greenhouse grown vs in vitro propagation derived plants), and (3) different tissues (rhizome, root, leaf, and shoot) of in vitro propagated HRT turmeric. One general comparison was performed, which included samples from all tissues (leaf, root, shoot, and rhizome) from the two lines under the different growth conditions. Four separate principal components resulted from this analysis and represented 100% of the variance (Supporting Information Table 2). Loading variables of >0.45 were selected as high-loading, as defined by Comrey and Lee (*32*), and used to generate clusters of compounds that appeared to be distinguished by the various principal components (see Supporting Information Tables 3 and 4). PC-2 and PC-3 could be effectively clustered and separated between the four different tissues (see **Figure 3f**), supporting the conclusions discussed

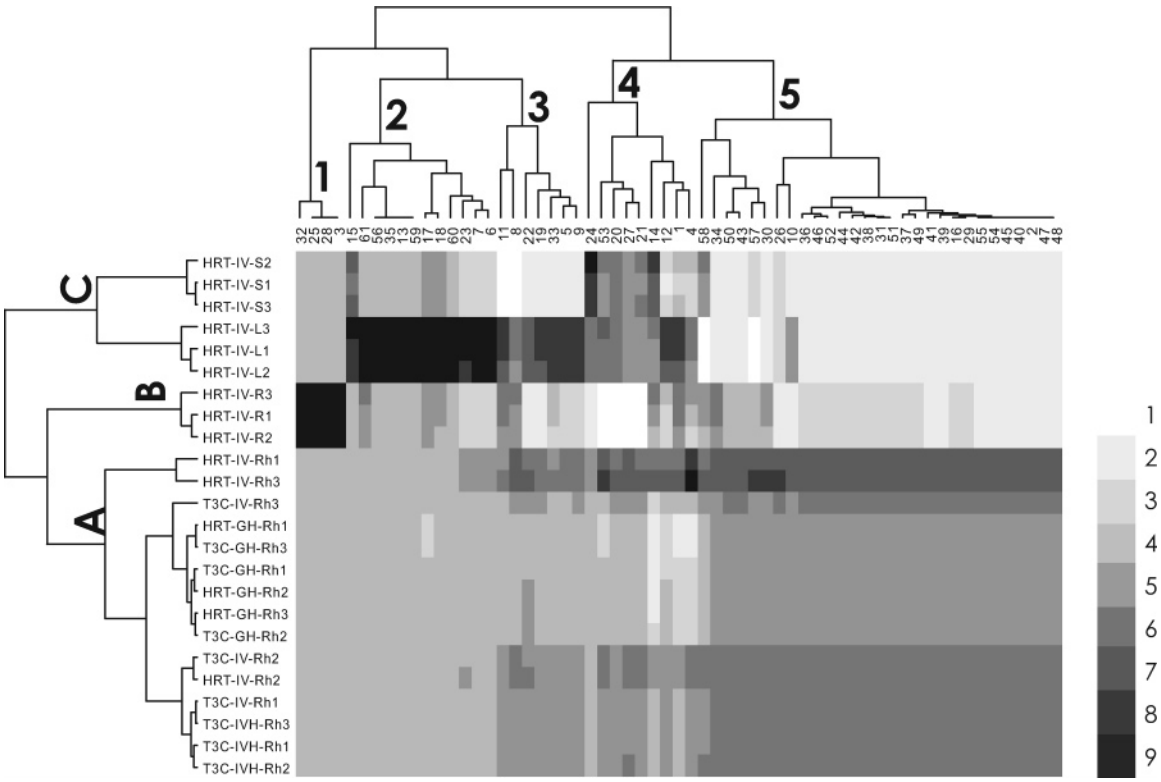

Figure 4. Hierarchical clustering results comparing metabolic profiles of turmeric samples to growth conditions and tissue comparisons. Sixty-one compounds from different tissues and growth and five treatments of two turmeric lines (T3C and HRT) were compared. Clusters 1–5 represent groupings of compounds. Clusters A–C show the cluster groups of 24 individual plant samples (biological triplicates of T3C-IV-Rh, T3C-IVH-Rh, T3C-GH-Rh, HRT-GH-Rh, HRT-IV-Rh, HRT-IV-R, HRT-IV-L, and HRT-IV-S). Abbreviations to define samples are as for **Figure 3**. Scale for heatmap indicates relative abundance of compounds (determined as peak area) for each corresponding sample.

above regarding the differences in metabolic profiles that exist between turmeric tissues. Interestingly, turmeric rhizome samples from the two different lines and from the different growth treatments for each turmeric line grouped together in this analysis, indicating that the two turmeric lines were indistinguishable and the in vitro propagation procedure had no significant effect on metabolism in turmeric rhizomes. This result was important because it clearly showed that the in vitro propagation procedure can be used to generate large numbers of plants while having no impact on major metabolic processes in the plants; that is, the in vitro propagated plants will be true chemical clones of the parents.

HCA of GC-MS Data. We used HCA of the GC-MS data to further explore the relationships between different lines, treatments, and tissues. A comparison of the HCA results of the different samples to the peak areas of the identified compounds was used to generate a heatmap (see **Figure 4**). Three major clusters among the sample treatments could be identified in this analysis: cluster A contained all of the rhizome samples from both lines including all growth treatments for each turmeric line; cluster B contained all root samples; and cluster C contained the leaf and shoot samples. Notably, the rhizome samples derived from in vitro propagated plantlets and traditionally greenhouse grown plants were closely aligned.

In addition, the 61 compounds clustered into five major groups in this analysis. Clusters 1, 2, 3, 4, and 5 have 4, 12, 7, 9, and 29 compounds, respectively (see **Figure 4** and Supporting Information Tables 5 and 6). Interestingly, the rhizome samples of cluster C possessed the highest levels of compounds in cluster 5, including 15% of total monoterpenoids, 84% of total sesquiterpenoids, and 50% of total others. In contrast, the leaf and shoot samples in cluster A contained high levels of compounds belonging to cluster 2, including 26% of total monoterpenoids, 4% of total sesquiterpenoids, 100% of total diterpenoids, and 38% of total others. Cluster 1 contained mostly compounds that were highly abundant in the root (cluster B), including 7% of total monoterpenoids and 8% of total sesquiterpenoids. Clusters 3 and 4 contained compounds that were found in multiple tissues and that belonged to multiple classes of compounds.

LC-ESI-MS-Based Comparison of Two Turmeric Lines. The bioactivities of fresh turmeric have been attributed to a series of homologous diarylheptanoids, of which curcumin, demethoxycurcumin, and bisdemethoxycurcumin are the three most abundant constituents. Diarylheptanoids belong to a class of natural products with a 1,7-diarylheptanoid skeleton (*33*). Diarylheptanoids have been found to possess a variety of biological and pharmacological activities including antioxidant, antihepatotoxic, anti-inflammatory, antiproliferative, antiemetic, chemopreventive, and antitumor activities (*13*, *34*−*41*), among others, leading to increased interest in recent years for this group of compounds, especially for curcumin. Fresh rhizomes of the two turmeric lines (T3C and HRT) were assayed in triplicate by LC-ESI-MS/MS to determine the composition and relative content of polar nonvolatile compounds (see **Table 5** and Supporting Information Figure 3). Nineteen compounds, including 5 phenolic acids, 13 diarylheptanoids, and 1 sesequiterpenoid, were readily 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 **Figure 5** and **Table 5**). All of these compounds were present in all samples. Moreover, curcumin, demethoxycurcumin, and bis-

Table 5. Relative Content of Phenolic Acids and Diarylheptanoids Identified by LC-ESI-MS-Based Metabolic Profiling of 1-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 Two Turmeric Lines (T3C and HRT)[a]

					T3C			HRT	
no.	RT	name	formula	MW	IV	IVH	GH	IV	GH
62	12.9	caffeic acid	$C_9H_8O_4$	180	1	1	1	1	1
63	13.2	hydro-*p*-coumaric acid	$C_9H_{10}O_3$	166	1	1	1	1	1
64	13.8	*p*-coumaric acid	$C_9H_8O_3$	164	1	1	1	1	1
65	14.5	hydroferulic acid	$C_{10}H_{12}O_4$	196	1	1	1	1	1
66	15.1	ferulic acid	$C_{10}H_{10}O_4$	194	1	1	1	1	1
67	20.7	3, 5-heptanediol, 1,7-bis(4-hydroxyphenyl)-	$C_{19}H_{24}O_4$	316	1	1	1	1	1
68	22.0	3-heptanone, 5-hydroxy-1,7-bis(4-hydroxyphenyl)-	$C_{19}H_{22}O_4$	314	1	1	1	1	1
69	22.1	4,6-heptadien-3-one, 1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-	$C_{20}H_{20}O_4$	324	1	1	1	1	1
70	22.6	1,4,6-heptatrien-3-one,1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-	$C_{20}H_{18}O_4$	322	1	1	1	1	1
71	23.1	1,4,6-heptatrien-3-one, 1,7-bis(4-hydroxy-3-methoxyphenyl)-	$C_{21}H_{20}O_5$	352	1	1	1	1	1
72	24.7	4-hepten-3-one, 5-hydroxy-1,7-bis(4-hydroxyphenyl)-	$C_{19}H_{20}O_4$	312	1	1	1	1	1
73	28.3	1,6-heptadiene-3,5-dione, 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-	$C_{19}H_{16}O_5$	324	1	1	1	1	1
74	30.6	1-heptene-3,5-dione, 1,7-bis(4-hydroxyphenyl)-	$C_{19}H_{18}O_4$	310	1	1	1	1	1
75	30.8	bisdemethoxycurcumin	$C_{19}H_{16}O_4$	308	3	3	3	3	3
76	31.4	1-heptene-3,5-dione, 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-	$C_{20}H_{20}C_5$	340	3	3	3	3	3
77	31.5	demethoxycurcumin	$C_{20}H_{18}O_5$	338	3	3	3	3	3
78	32.1	1-heptene-3,5-dione, 1,7-bis(4-hydroxy-3-methoxyphenyl)-	$C_{20}H_{20}O_5$	340	3	3	3	3	3
79	32.2	curcumin	$C_{21}H_{20}O_6$	368	3	3	3	3	3
80	33.5	turmeronol A	$C_{15}H_{20}O_2$	232	1	1	1	1	1

[a] Structures of compounds listed are shown in **Figure 5**. 1 indicates <0.5%, 2 indicates 0.5%–5%, and 3 indicates >5% of mass peak area of a particular sample.

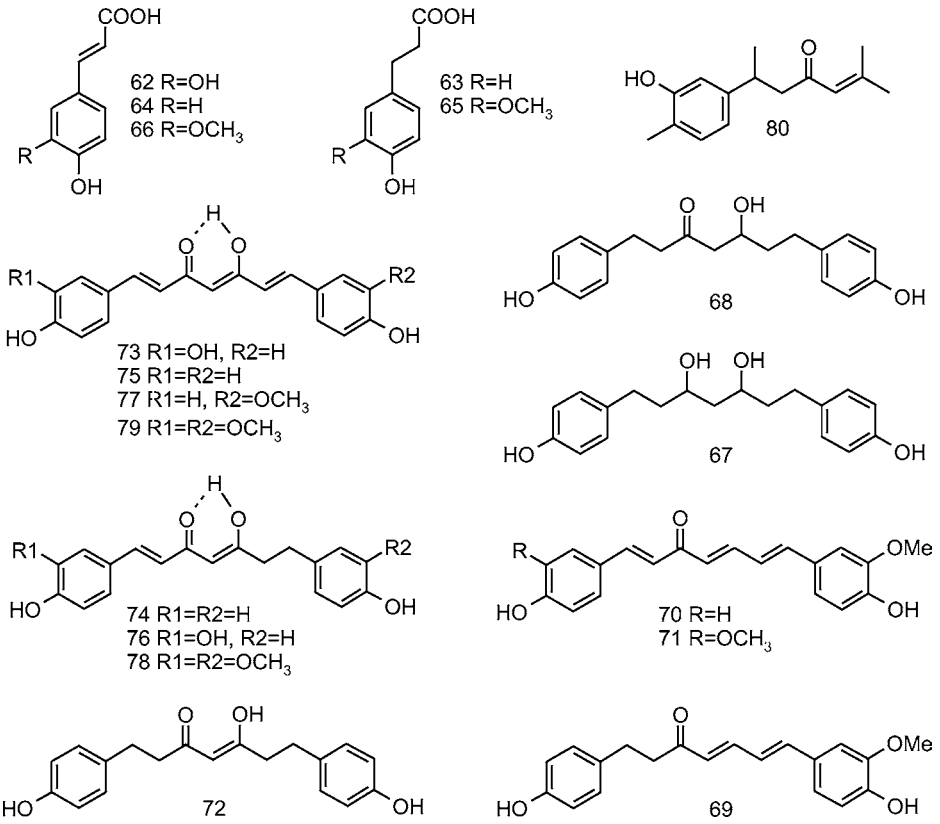

Figure 5. Structures of phenolic acids and diarylheptanoids identified by LC-ESI-MS.

demethoxycurcumin were identified as the major diarylheptanoids in all samples.

Remarkably, like the terpenoid constituents of the turmeric rhizomes (see **Table 2**), the levels of diarylheptanoids were practically identical between lines and treatments (see **Table 5**). In addition to these metabolic profiling based results, we also evaluated the concentrations of the three major curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) in a more quantitative manner using LC-MS. Calibration curves were prepared for quantitative analysis of the three major curcuminoids (see Supporting Information). LC-MS was then used to determine the content of curcuminoids in in vitro propagated and greenhouse-grown samples. The content in fresh turmeric rhizome varied from 5.86 to 8.84 mg g^{-1}, from 5.06 to 7.65 mg g^{-1}, and from 5.42 to 7.43 mg g^{-1}, respectively, for curcumin, demethoxycurcumin, and bisdemethoxycurcumin (see **Table 6**). Thus, although the metabolic profiling results indicated that no great chemical differences existed between the plants analyzed, in terms of overall metabolic capacity, these targeted analysis results suggested that some variation in specific metabolite concentrations does exist. Nevertheless, these differences were rather small in magnitude, although statistically

Table 6. Quantitative Comparison of Curcuminoid Content[a] in Rhizomes of 1-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 Two Turmeric Lines (T3C and HRT)

	T3C			HRT	
		in vitro propagated			
	greenhouse grown	pots	hydroponic	greenhouse grown	in vitro propagated
bisdemethoxy-curcumin	7.143 ± 0.035	8.837 ± 0.029	8.545 ± 0.045	5.862 ± 0.022	7.639 ± 0.022
demethoxy-curcumin	6.976 ± 0.024	7.654 ± 0.032	7.261 ± 0.018	5.057 ± 0.031	6.628 ± 0.031
curcumin	5.418 ± 0.027	7.432 ± 0.028	5.744 ± 0.024	5.726 ± 0.025	7.105 ± 0.025

[a] All values are given in mg g^{-1} of fresh weight, average of triplicate biological samples ± standard error.

significant. Thus, turmeric from different sources or grown under different conditions may possess different properties, both in flavor and in bioactivity. However, when turmeric plants from the same cultivar are grown under (near) identical conditions, these differences may be minor.

In conclusion, metabolic profiling, including PCA and HCA of the resulting metabolic profiles, of plants produced by the in vitro propagation procedure described in this paper demonstrated that this propagation method can be used for conservation and rapid amplification of highly productive turmeric stocks, such as stocks high in particular bioactive constituents such as curcumin or of new varieties of turmeric while maintaining confidence that these plants will not be significantly different in chemical properties from the parental lines. As part of the in vitro propagation procedure, we describe a safe and effective method for explant decontamination that overcomes the serious problem of microbial contamination in turmeric. Metabolic profiling of turmeric plants derived from these in vitro propagated plantlets showed no qualitative differences in major nonvolatile or volatile constituents when compared to traditionally propagated plants. Moreover, no significant differences existed in the metabolic profiles when in vitro propagated and traditionally greenhouse grown plants were compared, at least for the compounds that we were able to detect in our analysis. Finally, callus production and successful induction of plantlets from callus establish an ideal platform for future transgenic research in Zingiberaceae plants. Because turmeric rhizomes can now be rapidly produced in vitro, a highly controlled environment can be utilized for metabolism investigations in this important medicinal plant.

ACKNOWLEDGMENT

We thank Dr. Hongliang Jiang for assistance with compound identification, Brenda Jackson for assistance with LC-MS and GC-MS operation and upkeep, Dr. Robert P. Adams for the essential oil GC-MS mass spectra library, Dr. Steven P. McLaughlin for assistance with plant identification, Dr. Anita Hayden for discussion and suggestions regarding hydroponic culture media and growth conditions, and Montserrat Vasquez, Lili Huylebroeck, and Sheena Swapp for assistance with maintenance of cultures.

Supporting Information Available: Supplementary Figures 1−3 and Tables 1−6 and a description of micropropagation methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review June 13, 2006. Revised manuscript received September 25, 2006. Accepted September 26, 2006. This research was funded by Grant DBI-0227618 to D.R.G. from the National Science Foundation Plant Genome Program. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the National Science Foundation.

JF061658K