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Comparison of vetiver root essential oils from cleansed (bacteria- and fungus-free) vs. non-cleansed (normal) vetiver plants

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Abstract

'Karnataka' and 'Malaysia' cultivars of vetiver (*Vetiveria zizanioides* (L.) Nash, =*Chrysopogon zizanioides* (L.) Roberty) were subjected to meristem tissue culture in order to produce plants that were bacteria- and fungi-free. Tissue cultured (''cleansed'' or phytosanitary) vetiver was grown for five months in sterilized soil contained in pots, and the oil content of plants grown on the medium was compared to that of non-cleansed (normal) vetiver plants grown in unsterilized soil under the same conditions. Statistical analysis of 49 of the major oil components revealed numerous significant differences between tissue culture derived and natural plants for both genotypes.

Although oil yields differed, this may reflect the larger size of the initial plantlets obtained from natural sources. Tissue cultured vs. natural plantlets grown in sterilized soil resulted in the largest number of differences in compounds. The least number of differences of compounds were between tissue cultured vs. natural plantlets grown in non-sterile soil. The thesis that many of the compounds found in vetiver roots originate from endogenous fungi was not supported.

Keywords: Vetiver; *Vetiveria zizanioides*; *Chrysopogon zizanioides*; Poaceae; Essential oils; Mycorrhiza; Bacteria; Biotransformation

1. Introduction

Vetiver grass (*Vetiveria zizanioides* (L.) Nash, syn. *Chrysopogon zizanioides* (L.) Roberty), the roots of which produce an important essential oil, has been utilized in many parts of the world for soil and water management. Hedges of the non-seeding vetivers provide an effective living dam against erosion (NRC, 1993), and this technique is now in use

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in more than 100 countries. The origin of the non-seeding vetiver is not known. However, *V. zizanioides* seems to have originated in the area from India to Vietnam, and its fragrant roots, from which is extracted the essential ''Oil of Vetiver'', have been used for centuries for mats and perfumes (NRC, 1993).

Adams and Dafforn (1998) examined 121 accessions of pantropical vetiver and found that 86% appeared to be a single clone (no variation in the DNA examined). That clone was named 'Sunshine' (after a collection site in Sunshine, Louisiana, USA). Included in that analysis were plants from Haiti and Reunion that clustered with the 'Sunshine' group, indicating that the vetiver cultivars used for commercial essential oil production are 'Sunshine' or very similar cultivars. This work was expanded by Adams et al. (1998) to include the closely related genera, *Chrysopogon* and *Sorghum*. Based on an overlap of genetic and morphological data, Veldkamp (1999) combined *Vetiveria* and *Chrysopogon* under *Chrysopogon.* Although this has led to the recognition of *C. zizanioides* (L.) Roberty as a proper classification for *V. zizanioides* (L.) Nash, in this paper we will continue to use both names for clarity. Analysis of additional collections in Thailand cultivars from Bangkok (Adams et al., 1999) revealed that 'Sunshine' and its allied cultivars form the bulk of the vegetatively propagated cultivars in the world. Adams et al. (2003) reported on the growth and oils of 13 distinct DNA types of the 'Sunshine' group grown in test plots in Florida, Nepal and Portugal. No single DNA type (cultigen) was found to be superior in all plots. The oil yields (g/g root dry wt.) were highest in Portugal, followed by Nepal, then Florida. However, yields of oil per plant (g/plant) were much higher in Nepal (1.79 g), followed by Florida (1.23 g), then Portugal (0.85 g). The oil composition varied slightly by strains and by plots.

Weyerstahl et al. (1996, 1997, 2000a,b,c) exhaustively examined vetiver oil from Haiti. He stated (Weyerstahl et al., 2000c) that the composition is so complex (most GC peaks contained 2–4 components) that general, routine analyses of vetiver oils are probably not possible. Weyerstahl et al. (2000c) also noted that vetiver oil reminds him of agarwood oil that is obtained from fungus infected trees of *Aquilaria*, which contain constituents with eremophilane, eudesmane, spirovetivane, guaiane and 2-epi-prezizaane skeletons. These sesquiterpene families are also present in vetiver oil. Vetiver has been reported (Wong, 2003) to contain arbuscular mycorrhizal fungi (AMF). The endomycorrhiza could well be producing biotransformations of the vetiver oil. In addition, Viano et al. (1991) and Bertea and Camusso (2002) reported intracellular bacteria in association with essential oil cells in vetiver root (glands). It is also possible that bacteria could be making biotransformations of the essential oil. Adams et al. (2004) made a preliminary report on 'cleansed' vs. 'normal' vetiver and found that the single plant arising from tissue culture had considerable amounts of long chain hydrocarbons (C_{15}–C_{29}).

The purpose of this study was to conduct thorough research to compare the essential oils of plants cleansed (i.e., that do not contain internal bacteria or fungi) vs. non-cleansed, wild type plants with their normally associated internal microorganisms. However, as seen in the results, it is very difficult to grow completely sterile plants under ambient (non-sterile) conditions. In addition, these kinds of plants were grown on both sterile and non-sterile soils. To examine these effects, 'Karnataka' and 'Malaysia' vetivers, obtained from tissue culture (so they had no internal bacteria or fungi) were grown in both sterilized and not sterilized soils along side non-cleansed (normal) plants. The roots were harvested, the oil extracted and analyzed. This paper reports on the comparison of these oils.

2. Materials and methods

A portion of a single 'Karnataka' vetiver plant and a single 'Malaysia' plant from our test plot in ECHO, Ecological Concerns for Hunger Organization, Ft. Meyers, Florida (Adams et al., 2003) were removed and subjected to meristem tissue culture. Fungi- and bacteria-free 'Karnataka' (KRN) and 'Malaysia' (MAL) vetiver plantlets were generated using the shoot apical meristem culture method (Smith, 2000). From these plantlets, five individuals of each type (KNR, MAL) were transferred to individual PVP plastic pipe containers (10 cm diam. × 3 m long, with 4 cm of sterile gravel in the bottom to facilitate drainage) and filled with either heat-sterilized soil or non-sterilized soil. The soil was a sandy-loam, pH 4.7, N 4.0, K 91.0, Ca 584.0, Mg 76.0, salinity 449.0, Na 199.0, S 47.0. Five additional (non-cleansed) plantlets of each type (KNR, MAL) were taken from the original ECHO vetiver plot and re-planted in other PVC pipe containers in either heat-sterilized soil or non-sterilized soil. Thus, all plants were grown in the same soil in identical PVC pipe containers, outside, in a complete randomized design, in Waco, Texas, USA under ambient conditions, with supplemental watering as needed, and not fertilized. After the summer growing season (158 days, until mid-October), each plant was removed from its pot. The roots were separated from the culm and washed to remove the soil. Root portions were cleared and stained as described in Habte and Osorio (2001) and examined by microscopy to

determine the extent to which the roots were colonized by arbuscular mycorrhizal fungi (AMF). Internal bacteria content of washed roots was also microscopically ascertained. The balance of the roots was used for steam distillation on the same day as harvested.

The roots were steam distilled for 24 h using a circulatory Clevenger-type apparatus (Adams, 1991). The oil samples were concentrated (ether trap removed) with nitrogen, and the samples stored at −20 °C until analyzed. The extracted roots were oven dried (48 h, 100 °C) for dry weight basis determination of oil yields.

The essential oil compositions were analyzed on a Hewlett–Packard 5972 MSD, directly coupled to a HP5980 gas chromatograph. EI mass spectra were collected at 70 eV ionization voltage over the mass range *m/z* 41–425. Oil samples of 0.1 μL (5% concentration) were injected and split 1/10. Analytical conditions: column: J & W DB-5, (0.26 mm × 30 m, 0.25 μm film thickness); carrier gas: helium at 1 mL/min; injector temperature: 220 °C; split ratio: 10:1; oven programming: initial temperature 60 °C, gradient 3 °C/min, final temperature 246 °C. Identifications were made by library searches of our volatile oil library (Adams, 2006), coupled with retention time data of reference compounds. For compound quantitation, the oils were analyzed using the same type column (DB-5) and conditions (as above) on a HP9580 gas chromatograph equipped with FID. The percentages of each compound are FID responses integrated with an HP Chemstation integrator.

Hypotheses were tested on each oil component separately using analysis of variance and *t*-tests (SPSS, version 14, SPSS Inc., Chicago, IL). Patterns of change for the differing varieties and soil types were measured both by mean comparison and by examining the interactions between varieties, soils, and tissue culture conditions.

3. Results and discussion

Table 1 shows the essential oil compositions of ''Malaysia'' genotype (MAL) and ''Karnataka'' genotype (KRN) from different sources and in different soils. Even though Weyerstahl et al. (2000a,b,c) made a heroic effort to identify all the vetiver oil components and he provided the senior author with his oil fractions, mass spectra and retention indices, it is very difficult to identify many of the oil components by GC–MS/retention time data. Many of the minor, unknown compounds (<0.05%) were not reported in the previous study (Adams et al., 2004), but are included in this paper to determine if they might originate in non-sterile plants and/or non-sterile soil (from fungi and bacteria). Two sesquiterpene alcohols (KI 1625, KI 1675) are major components. The KI 1625 sesquiterpene alcohol ranges from 4.57 to 7.4% and was found from 1.6 to 4.5% (KI 1621 in Adams et al., 2004) in genotype ''Sunshine'' (SS). The KI 1675 sesquiterpene alcohol ranges from 14.36 to 17.23% in MAL and 6.24–6.88 in KRN (Table 1), but was less than 0.05% in SS (Adams et al., 2004).

There were 20 significant differences between MAL from tissue culture vs. natural (ECHO field plot) grown in sterilized soil (Tables 1 and 2). There were fewer (11) differences between MAL from tissue culture vs. natural (ECHO field plot) grown in non-sterilized soil (Tables 1 and 2). There were 32 significant differences between KRN from tissue culture vs. natural (ECHO field plot) grown in sterilized soil (Tables 1 and 2).

For the MAL genotype, the incidence of colonization by arbuscular mycorrhizal fungi (AMF) was significantly lower in plants from either tissue culture or nature than from plants grown in non-sterile soil (Table 2). It appears that the AMF in the natural, sandy, non-sterilized soil effectively inoculated both the TC and ECHO plants (23 and 29%, Table 2). Whereas, the TC and ECHO plants had no significant difference in colonization rates (8.4 and 5%, Table 2). In contrast, it appears that, for the KRN genotype, the transplanted ECHO plantlets seem to have carried endogenous AMF because the ECHO plantlets grown in sterilized soil had 35% colonization by AMF vs. 10% for TC plantlets (Table 2). There was no correspondence between AMF colonization and oil yield (Table 2). However, in soils that do not have sufficient P for mycorrhiza-free growth, AMF have been shown to aid plant growth which would increase root biomass (Habte and Manjunath, 1987) This could result in larger yields per hectare and would definitely aid in soil erosion control.

For both MAL and KRN genotypes, there were noticeably more compounds that were of greater relative abundance from TC (sterilized) than ECHO (natural) plants grown in sterilized soil (Table 3), but there were no obvious terpenoid class differences. The number of compounds differing between MAL, TC vs. ECHO sources, grown in non-sterilized soil was smaller than in other groups (Table 3). The types of compounds normally associated with fungi (eremophilane, eudesmane, spirovetivane, guaiane and 2-epi-prezizaane skeletons) do not seem to be associated with AMF colonization.

Table 1
Comparisons for 50 essential oil components from 'Karnataka' (KRN), and 'Malaysia' (MAL) types of vetiver, using tissue cultured vs. normal, grown in sterilized vs. non-sterilized soils using five reps for each treatment

KI	Compound	MAL genotype						KRN genotype		
		Sterile soil			Non-sterile soil			Sterile soil		
		TC		ECHO	TC		ECHO	TC		ECHO
1449	(*E*)-Isoeugenol + prezizaene	0.81		0.93 ns	0.90		0.93 ns	0.75	>	1.18*
1453	Khusimene	1.16		1.54 ns	0.97	<	1.67*	0.67		0.82 ns
1460	C_{15}HC, *105*, 91, 161, 204	0.07		0.08 ns	0.12		0.16 ns	0.48	>	0.20*
1484	α-Amorphene	0.34	>	0.21*	0.41		0.53 ns	0.54	>	0.41*
1493	C_{15}HC, *105*, 91, 161, 204	0.23		0.18 ns	0.24	<	0.37*	0.48	>	0.30*
1496	Valencene	0.07	>	0.00*	0.10		0.12 ns	0.24	>	0.15*
1498	*149*, 95, 107, 121, 192	0.43		0.99 ns	0.51		0.50 ns	0.08		0.05 ns
1500	α-Muurolene	0.02		0.00 ns	0.03		0.00 ns	0.53	>	0.02*
1507	C_{15}HC, *131*, 119, 145 202	0.22	>	0.11*	0.20		0.19 ns	0.32		0.22 ns
1512	δ-Amorphene	0.17		0.13 ns	0.18	<	0.35*	0.38	>	0.27*
1524	C_{15}HC, *161*, 91, 105, 204	0.18		0.18 ns	0.22		0.37 ns	0.42		0.30 ns
1538	C_{15}HC, *178*, 135, 41, 204	0.16		0.20 ns	0.14		0.16 ns	0.53	>	0.36*
1548	Elemol	0.20	>	0.07*	0.16		0.22 ns	0.27		0.22 ns
1554	β-Vetivenene	0.42	>	0.25*	0.44		0.57 ns	0.60		0.59 ns
1565	C_{15}HC, *59*, 43, 149, 222	1.57	>	1.24*	1.81		1.63 ns	1.56		1.48 ns
1574	C_{10}OH, *152*, 91, 105	0.27		0.29 ns	0.36		0.38 ns	0.40		0.40 ns
1577	*trans*-Sesquisabinene hydrate	0.16	<	0.29*	0.21		0.23 ns	0.27		0.29 ns
1584	C_{15}HC, *59*, 109, 205, 220	0.05		0.05 ns	0.10		0.13 ns	0.45	>	0.21*
1589	C_{15}HC, *202*, 187, 131, 145	0.47		0.29 ns	0.44		0.61 ns	0.74	>	0.56*
1594	C_{15}OH, *43*, 161, 105, 222	0.19		0.23 ns	0.25	<	0.30*	0.39	>	0.25*
1604	Khusimone	0.44	<	0.96*	0.65		0.63 ns	0.22	<	0.98*
1605	C_{15}HC, *151*, 111, 119, 202	0.00		0.00 ns	0.00		0.00 ns	0.45	>	0.00*
1613	C_{15}HC, *134*, 41, 79, 204	0.00		0.20 ns	0.04		0.03 ns	0.09	<	0.85*
1625	C_{15}OH, *81*, 43, 161, 222	7.40	>	4.57*	7.09		7.19 ns	6.24		6.88 ns
1630	C_{15}OH, *59*, 149, 43, 220	0.61		0.51 ns	0.80		0.82 ns	1.14	>	0.71*
1647	C_{15}O, *133*, 67, 91, 218	0.21		0.32 ns	0.23	<	0.37*	0.24	<	0.57*
1651	C_{15}OH, *43*, 71, 95, 222	0.93	<	1.40*	1.00	<	1.22*	1.02		1.02 ns
1653	α-Cadinol	1.84		1.45 ns	2.20		1.92 ns	3.11	>	2.26*
1662	C_{15}O, *43*, 109, 204, 218	0.28		0.70 ns	0.38		0.43 ns	0.46		0.48 ns
1669	epi-Zizanone	6.38		6.81 ns	7.12		6.03 ns	3.20	>	4.78*
1675	C_{15}OH, *150*, 131, 202, 220	17.23		15.10 ns	16.14		14.36 ns	2.79	<	4.27*
1679	C_{15}OH, *81*, 91, 107, 220	0.00		0.00 ns	0.00		0.00 ns	1.56		0.00 ns
1682	C_{15}OH, *177*, 41, 222, 107	2.81	<	3.35*	3.14		2.93 ns	1.49	<	2.91*
1688	C_{15}O, *119*, 148, 189, 218	1.14		1.74*	0.94	<	1.48*	0.71	<	1.69*
1697	Zizanal	0.42	>	0.00*	0.27		0.28 ns	0.00		0.00 ns
1698	C_{15}OH, *135*, 41, 91, 220	2.00	<	2.70*	2.18		2.31 ns	3.14		3.00 ns
1715	Nootkatol	1.79		1.47 ns	2.08		2.08 ns	2.08		2.18 ns
1730	Vetiselinenol	2.20		2.18 ns	2.76	>	2.44*	8.76	>	2.73*
1736	C_{15}OH, *136*, 121, 202, 220	1.28		1.04 ns	1.32		1.49 ns	1.83	>	2.06*
1742	Khusimol (=zizanol)	21.57	<	25.68*	18.93	<	20.36*	18.92	<	23.06 ns
1758	C_{15}O, *68*, 79, 91, 218	0.69	<	1.12*	0.73		0.94 ns	0.44	<	1.14*
1767	13-Hydroxy valencene	0.27		0.26 ns	0.40		0.34 ns	1.68	>	0.16*
1773	α-Costol	0.36	>	0.11*	0.34		0.30 ns	2.24	>	0.17*
1793	(*E*)-Isovalencenol	8.78	>	7.11*	8.38		8.50 ns	10.60		8.31 ns
1801	C_{15}OH, *120*, *119*, 93, 220	2.04	>	1.50*	1.94		2.19 ns	2.56		2.31 ns
1806	Nootaktone	1.24	>	0.92*	1.27		1.42 ns	1.78		1.68 ns
1823	β-Vetivone	0.96		1.07 ns	1.14		1.15 ns	2.51	>	1.63*
1834	C_{15}O, *91*, 41, 105, 218	2.59	>	1.89*	2.48		2.63 ns	1.89	<	2.82*
1842	α-Vetivone	2.36		2.34 ns	2.71	>	1.76*	3.50	>	2.95*
1902	C_{15}O, *218*, 136, 147, 203	0.40	>	0.20*	0.57	>	0.40*	0.48	<	0.78*
1933	Cyclohexadecanolide	0.00		0.00 ns	0.01		0.02 ns	0.00		1.16 ns
1960	Hexadecanoic acid	0.17		0.42 ns	0.95		0.58 ns	1.68	<	4.22*
Percent oil (100 × g oil/g dry wt.)		0.32	<	1.04*	0.51		0.66 ns	0.82		0.66 ns

KI = Kovats index on DB-5; TC = tissue cultured plantlets; ECHO = plantlets originated from ECHO outdoor plot in Florida, *significant at $P = 0.05$.

Table 2
Comparison of arbuscular mycorrhizal fungi (AMF) colonization on vetiver samples

Test	Genotype	Origin	Soil	Colonization (%)	Oil yield (%)	No. of cpds diff.	No. of cpds larger
1a	MAL	TC	Sterile	8.4[a]	0.32[a]	20 (1a vs. 1b)	14 in MAL/TC/st.
1b	MAL	ECHO	Sterile	5.2[a]	1.04[c]		8 in MAL/ECHO
2a	MAL	TC	Non-sterile	26.0[b]	0.51[a,b]	11 (2a vs. 2b)	3 in MAL/TC/n-st.
2b	MAL	ECHO	Non-sterile	28.8[b]	0.66[b]		8 in MAL/ECHO/n-st.
3a	KRN	TC	Sterile	10.2[a]	0.82[b]	32 (3a vs. 3b)	19 in KRN/TC/st.
3b	KRN	ECHO	Sterile	34.8[b]	0.66[b]		13 in KRN/ECHO/st.

Any values with a common superscript are not significantly different ($P = 0.05$, SNK test).
TC = tissue cultured plantlets; ECHO = plantlets from ECHO, Ft. Meyers, FL.

There were, however, a couple of anomalities. For the MAL genotype, zizanal was absent only in the ECHO (natural plants) grown in sterilized soil (Table 1). For the KRN genotype, KI 1605 (sesquiterpene hydrocarbon) and KI 1679 (sesquiterpene alcohol) were present in the TC plants, but absent in normal (ECHO) plants. These compounds may have arisen by a somatic mutation in tissue culture, but this kind of event is unusual in apical meristem tissue culture (Morel, 1972).

In conclusion, it was not possible to obtain completely AMF free plants when growing these under ambient conditions. It may be that some AMF were contaminated due to rain splash or wind, incomplete sterilization of the soil or tissue cultured plantlets. For MAL the lowest incidence of AMF is 5.2% and it's highest at 28.8%. There were many significant differences between sterilized plants vs. natural plants grown in either sterilized or non-sterilized soil. However, oil yields (%) were not correlated with the amount of AMF colonization. So even though the study contained some AMF colonization, there were sufficient differences. AMF colonization did not appear to produce unusual compounds characteristic of fungi, nor did bacterial biotransformations appear to change the oils. The oil composition profile may be influenced by biological entities in the soil, but it appears that vetiver genes control the major portion of the composition of vetiver oil.

Table 3
Compounds found in significantly larger amounts in various paired treatments

MAL genotype				KRN genotype	
Grown in sterile soil		Grown in non-sterile soil		Grown in sterile soil	
TC (sterilized)	ECHO (non-sterilized)	TC (sterilized)	ECHO (non-sterilized)	TC (sterilized)	ECHO (non-sterilized)
8.4% AMF	5.2% AMF	26.0% AMF	28.8% AMF	10.2% AMF	34.8% AMF
α-Amorphene	*trans*-Sabinene hydrate	Vetiselinenol	Khusimene	KI 1460 C_{15}HC	Khusimone
Valencene	Khusimone	α-Vetivone	KI 1493 C_{15}HC	α-Amorphene	KI 1613 C_{15}HC
KI 1507 C_{15}HC	KI 1651 C_{15}OH	KI 1902 C_{15}O	δ-Amorphene	KI 1493 C_{15}HC	KI 1647 C_{15}O
Elemol	KI 1682 C_{15}OH		KI 1594 C_{15}OH	Valencene	epi-zizanone
β-Vetivene	KI 1688 C_{15}O		Khusimone	δ-Amorphene	KI 1675 C_{15}OH
KI 1565 C_{15}HC	KI 1698 C_{15}OH		KI 1647 C_{15}O	α-Muurolene	KI 1682 C_{15}OH
KI 1625 C_{15}OH	Khusimol		KI 1651 C_{15}OH	KI 1538 C_{15}HC	KI 1688 C_{15}O
Zizanal	KI 1758 C_{15}O		KI 1688 C_{15}O	KI 1584 C_{15}HC	KI 1736 C_{15}O
α-Costol				KI 1589 C_{15}HC	KI 1758 C_{15}O
(*E*)-Isoeugenol				KI 1594 C_{15}OH	KI 1834 C_{15}O
KI 1801 C_{15}OH				KI 1605 C_{15}HC	KI 1902 C_{15}O
Nootkatone				KI 1630 C_{15}OH	Hexadecanoic acid
KI 1834 C_{15}O				α-Cadinol	
KI 1902 C_{15}O				Vetiselinenol	
				13-Hydroxyvalencene	
				α-Costol	
				(*E*)-Isovalencenol	
				β-Vetivone	
				α-Vetivone	

TC = plantlets obtained from tissue culture; ECHO = plantlets obtained from ECHO test plot on native soil; Ft. Meyers, FL.

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