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Vetiveria zizanioides: an approach to obtain essential oil variants via tissue culture

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Summary

Vetiver oil, isolated from the roots of the tropical grass *Vetiveria zizanioides*, is an important raw material for the perfume industry. Since it is a very complex essential oil, vetiver oil could up to now not be produced artificially. Therefore, it would be of interest to obtain vetiver variants with a different odor (oil composition) or a higher oil yield.

Such variants can not be produced via traditional breeding, since the used vetiver variant is not flowering. Therefore plant regeneration via callus or liquid culture starting from crown or leaf slices was chosen to obtain somaclonal variants. A successful regeneration within 18 weeks was reached via callus on up to 55 % of the crown slices and up to 60 % of the leaf slices with up to 100 plantlets per slice by changing growth regulator concentrations (2,4-dichlorophenoxy acetic acid, 6-benzylaminopurine), sucrose concentrations and cultivation conditions (light, dark). Other regeneration methods, described in literature, did not work with the used non-flowering vetiver from Java.

Since, starting from *in vitro* plantlets, more than 15 - 22 months are needed until the plant contains the complete vetiver oil, a pre-screening of the plantlets in an early stage would be advantageous. Unfortunately, the genes involved in the biosynthesis of vetiver oil are not known. As a result it is necessary to screen for phenotypical changes and especially for the production of more or altered oils. To assess quantitative and qualitative changes in the vetiver oil composition of the plant material already in tissue cultures, methods to extract and analyze the vetiver oil from small samples had to be optimized and compared. Methods based on olfactive detection, inhibition of microbial growth and analysis by thin layer chromatography (TLC) and gas chromatography (GC) were therefore compared. The olfactive detection was useful for pre-screening, but the analysis was subjective and not accurate enough. GC analysis provided more detailed information, while TLC was preferred for a preliminary analysis of many samples.

To extract the oil, water distillation and solvent extraction were optimized and compared. The distillation times could be reduced by using 0.5 M phosphate buffer at pH 8 instead of water. Furthermore, this substitution resulted in the distillation of less acidic compounds. By combining water distillation with solid

phase extraction, an approach was developed to distill several small samples (about 100 mg) in parallel. The procedure for solvent extraction at room temperature could easily be miniaturized for extracting 100 mg vetiver roots in 1.5 ml hexane. Unfortunately additional non-volatile compounds were extracted, which caused base line shifting and increased noise during GC analysis. Neither TLC nor column chromatography were able to remove the non-volatile compounds from small scale samples. The choice of hexane extraction is favorable since it is less labor intensive than water distillation combined with solid phase extraction.

In this study, we were able to regenerate plantlets via *in vitro* culture and optimized methods to extract and analyze large numbers of very small samples in parallel. This means that we now have all tools to develop somaclonal variants *in vitro* and test the oil produced in such plantlets. The next stage in this work will be dependent on the ability to induce oil production and accumulation in plantlets or *in vitro* tissue in an early stage. Future work should include the development of such an induction method and the application and further development of the extraction and analysis methods.

Zusammenfassung

Vetiveröl, welches aus den Wurzeln des tropischen Grases *Vetiveria zizanioides* isoliert wird, ist ein wichtiges Rohmaterial der Parfümindustrie. Da es ein sehr komplexes ätherisches Öl ist, konnte es bis jetzt noch nicht künstlich hergestellt werden. Es besteht deshalb ein grosses Interesse an Vetiver-Varianten mit unterschiedlichem Geruch d.h. Ölzusammensetzung oder höherem Ölgehalt.

Bei der verwendeten Vetiver-Kultivar aus Java versagen die traditionellen Züchtungsmethoden, da die Pflanze nicht blüht. Aus diesem Grunde wurde der Weg über die Produktion somaklonaler Varianten via Kallus- oder Flüssigkultur gewählt, wobei Rhizom- und Blattscheiben als Ausgangsmaterial benutzt wurden. Eine erfolgreiche Regeneration via Kalli wurde durch Optimieren der Wachstumregulator-Konzentrationen (2,4-Dichlorophenoxyessigsäure, 6-Benzylaminopurin) und des Zuckergehaltes im Medium sowie durch Ändern der Kulturbedingungen (Kultur im Licht oder im Dunkeln) erreicht. Mit der optimierten Methode konnten innerhalb 18 Wochen auf bis zu 55 % Rhizomscheiben und auf bis zu 60 % Blattscheiben Pflanzen regeneriert werden, mit bis zu 100 Pflanzen pro Scheibe. Andere in der Literatur beschriebene Regenerationsmethoden führten bei dem nicht-blühenden Vetiver-Kultivar aus Java zu keinen positiven Resultaten.

Ausgehend von den regenerierten *in vitro* Pflanzen dauert es 15 - 22 Monate oder länger, bis die Wurzeln das vollständige Vetiveröl enthalten. Für die Züchtung neuer wertvoller Vetiver-Varianten wäre deshalb eine Vor-Selektion in einem frühen Stadium vorteilhaft. Leider sind die für die Vetiveröl Biosyntese verantwortlichen Gene nicht bekannt. Deshalb muss nach phänotypischen Veränderungen insbesondere des Ölertrags und des Ölqualität gesucht werden. Zur qualitativen und quantitativen Bestimmung der Vetiveröl-Zusammensetzung wurden für kleine Probenvolumen die nötigen Extraktionsmethoden und Analytik entwickelt. Zur Analyse wurden Methoden basierend auf olfaktorischer Detektion, bakterieller Wachstumshemmung, Dünnschichtchromatographie (DC) und Gaschromatographie (GC) getestet. Die olfaktorische Detektion wurde zum Vor-Screenen auf das Vorhandensein von

Vetiveröl genutzt. GC-Analysen ergaben detaillierte Informationen, während DC für Voranalysen vieler Proben bevorzugt wurde.

Zur Extraktion des Öles wurden Wasserdestillation und Lösungsmittel-Extraktion optimiert und verglichen. Die Destillationsdauer konnte reduziert werden, indem das Wasser durch 0.5 M Phosphatpuffer (pH 8) ersetzt wurde. Gleichzeitig wurden dadurch weniger Säuren destilliert. Die Kombination von Wasserdestillation und Festphasen-Extraktion ermöglicht die gleichzeitige Destillation vieler kleiner Proben (ca. 100 mg). Die Lösungsmittel-Extraktion bei Raumtemperatur konnte leicht auf kleine Mengen übertragen werden (100 mg Vetiver-Wurzeln in 1.5 ml Hexan). Gleichzeitig werden dabei auch nicht-flüchtige Substanzen extrahiert, welche die GC Analytik erschweren und weder durch DC noch durch Säulenchromatographie von den kleinvolumigen Proben getrennt werden können. Gegenüber der mit Festphasen-Extraktion kombinierten Destillation hat die Hexan-Extraktion den Vorteil, dass sie weniger arbeitsintensiv ist.

In dieser Arbeit gelang es, Pflanzen via *in vitro* Kulturen effizient zu regenerieren und Methoden zu entwickeln für eine parallele Extraktion und Analyse sehr kleiner Probemengen. Somit sind nun alle Werkzeuge zur Produktion somaklonaler Varianten und zur Analyse der Öle dieser regenerierten Pflanzen vorhanden. Der nächste Schritt ist abhängig davon, ob die Ölsynthese und Akkumukation in einem frühen Stadium der Pflanze induziert werden kann. Zukünftige Arbeiten sollten neben der Entwicklung einer solchen Induktionsmethode auch die Anwendung und Weiterentwicklung der Extraktions- und Analyse-Methoden beinhalten.

Abbreviations

AA medium	Müller and Grafe medium (Müller and Grafe 1978)
AFLP	amplified fragment length polymorphism
BA	6-benzylaminopurine
CAPS	cleaved amplified polymorphic sequence
CC	column chromatography
cfu	colony forming units
Chl	chloroform
CoA	Co-enzyme A
2,4-D	2,4-dichlorophenoxy acetic acid
DOXP	1-deoxy-D-xylulose-5-phosphate
DMAPP	dimethylallyl diphosphate, IPP isomer
DMSO	dimethyl sulphoxide
dw	dry weight
FID	flame ionization detector
FPP	farnesyl diphosphate
GC	gas chromatography
GGPP	geranylgeranyl diphosphate
GPP	geranyl diphosphate
Hex	hexane
HexN	hexane extract of vetiver roots evaporated with nitrogen
HexSil	hexane extract of vetiver roots loaded on a silica column
HMG-CoA	(S)-3-hydroxy-3-methylglutaryl-CoA
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HPLC	high pressure liquid chromatography
IAA	indole acetic acid
IPP	isopentenyl diphosphate
IS	internal standard
Kin	kinetin
LB medium	Luria-Bertani medium (Sambrook *et al.* 1989)
M65 medium	Streptomyces medium (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany)

material X	estimated amount of oil in segment X based on GC analysis
MS	mass spectroscopy
MS medium	Murashige and Skoog medium (Murashige and Skoog 1962)
MTBE	methyl *tert*-butyl ether
MVA	mevalonate
N6 medium	medium described by Chu *et al.* (1975)
NAA	α-naphthalene acetic acid
P-buffer	phosphate buffer
PCR	polymerase chain reaction
Px/pHy	distillation with a phosphate buffer (x M), at a pH y
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rpm	rotations per minute
TLC	thin layer chromatography
UV	ultra violet light
v/v	volume per volume

Chapter 1: Introduction

1. *Vetiveria zizanioides*

1.1. Plant

synonyms: *Phalaris zizanioides* L. (1771), *Andropogon muricatus* Retzius (1783), *A. zizanioides* (L.) Urban (1903) (de Guzman and Oyen 1999)

Vetiveria zizanioides , also known as vetiver (grass), khus or khus-khus, is a perennial tropical grass. It belongs like maize, sorghum, sugarcane and lemon-grass to the family of *Poaceae* (*Gramineae*). The generic name *Vetiveria* comes from the Tamil word "vetiver" meaning "root that is dug up". The specific name *zizanioides* was first given by the Swedish taxonomist Carolus Linnaeus in 1771, meaning "by the river side" (Vietmeyer and Ruskin 1993).

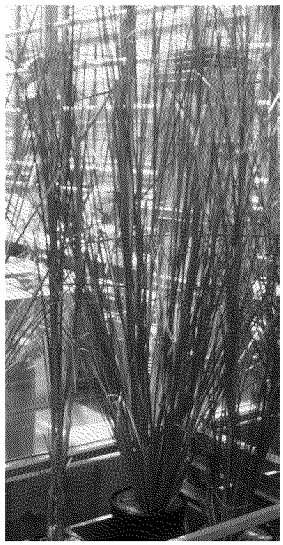

Figure 1.1: Vetiveria zizanioides in a green-house

Vetiver is native to India. The exact location of origin is not precisely known; some say that it is native to northern India, others say that it is native around Bombay (Vietmeyer and Ruskin 1993). As the specific name suggests, vetiver grows particularly on river-banks and in rich marshy soil (Anonymous 1976). However, it can not only stand long periods of inundation, it can also withstand extreme drought, survives temperatures between -9°C and 45°C, is fire-resistant and is able to grow in any type of soil regardless of fertility, salinity or pH (pH 4.0 - 9.6) (Anonymous 1976; de Guzman and Oyen 1999; Sreenath *et al.* 1994).

Vetiver grows in large, densely tufted clumps from a stout, compact rhizome (crown) with erect culms up to 3 meters high. The roots bind the soil beneath the plant, reaching depths of up to 4 meters (de Guzman and Oyen 1999; Sreenath *et al.* 1994; Vietmeyer and Ruskin 1993). The wild type from North India flowers under suitable marshy conditions regularly and sets fertile seeds. It is a "colonizer" and therefore might become a weed (de Guzman and Oyen 1999). The "domesticated" type from South India consists of flowering and non-flowering vetiver plants (Anonymous 1976). If they flower they produce no

viable seeds. It might be that the seeds are sterile or the conditions for germination are seldom met (Vietmeyer and Ruskin 1993). These vetiver plants replicate by vegetative propagation via side shoots.

1.2. Use of vetiver

Vetiver has been cultivated in India for centuries and is now found throughout the tropics and in many subtropical areas (de Guzman and Oyen 1999). Besides many others, there are two main reasons why vetiver is cultivated: it is used as protection against erosion and the roots contain an essential oil.

1.2.1. Protection against erosion

Due to its densely packed, stiff and tough grass stems and the deeply penetrating root system that works as an anchor, non-seeding vetiver plants are used as living dams to slow down run-off water, to trap nutrient-rich top soil and generally therefore, to combat erosion and increase moisture conservation (Adams *et al.* 1998; de Guzman and Oyen 1999; Vietmeyer and Ruskin 1993). Since 90 % of the roots are found within a radius of 20 cm from the vetiver plant (de Guzman and Oyen 1999; Salam *et al.* 1993), vetiver does not interfere with plants cultivated nearby and can be used in natural hedges beside crops.

Erosion is combated with vetiver hedges in more than 160 countries (Adams *et al.* 1998). Since a lack of genetic diversity increases susceptibility by diseases or insects, Adams *et al.* (1998) tested vetiver plants from around the world by random amplified polymorphic DNA (RAPD) for additional nonfertile germplasm to broaden the genetic base for erosion-control projects. They found that the vetiver cultivated outside of South Asia have been derived from a single genotype. More nonfertile vetiver lines will be analyzed to assure the genetic diversity of cultivated vetiver (Adams *et al.* 1998).

1.2.2. Roots contain an essential oil

Vetiver contains an essential oil, called vetiver oil. The oil occurs primarily in the roots, but traces of it in the foliage may nevertheless account for the plant's inherent resistance to pest and diseases (Vietmeyer and Ruskin 1993). The aroma of the essential oil is basically of a heavy, woody, earthy character, pleasant and extremely persistent (de Guzman and Oyen 1999; Sreenath *et al.* 1994). These aromatic roots are either used directly to weave them into mats, fans, sachets and ornaments or put among clothes to keep insects away (de Guzman and Oyen 1999; Vietmeyer and Ruskin 1993), or they are extracted by steam distillation to yield the essential oil, which is used in perfumes, deodorants, soap and other toilet articles (de Guzman and Oyen 1999). Vetiver oil is of interest to the cosmetic and perfumery industry, not only due to its scent, but also due to its ability as fixative, keeping more volatile oils from evaporating (Anonymous 1976).

1.2.3. Phytoremediation of polluted areas

Since vetiver is highly tolerant to arsenic, cadmium, chromium, copper, lead, nickel and zinc in the soil, it is suitable for the rehabilitation of lands contaminated with these elements. The vetiver plants do not only prevent erosion and thus retain the polluted soil at these sites, but when harvested and removed from the sites, and disposed of safely elsewhere, the level of heavy metals in the soil can be gradually lowered with time (Chen *et al.* 2000; Truong 1999; Truong and Claridge 1996).

1.2.4. Medicinal applications

In medicine, both the plant and its essential oil are used. A paste made of the roots is claimed to have an almost magic effect, when applied to bruises, swellings or burns. A stimulant drink is made from fresh rhizomes in India, and in Madya Pradesh (India) the plants are used as an anthelmintic (de Guzman and Oyen 1999; Sreenath *et al.* 1994).

1.2.5. Other uses

Young leaves of vetiver can be used as fodder for cattle and goats, whereas the dried grass is used for making brooms or for thatching of huts. The grass can also be used for making writing and printing paper. Since the pulp is short-fibered, 30 - 40 % of a long-fibered pulp (e.g. *Eulaliopsis binata*) has to be added (Anonymous 1976; de Guzman and Oyen 1999).

2. Variants of plants

2.1. Production of variants

2.1.1. Traditional breeding and selection

Traits of different variants can be combined by traditional breeding and the resulting plants can then be screened for the desired trait. However, the desired traits have to be present in a sexually compatible plant.

2.1.2. Tissue culture: somaclonal variation and mutation

Plant cells propagated in culture can undergo variations. Although only a fraction of the variability present in the cell cultures is actually recovered in the regenerated plants, due to some degree of selection during regeneration (Vasil and Vasil 1986), regeneration via tissue culture provides another way to obtain variants.

The variations are divided in genetic and epigenetic. Genetic variation is used to describe heritable variation that is sexually transmitted to the progeny of regenerated plant. This phenomenon is called somaclonal variation. Epigenetic variation is used to describe non-hereditary variation, which is at the same time reversible and is often the result of a changed gene expression (Pierik 1987).

The frequency of variations depends on factors like cultivar, source of the tissue, the culture method (e.g. callus culture, suspension culture, regeneration via organogenesis or somatic embryogenesis), duration of the disorganized

phase and growth regulators used (Karp 1994; Maddock 1985; Pierik 1987). In somaclonal variants, changes in ploidy level, chromosome breakage and rearrangements, gene amplification, single-gene mutation, variation in quantitative traits, and activation of transposable elements have been reported (Maheshwari *et al.* 1995). Nevertheless, there is no guarantee that any character of specific interest will be among somaclones, since even in cases where somaclonal variation has occurred at high frequencies and changes have been observed in a whole range of characters, some characters do not change (Karp 1994).

Since many of the variations observed in somaclones are similar to those obtained by traditional breeding (Karp 1994; Vasil and Vasil 1986), somaclonal variation as a novel source of variability for plant improvement remains a controversial issue. However, in asexually propagated species where alternative breeding approaches are limited, the prospects of somaclonal variation remain promising (Karp 1994).

2.1.3. Genetic engineering

A third possibility to obtain plants with a desired trait is by genetic engineering. By introducing and expressing or inactivating specific genes, plants with useful phenotypes unachievable by conventional plant breeding can be generated.

Essential requirements for the production of transgenic plants are (Birch 1997; Maheshwari *et al.* 1995):

a) availability of a target tissue including cells competent for plant regeneration, which are also accessible to the gene transfer treatment. Tissue culture is often employed to achieve a workable efficiency of gene transfer, selection, and regeneration of transformants. To avoid somaclonal variation, the tissue culture phase, especially in the unorganized state, should be minimized or even eliminated, by transferring genes into intact tissue explants and regenerating these without substantial *in vitro* culture.

b) a method to introduce DNA which can be expressed in plants, into the regenerable cells. Depending on the plant material, methods like

Agrobacterium-mediated transformation, bombardment with DNA-coated microprojectiles, and electroporation or polyethylene glycol treatment of protoplasts, are used to introduce DNA into the plant cells. Efficient expression of a foreign gene in plants requires the adaptation of the DNA to plant genes and selection of an appropriate promoter as well as polyadenylation sequences, since the plant transcription machinery does not recognize foreign gene control sequences, particularly promoter sequences of many bacterial genes, while the codon usage of plants and bacteria differs significantly. The introduction of introns has been shown to enhance expression of genes several-fold in a number of systems.

c) a procedure to select for transformed cells and to regenerate plantlets in a satisfactory frequency has to be available. To recover transformed cells, genes conferring resistance to a selective chemical agent, genes conferring a phenotype allowing visual or physical screening, or even PCR screening to identify plants containing transferred genes can be used.

2.2. Detection of variants

2.2.1. Detection of variants by comparing phenotypes

Depending on the trait of interest, different methods have to be chosen to detect variants. Selection for morphological changes like herbage yield, tiller number, diameter of the bush, length and area of the longest leaf, morphology of the flowers or leaves, fresh and dry weight ratio and harvest of crops, is the easiest, since these changes can be monitored during normal growth. However, to select for traits like resistance against stress (temperatures, water, chemicals or fungi, bacteria or insect attack) or for changed production rate or spectrum of secondary metabolites which are only produced or accumulated under specific conditions or in a specific developmental stage, the plantlets can only be tested under these specific conditions. The results can be influenced by developmental and environmental factors and it is not possible to distinguish between soma-clonal and epigenetic variants.

2.2.2. Detection of variants at the molecular level

Somaclonal variations might be detected at the molecular level. By screening the plants for changes within the DNA, the results are not influenced by developmental and environmental factors. There are several molecular techniques available for checking the genome, based on analysis of random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), microsatellite repeat polymorphism (also known as simple sequence repeat) and cleaved amplified polymorphic sequences (CAPS) (Jones *et al.* 1997; Rafalski and Tingey 1993; Ridout and Donini 1999). However, after detection of a change in the DNA, it is not known what kind of effect it has on the plant or even whether there is a detectable effect.

3. Essential oil

Essential oils are a mixture of fragrant, highly volatile substances isolated by a physical process from an odiferous plant of a single botanic species or variety (Encyclopaedia Britannica 1986, Oyen and Dung 1999). The essential oil is named after the aromatic plant from which it has been derived. They are often stored in specialized storage and secretory sites like glandular hairs, resin canals or oil ducts and schizogenous glands (Banthorpe 1988; Charlwood and Charlwood 1991). Essential oils may be found in any part of a plant and can be extracted from roots, wood, bark, leaves, flowers, fruit and seed for commercial purposes (Oyen and Dung 1999).

Essential oils have many uses for man. Most obvious is their role in fragrance materials, but they are also used as flavoring materials and in medicine (Oyen and Dung 1999).

3.1. Extraction methods

Depending on the plant material, distinct processes like distillation, solvent extraction, enfleurage and expression are used to produce essential oil (Oyen and Dung 1999; Roth and Kornmann 1997; Weiss 1997b).

3.1.1. Water or steam distillation

Essential oils are most often obtained from plant materials by water or steam distillation. During water distillation, water and plant material are boiled together in a pot by direct fire, whereas during steam distillation, the steam is produced in a separate steam generator. As a result, the plant material does not come in contact with the hot wall of the pot and therefore charring and decomposing of compounds can be avoided (Oyen and Dung 1999; Roth and Kornmann 1997; Weiss 1997b).

3.1.2. Solvent extraction

Certain oils are obtained by solvent extraction, a process which also extracts non-volatile compounds, yielding after removal of the solvent a substance called concrete. Solvent extracted oils are generally considered to reflect a plant's natural odor more accurate than distilled oils. Repeated washing of the concrete with alcohol to remove waxes and other inert matter produces an absolute. If ethanol is used to extract the plant material, the resulting extract is called resinoid. By using carbon dioxide or supercritical fluid extraction the problems associated with solvent removal can be avoided, since carbon dioxide is odorless, tasteless and inexpensive (Oyen and Dung 1999; Roth and Kornmann 1997; Weiss 1997b).

3.1.3. Enfleurage

Enfleurage is a method to capture the odor of delicate, heat sensitive flower oils by absorption on wax or fat. The enfleurage process is mainly advantageous for flowers, like jasmine, that continue to produce aroma compounds for several days after they have been picked. The resulting product is called pomade (Oyen and Dung 1999; Roth and Kornmann 1997; Weiss 1997b).

3.1.4. Expression

Citrus peel oils, like those of bergamot, grapefruit, lemon, lime and orange, are extracted by expression. In expression methods the fruit peel is compressed, lacerated or abraded to rupture the oil cells (Oyen and Dung 1999; Roth and Kornmann 1997; Weiss 1997b).

For small scale extraction of essential oils, methods like simultaneous distillation - solvent extraction (Bicchi *et al.* 1983; Godefroot *et al.* 1981), headspace solid-phase micro extraction (Field *et al.* 1996), microwave extraction (Craveiro *et al.* 1989) and supercritical fluid extraction (Blatt and Ciola 1991; Sugiyama and Saito 1988) have been described. Depending on the extraction method used, the composition and yield of essential oils may vary to a large extent (Boutekedjiret *et al.* 1997; Pino *et al.* 1996; Scheffer 1996; Simándi *et al.* 1999). For example, the supercritical carbon dioxide extraction of ground fennel seeds resulted in a higher yield (10 %) than steam distillation (3 %), almost the same yield as hexane extraction (10.6 %), and lower yield than alcohol extraction (15.4 %). Sensory evaluation showed that the carbon dioxide extraction product and the distilled oil were more intense in odor and taste than the alcohol and hexane extracts (Simándi *et al.* 1999). These differences can be explained by the fact that not all components are extracted equally well by each process and because individual components may undergo changes during the process (Oyen and Dung 1999).

3.2. Analysis methods

Until a few decades ago, the human nose, supported by the measurement of physical characteristics and a few simple chemical analyses were the only means of verifying the density, purity and the naturalness of essential oils. The physical characteristics most commonly used to characterize essential oils are relative density, refractive index, miscibility (usually with aqueous alcohol) and optical rotation (Oyen and Dung 1999). However, the information on the chemical composition of essential oils was greatly improved by the development

of analyses methods involving chromatographic separation and pure natural essential oil and modified oils can now be more easily distinguished (Oyen and Dung 1999). Different methods like gas chromatography (GC), high pressure liquid chromatography (HPLC), column chromatography and thin layer chromatography (TLC) have been used for the analysis of essential oils (Banthorpe 1991; Croteau and Ronald 1983; Harborne 1984b; Kubeczka 1985).

3.2.1. Gas chromatography (GC)

GC has been the classical tool for analysis and isolation of volatile compounds, providing qualitative and quantitative data (Banthorpe 1991; Harborne 1984b; Kubeczka 1985). To detect the compounds, a flame ionization detector (FID) is commonly used. However the human nose or mass spectroscopy (MS) can also be used to obtain more information about the odor or the structure of the compounds (Gardner and Bartelett 1999). Since the very complex nature of many essential oils does not allow a complete chromatographic separation even on a high resolution capillary column, gas chromatography-tandem mass spectrometry and comprehensive gas chromatography (GCxGC) have been used (Cazaussus *et al.* 1988; Marriott *et al.* 2000; Sellier *et al.* 1991). By using gas chromatography-tandem mass spectrometry, Cazaussus *et al.* 1988 and Sellier *et al.* 1991 were able to analyze each component of overlapping peaks separately. Marriott *et al.* (2000) obtained a much superior resolution of vetiver essential oil with comprehensive gas chromatography than by normal GC. Another option to obtain a total delineation of the components is by a prior fractionation of the essential oil, by chemical class separation, fractional distillation or liquid chromatography (Kubeczka 1985).

3.2.2. Thin layer chromatography (TLC)

Due to its simplicity and rapidity, TLC is still one of the most important methods for the analysis of essential oils (Kubeczka 1985). Furthermore, TLC is also useful in combination with GC to obtain a better separation (Harborne 1984b) or as a pilot technique for column chromatography separation of essential oils (Kubeczka 1985). To detect the TLC spots, UV or different

staining methods can be used (Cosicia 1984; Croteau and Ronald 1983; Gibbons and Gray 1998; Merck 1970).

3.2.3. High pressure liquid chromatography (HPLC)

HPLC is not often used for the analysis of volatile compounds, since relatively good separations were obtained by GC. However, in contrast to GC, HPLC is useful for separation of temperature labile and of non-volatile compounds (Kubeczka 1985).

Depending on the mix, different columns based on normal phase, reversed phase, size exclusion, partition and affinity materials can be chosen (Banthorpe 1991) and by changing from isocratic to gradient elution, better resolution has been obtained (Schwanbeck *et al.* 1982). For the detection of the eluates, UV absorption, refraction index changes or mass spectrometry can be used (Banthorpe 1991). However, since most of the essential oil components lack chromophoric groups, low UV monitoring from 200 to 220 nm is necessary, which limits the application of such analysis to HPLC separations with only a few solvents (Kubeczka 1985).

3.2.4. Sensory analysis

The human nose is a useful tool to analyze qualitative changes in essential oil, since the human nose is still about 1000 times more sensitive than modern analyzers (Oyen and Dung 1999). However, the measurement is limited by the onset of olfactory fatigue after around 30 minutes and the sensitivity to odorants varies widely both with the nature of the odorant and from person to person. In addition, the sensitivity of individuals was found to vary with their state of health and their endocrinological condition (Gardner and Bartelett 1999).

Therefore, the development of electronic noses (Gardner and Bartelett 1999; Weiss 1997b) or a detector made of cell cultures with specific olfactory receptors (Metzger 2001; Monastyrskaia *et al.* 1999) would be interesting.

3.3. Chemical components

Essential oils are complex mixtures of sometimes hundreds of chemical compounds. Most of these compounds can be grouped into 4 major groups: aliphatic compounds (acyclic organic compounds), terpenes and terpene derivatives, benzene derivatives and miscellaneous compounds (Oyen and Dung 1999).

3.4. Terpenoids

3.4.1. General

In primary metabolism, terpenoids are involved in photosynthesis (carotenoids, chlorophyll, phylloquinone, plastoquinone), in respiration (ubiquinone, cytochrome a), lipid membrane structure (triterpenoids, sterols), regulation of development and growth (gibberellins, abscisic acid, brassinosteroids, some cytokinins) and in cell cycle control (prenylated proteins). Terpenoids, which are not essential for viability (e.g. monoterpenoids, sesquiterpenoids and diterpenoids as present in essential oils) are classified as secondary metabolites. Some are important for the plant to adapt to environmental conditions e. g. plant-plant, plant-insect and plant-microorganism interactions. However, for most of the terpenoids the function is unknown (Cane 1999a; Champenoy *et al.* 1999; Kribii *et al.* 1999; Newman and Chappell 1999).

Terpenoids share a common characteristic: they all derive from the same building block, the isoprene unit (C5). They are therefore also called isoprenoids. Some terpenoids are acyclic, but most are cyclic, with a wide variety of carbon skeletons (Kleinig 1989). For sesquiterpenoids (C15), already more than 300 sesquiterpene carbon skeletons have been identified and thousands of naturally occurring oxidized or otherwise modified derivatives have been isolated from marine, terrestrial plant, and microbial sources (Cane 1999b).

3.4.2. Terpenoid biosynthesis

3.4.2.1. Biosynthesis of the isoprene unit

Two biosynthesis pathways have been described for the production of the precursor isopentenyl diphosphate (IPP): the mevalonate pathway starting from acetyl-CoA via mevalonate (MVA) (Figure 1.2) and the non-mevalonic pathway or DOXP pathway from pyruvate and glyceraldehyde-3-phosphate via 1-deoxy-D-xylulose-5-phosphate (DOXP) (Figure 1.3). Only a minority of the bacteria use the MVA pathway, while the DOXP pathway is more common. The DOXP pathway is found either alone (e.g. *Bacillus subtilis, Escherichia coli*), with some genes of the MVA pathway or in addition to the complete MVA pathway (Boucher and Doolittle 2000). Non-photosynthetic eukaryotesuse the MVA pathway exclusively. In most plants and algae both pathways exist, except in unicellular green algae (e.g. *Scenedesmus obliquus, Chlamydomonas reinhardtii* and *Chlorella fusca*) which seem to have lost the MVA pathway after acquisition of the DOXP pathway (Boucher and Doolittle 2000). Within plant cells, the terpenoid biosynthesis was observed in the cytoplasm, the mitoplasm (the mitochondrial matrix), and the plastoplasm (the plastid stroma) (Kleinig 1989). In the cytoplasm, IPP is produced via the MVA pathway, whereas the DOXP pathway is located in the plastids. For the biosynthesis of the prenyl side-chain of the ubiquinones, the mitochondria depend on cytosolic IPP formation (Lichtenthaler 1999). Whether the two cellular IPP pools of the cytoplasm and the plastids cooperate and exchange IPP or other prenyl-diphosphates, such as geranyl diphosphate (GPP), farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP), is not known at present. Several observations suggest at least some exchanges (see also Lichtenthaler 1999).

3.4.2.1.1. MVA pathway (Figure 1.2)

In the first two reactions of the MVA pathway, three acetyl-CoA condense resulting in (*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In animals and yeasts these two steps are catalyzed by two separate enzymes, acetyl-CoA acetyltransferase (EC 2.3.1.9) and HMG-CoA synthase (EC 4.1.3.5) (McGarvey

and Croteau 1995), whereas in radish seedlings this double condensation reaction is catalyzed by a single monomeric protein (Weber and Bach 1994).

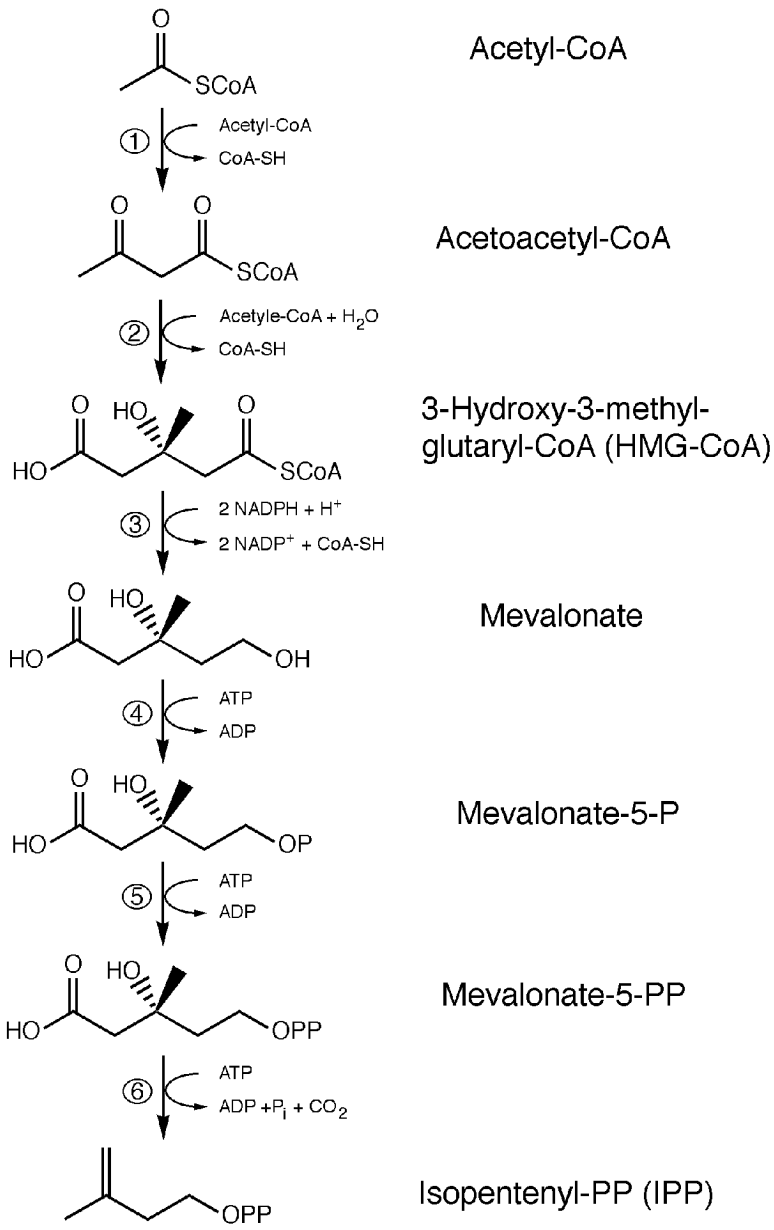

Figure 1.2. Mevalonate (MVA) pathway

1: Acetoacetyl-CoA synthase
2: HMG-CoA synthase
3: HMG-CoA reductase
4: Mevalonate kinase
5: Phosphomevalonate kinase
6: Phosphomevalonate decarboxylase

The next step from HMG-CoA to (*R*)-mevalonate is the first reaction unique to terpenoid biosynthesis and is irreversible. This reaction is catalyzed by HMG-CoA reductase (HMGR, EC 1.1.1.34), a membrane associated enzyme. In animals, fungi and perhaps insects, HMGR is the key regulatory step controlling isoprenoid metabolism (Newman and Chappell 1999). In plants, it is tightly regulated, specially in the case of sterol and phytoalexin synthesis (McCaskill and Croteau 1998). Plants produce much more diverse products from the mevalonate pathway in various tissues and organelles. They contain multiple genes for HMGR, that vary in number from only two in Arabidopsis to over 12 in potato (McCaskill and Croteau 1998; Stermer *et al.* 1994). In all plants investigated, HMGR is encoded by a small family of genes which are expressed differentially during development and in response to external stimuli including light, plant growth regulators, wounding, pathogen attack and exogenous sterols (Lange *et al.* 1998; McGarvey and Croteau 1995; Stermer *et al.* 1994; Weissenborn *et al.* 1995). There is also evidence that the use of different promoters allows the use of alternate transcription start sites within a single gene, with the subsequent expression of different forms of HMGR with variably extended N-terminal regions (Lumbreras *et al.* 1995; McCaskill and Croteau 1998).

The phosphorylation of mevalonate to mevalonate-PP is catalyzed by two separate soluble kinases, mevalonate kinase (EC 2.7.1.36) and phospho-mevalonate kinase (EC 2.7.4.2). The following reaction is catalyzed by diphosphomevalonate decarboxylase (EC 4.1.1.33), resulting in the terpenoid building block IPP (McGarvey and Croteau 1995).

3.4.2.1.2. DOXP pathway (Figure 1.3)

In the first step of the DOXP pathway, a C2-unit derived from pyruvate is transferred to glyceraldehyde 3-phosphate, forming DOXP. This transketolase-type condensation reaction is thiamine-dependent and is catalyzed by the enzyme DOXP-synthase (Lichtenthaler 1999; Lois *et al.* 2000). DOXP is not only an intermediate of IPP synthesis but is also involved in the biosynthesis of thiamine and pyridoxol in plastids. Analysis of the DOXP-synthase expression in tomato indicated developmental and organ-specific regulation of mRNA

accumulation and showed a strong correlation with carotenoid synthesis during fruit development (Lois *et al.* 2000).

The next transformation step from DOXP to 2-C-methyl-D-erythritol 4-phosphate is catalyzed by DOXP reductoisomerase, requiring NADPH and manganese (or magnesium) (Lange and Croteau 1999). In plants, the remaining enzymatic reactions leading to the synthesis of IPP have not yet been fully elucidated (Lois *et al.* 2000) and whether IPP or its isomer dimethylallyl diphosphate (DMAPP) is formed as the first isoprenoid C5-unit is unresolved (Lichtenthaler 1999).

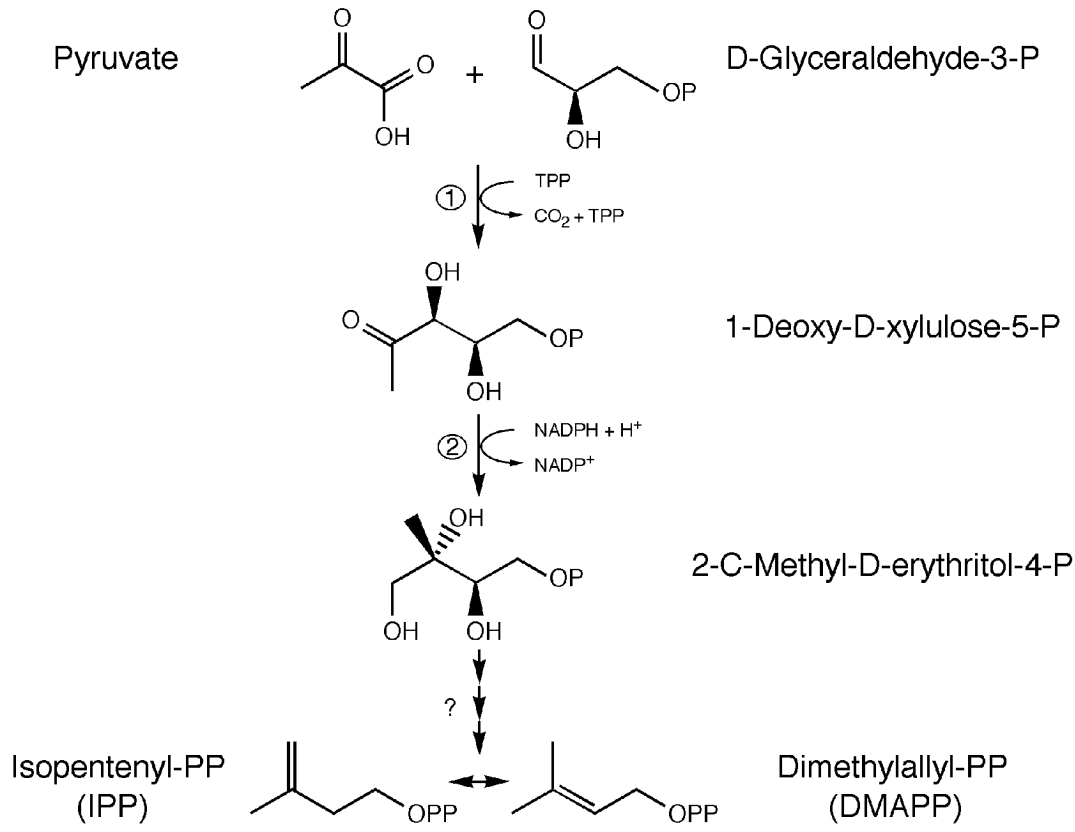

Figure 1.3. 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway
TPP: thiamine diphosphate
1: 1-Deoxy-D-xylulose-5-phosphate synthase
2: 1-Deoxy-D-xylulose-phosphate reductoisomerase

3.4.2.2. Elongation

IPP is not reactive enough to initiate the condensation to higher terpenoids. It first needs an activation step catalyzed by IPP isomerase (EC 5.3.3.2), which converts IPP to its allylic DMAPP (Figure 1.4) (Koyama and Ogura 1999; McGarvey and Croteau 1995). The isomerization is reversible, but the equilibrium is towards DMAPP formation (Ramos-Valdivia *et al.* 1997). Prenyltransferases catalyze the multistep elongation reaction beginning with DMAPP and IPP to form linear terpenoids. Depending on the prenyltransferase, the product may be released from the enzyme surface, or may serve as the substrate for additional condensations. The isoprenoid chain length is tightly controlled by the relevant enzyme (Cane 1999a). Different types of prenyltransferases have been recognized, with two types involved in pure terpenoid reactions, i.e. catalyzing either head-to-tail (1'-4 prenyltransferase: e.g. geranyl diphosphate synthase (GPP synthase or dimethylallyl*trans*transferase, EC 2.5.1.1), farnesyl diphosphate synthase (FPP synthase or geranyl*trans*-transferase, EC 2.5.1.10) and geranylgeranyl diphosphate synthase (GGPP synthase or farnesyl*trans*transferase, EC 2.5.1.29)), or head-to-head condensation of prenyl diphosphates (1'-1 prenyltransferase: e.g. squalene synthase (or farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21) and phytoene synthase (or geranylgeranyl-diphosphate geranylgeranyltransferase, EC 2.5.1.32)). Furthermore, other types of prenyltransferase are involved in the prenylation of proteins and in the reaction of an intermediate of the terpenoid pathway with an intermediate from another biosynthetic pathway to form mesoterpenoids (e.g. ubiquinones, plastoquinones, chlorophyll) (Koyama and Ogura 1999; Kribii *et al.* 1999; Ramos-Valdivia *et al.* 1997).

In plants, FPP and GGPP synthases are encoded by multigene families, e.g. *A. thaliana* contains at least two genes for FPP synthase (Cunillera *et al.* 1997) and 5 genes for GGPP synthase (Okada *et al.* 2000). Each gene is expressed in a different tissue during plant development (Cunillera *et al.* 1997; Okada *et al.* 2000).

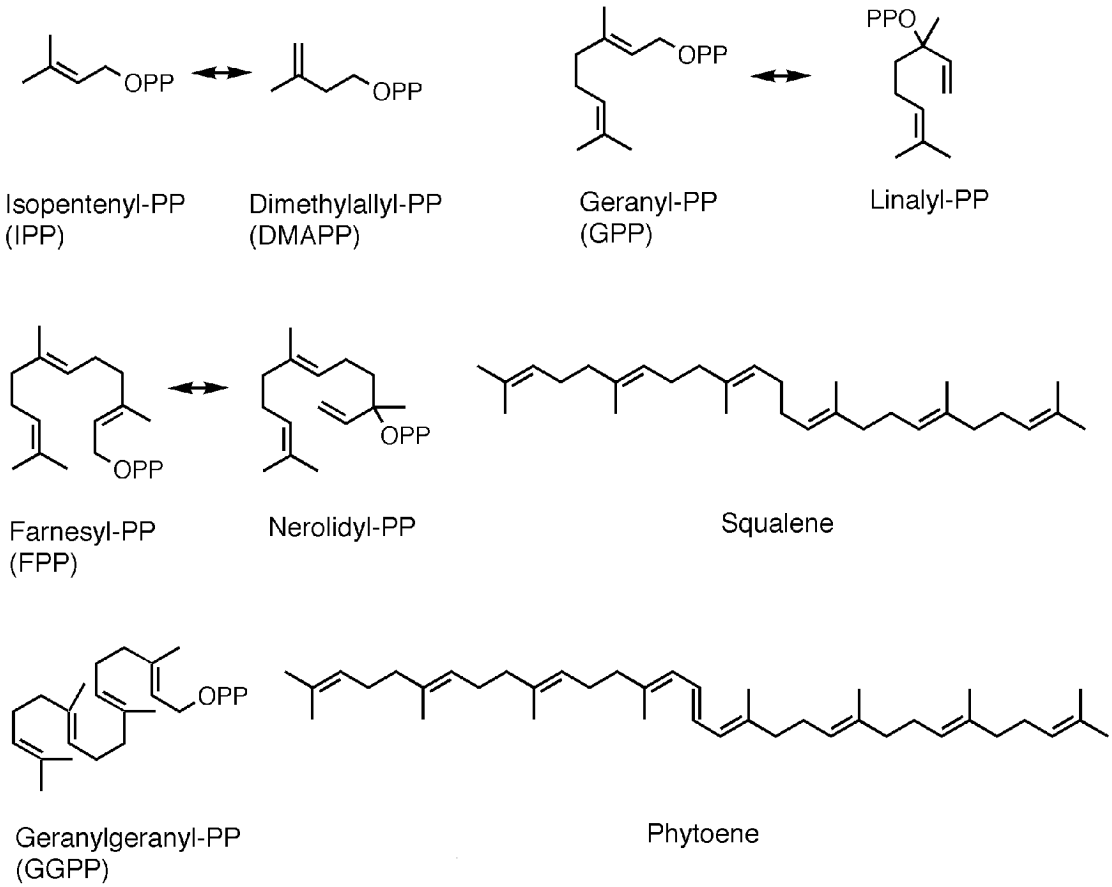

Figure 1.4. Names and structures of the parent compounds of the central terpenoid pathway (C5 - C20)

3.4.2.3. Cyclization

The next metabolic branch point of the terpenoid biosynthesis is the cyclization of the prenyldiphosphates. Due to some geometric barrier of GPP and FPP to form cyclohexanoid compounds, a preliminary isomerization to the corresponding tertiary allylic ester (Figure 1.4) was proposed for such cases before the ionization-dependent cyclization , rearrangements including methyl migration and proton elimination take place (Bohlmann *et al.* 1998; Cane 1999b). These transformations are catalyzed by terpenoid synthases (cyclases), leading to a broad range of acyclic and cyclic parent skeletons of each class (Bohlmann *et al.* 1998).

The monoterpene synthases and diterpene synthases show strict substrate specificity in accepting GPP or GGPP, respectively, whereas several sesquiterpene synthases accept GPP, although inefficiently, as an alternate to FPP (Bohlmann *et al.* 1998).

Many terpenoid synthases of secondary metabolism are represented by multiple-gene copies, that arose by duplication and then provided the basis for diversification. In grand fir at least seven very closed related genes encode monoterpene synthases with distinct product patterns.

Since genes of the secondary metabolism are not essential for growth and development, functional mutations are tolerated, leading to product diversity, which may be beneficial in interactions with other organisms (Bohlmann *et al.* 1998).

The expression of terpenoid synthases is often tissue or cell-specific. It is up-regulated in specialized cells (e.g. glandular trichomes in mint), or restricted to certain developmental stages or short periods of transient defense reactions (Bohlmann *et al.* 1998).

3.4.2.4. Transformations

Subsequent transformations of the basic parent skeletons involve various modifications and isomerisations to form many thousands of different terpenoids, e.g. alcohols, epoxides, aldehydes, ketones, acids and lactones and even halogen-, sulfur, and nitrogen-containing forms have been described. Modifications of the carbon skeleton itself that exhibit more or fewer carbon atoms than the original structure have also been observed. Finally, composite structures such as esters and glycosides are also known (Kleinig 1989). Many of the hydroxylations or epoxidations involved are performed by cytochrome P-450 mixed function oxidases (Bohlmann *et al.* 1998; McGarvey and Croteau 1995).

3.5. Vetiver oil

Vetiver oil is an essential oil extracted from the roots of *Vetiveria zizanioides*. It is of interest to the cosmetic and perfumery industry, not only due to its scent, but also due to its ability to fix some more volatile essential oils (Anonymous 1976). Vetiver oil is used unprocessed as a component for perfumes, deodorants, soap and other toilet articles, and it is raw material for vetiverol (bi- and tricyclic primary, secondary, and tertiary sesquiterpene alcohols (Anonymous 1976)) and vetiveryl acetate (more than 100 GC/MS peaks (Demole *et al.* 1995)), resulting from vetiverol after acetylation (Weiss 1997a). Since vetiverol and vetiveryl acetate have milder odors than the basic oil and equally good fixative properties, they are used in more expensive products (Anonymous 1976; Sreenath *et al.* 1994).

For its essential oil, Vetiver is mainly cultivated in Haiti, West Java, India, Réunion, China and Brazil. The essential oils of the two vetiver types of India vary in quality and quantity. The oil from wild vetiver (mainly the North Indian type), called khus oil, is poor in yield but superior in oil quality, whereas cultivated vetiver contains more oil, but the oil, called vetiver oil, suffers from inferior quality (Lavania 1988). Depending on the origin, the quality of vetiver oil varies. The best quality oil, and therefore the most expensive one (Table 1.1), originates from Réunion and is called Bourbon oil (de Guzman and Oyen 1999).

Table 1.1: Prices of vetiver oil

	in the 1990's [1] [US$ kg^{-1}]	2000 [2] [US$ kg^{-1}]
vetiver oil, bourbon	35-155	137
vetiver oil, Haiti origin	90-100	93
vetiver oil, Indonesia origin	54-62	
vetiver oil, Java origin		71
vetiveryl acetate	160	160.50

[1]: Data from de Guzman and Oyen 1999
[2]: Data from Chemical Market Reporter (2000) Vol. 258, No 5

The occurrence and distribution of the vetiver oil in the roots was studied by microscopical analyses. In young roots no oil was detected and only in about six months old roots, the volatile oil appeared in the form of oil drops mainly in the first cortical layer outside the endodermis. In older roots, cells in the cortical parenchyma were lysed forming lysigenic lacunae, which are filled with essential oil, resulting in oil ducts. Specialized oil cells could not be observed (Kartusch and Kartusch 1978; Viano *et al.* 1991a; Viano *et al.* 1991b).

The roots should be harvested at the age of 15 - 18 months (Anonymous 1976), since it is uneconomical to harvest prior to a minimum maturity of 15 months, while a period of 21 - 24 months is also uneconomical (Virmani and Datta 1975; Weiss 1997a).

Vetiver oil is mainly extracted from fresh or air-dried vetiver roots by steam distillation. Occasionally, roots are also extracted with benzene, yielding a vetiver concrete (Anonymous 1976; de Guzman and Oyen 1999; Weiss 1997a).

The yield of oil (up to 3 % (dw) (de Guzman and Oyen 1999)) depends not only on the vetiver type cultivated, but also on the climate, the soil, frequent cutting of the grass, the harvesting time, methods of distillation, time of distillation. For example, on soil with lower clay content, a marked improvement in quality is observed but simultaneously the oil yield decreases. On sandy pond and river banks the decrease is so extreme that distillation becomes uneconomical (Virmani and Datta 1975).

3.5.1. Composition

The vetiver oil is extremely complex, containing more than 300 components. It consists mainly of bicyclic and tricyclic sesquiterpenoids (hydrocarbons, alcohols, ketones, aldehydes, acids) (Cazaussus *et al.* 1988; de Guzman and Oyen 1999; Vietmeyer and Ruskin 1993), but monoterpenoids (Nikiforov *et al.* 1992) and phenols (Garnero 1971; Shibamoto and Nishimura 1982) have also been detected.

Many of the vetiver oil components have been isolated, identified and even synthesized (references in Smadja 1994; Weyerstahl *et al.* 1999; Weyerstahl *et al.* 2000a ; Weyerstahl *et al.* 2000b; Weyerstahl *et al.* 2000c; Wolf 1996). In the typical vetiver oils (Haiti, Réunion, Java, Brazil and South India) sesquiterpen-

oids with valencane, eremophilane, eudesmane, epieudesmane, vetispirane, zizaane (or tricyclovetivane), prezizaane, guaiane and cyclocopacaphane skeletons were found (Anonymous 1976; Garnero 1971; Weyerstahl *et al.* 2000a; Weyerstahl *et al.* 2000b). However, the major representatives of the vetiver oil, (+)-α-vetivone, (-)-β-vetivone and khusimol (Figure 1.5), can be considered as the "fingerprint" of vetiver oil (de Guzman and Oyen 1999; Demole *et al.* 1995).

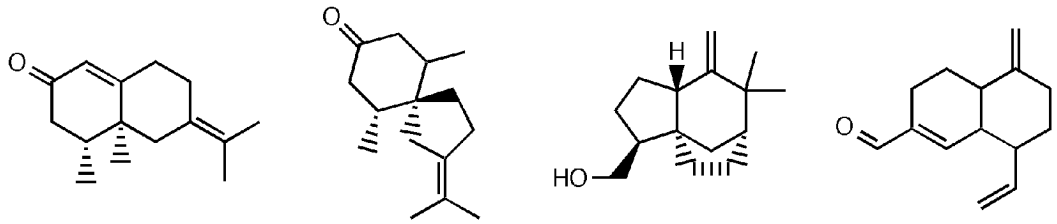

Figure 1.5. Characteristic components of typical vetiver oils or khus oil

The khus oil from Northern India differs markedly from the typical vetiver oil. It is characterized by the presence of antipodal cadinane and eudesmane sesquiterpenoids (e.g. khusol, khusinol, laevojunenol, epikhusinol and isokhusinol-oxide) and contains a large amount of khusilal (Figure 1.5), a laevorotatory C14-terpenoid. The khus oil has laevorotatory characteristics, while the vetiver oil from all other sources is dextrorotatory (Table 1.2) (Andersen 1970; Anonymous 1976; de Guzman and Oyen 1999; Garnero 1971; Kalsi *et al.* 1964; Kalsi *et al.* 1985).

Table 1.2: Physical properties of Vetiver oil

	wild North Indian vetiver [1]	cultivated South Indian vetiver [1]	Bourbon [2,3]	Java [2,3]	Haiti [4]
relative density	0.990-1.032	0.992-1.015	0.986-1.015	0.980-1.022	0.986-0.998
refraction index	1.512-1.523	1.516-1.530	1.521-1.530	1.521-1.530	1.521-1.526
optical rotation	-50° to -130°	+10 to +25°	+14° to +32°	+17° to +46°	+22° to +38°
Miscibility in 80% ethanol (vol)			1 : 1-2	1 : 2 [2] insoluble [3]	1 : 2

[1]: Data from Anonymous 1976
[2]: Data from de Guzman and Oyen 1999
[3]: Data from Masada 1976
[4]: Data from Bauer et al 1988

The odor of vetiver oil is very complex. It is unclear, which compounds are responsible for the typical vetiver scent (de Guzman and Oyen 1999; Demole *et al.* 1995; Kraft *et al.* 2000). It appears that the scent does not depend on a single group of compounds but rather on a combination of skeletons and functional groups contributing to the total sensory impression (Nikiforov *et al.* 1992; Weyerstahl *et al.* 2000c; Wolf 1996). Therefore and due to the problem to chemically synthesize economically pure sesquiterpenoids, it has not been possible up to now to produce a synthetic vetiver odorant (Kraft *et al.* 2000; Spreitzer *et al.* 1999).

4. Scope of this work

Since a synthetic vetiver oil cannot yet be manufactured (Kraft *et al.* 2000; Spreitzer *et al.* 1999; Weyerstahl *et al.* 2000c), there is an interest in the perfume industry in new vetiver variants with higher oil yields or different odor tonalities. New vetiver variants have been obtained with a higher oil yield or an odor with a different note by traditional breeding and selection (Gupta *et al.* 1983; Lal *et al.* 1997; Lal *et al.* 1998; Sethi 1982; Sethi and Gupta 1980). However, traditional breeding with non-flowering South Indian type vetiver is not possible. Therefore regeneration via *in vitro* cultures was chosen to obtain variants. This decision was supported by the finding of Shreenath and Jagadishchandra (1991) that regenerants of commercially important *Cymbopogon* species (*C. flexuosus*, *C. nardus*, *C. winterianus*, *C. martinii* var. *motia* and *C. jwarancusa*) showed significant variations in oil content (Sreenath and Jagadishchandra 1991) and of Mathur *et al.* (1988) who recorded variations for six major constituents of the essential oil of the aromatic grass *C. winterianus* (citronellal, citronellol, geraniol, citronellylacetate, geranylacetate, and elemol) (Mathur *et al.* 1988).

The aim of this study is to extend this work to vetiver, and to obtain vetiver variants with a higher vetiver oil yield or a different oil composition via tissue culture. To reach this goal, the project was divided in two parts: first to develop

the necessary tools and technologies and second to obtain plantlets and identify variants.

In the first part, we optimized methods to regenerate vetiver plantlets via *in vitro* cultures. To obtain compact calli for subsequent regeneration, the influence of different factors like starting material, medium composition and culture conditions on callus induction were tested (Chapter 2). The influence of growth conditions and callus induction media on the subsequent plantlet regeneration were examined to achieve an optimal plantlet regeneration. The optimized media and conditions were compared with earlier procedures for vetiver regeneration described in the literature (Chapter 3). To regenerate plantlets via liquid cultures, studies on the influence of callus and liquid culture media composition on the liquid culture production and the subsequent plantlet regeneration were performed (Chapter 4).

We also compared different methods to identify oil variants. To pre-screen the regenerated plants, the oil synthesis has to be induced in an earlier stage and plant material has to be analyzed for oil content and composition. Therefore, methods to extract and analyze large numbers of small samples of *in vitro* tissue are needed. To find a balance between the time required per analysis, the accuracy of the analysis and the amount of oil necessary, different analysis methods were compared (Chapter 5). Water distillation and solvent extraction were first optimized and compared on a larger scale (≈ 5 g vetiver roots) (Chapter 6) before small scale extraction experiments were started (Chapter 7).

To induce the oil production in tissue cultures, initial studies on the usefulness of different tissue such as *in vitro* plantlets, root cultures and callus and the feasibility to produce enough tissue to analyze the oil content were done (Chapter 8).Finally, general conclusions on the results presented in this thesis and considerations of how the oil could be induced and accumulated in tissue cultures are written in chapter 9.

Chapter 2: Compact callus induction from *in vitro* plantlets of vetiver (*Vetiveria zizanioides*) from Java

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ABSTRACT

Crown and leaf slices of *in vitro* plantlets of a non-flowering *Vetiveria zizanioides* from Java were cultured on agar medium containing various concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D, 0.1 - 10 mg l^{-1}), sucrose (10 - 100 g l^{-1}), 6-benzylaminopurine (BA, 0 - 2 mg l^{-1}) and α-naphthalene acetic acid (NAA, 0 or 0.1 mg l^{-1}) in the dark (23°C). After 4 - 6 weeks in culture, compact calli were observed. A relatively low 2,4-D concentration (0.5 mg l^{-1}) was best for compact callus induction. The induction of compact calli could be increased by adding 0.5 mg l^{-1} BA and increasing the sucrose concentration up to 75 g l^{-1}. The auxin NAA did not increase the compact callus induction significantly. Combining the above results, the optimal medium for the generation of compact calli from *Vetiveria zizanioides* consisted of a modified Murashige and Skoog medium supplemented with 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA and 75 g l^{-1} sucrose.

INTRODUCTION

Vetiveria zizanioides is a tropical grass. It belongs to the subfamily of *Panicoideae*, which includes maize, sorghum, sugarcane and lemongrass (Vietmeyer and Ruskin 1993). There are flowering and non-flowering vetiver plants. The wild-growing variety commonly found in North India is mostly of the former type, whereas in South India both types are found (Anonymous 1976). As not all vetiver plants flower and the germination rate of the seeds is low, vetiver propagates itself through axillary shoots (Vietmeyer and Ruskin 1993).

There are two reasons why vetiver is of interest. A traditional and well established use of vetiver is based on its deep and fibrous roots, which allow the plant to be used for protection against soil erosion and loss of moisture (Erskine 1992; Vietmeyer and Ruskin 1993). Another reason why vetiver is cultivated in the tropics, is that the roots contain an essential oil which consists of more than 150 sesquiterpenoids (Akhila *et al.* 1981). This oil is used as a component for perfumes, scenting soaps and as a fixative to prevent the evaporation of more volatile oils (Vietmeyer and Ruskin 1993). Because a

completely synthetic vetiver oil cannot be manufactured at a realistic price (Vietmeyer and Ruskin 1993), vetiver variants with more oil or with a different oil composition are of interest. However, since not all vetiver plants flower and the germination rate of the seeds is low, it is difficult to produce variants via traditional sexual breeding. An alternative is to produce somatic variants via tissue culture. This requires that *in vitro* tissue is available, from which plantlets can be regenerated. Generating such tissue in monocotyledons is not trivial, as the methods developed for dicotyledonous species have not always proven suitable for monocotyledonous species, and this is particularly so for grasses (Vasil and Vasil 1986). Although plants can be regenerated from a wide variety of explants, the efficiency of regeneration depends largely on the nature of the explant used. Best results are obtained when explants from immature organs or meristematic and undifferentiated tissue, such as mature and immature embryos, inflorescences, anthers, ovary, the base of young leaves and shoot apices are used (Vasil and Vasil 1994; Vasil and Vasil 1986).

Somatic embryogenesis has been reported for all of the major species of cereals and grasses (Vasil and Vasil 1986), and it has been suggested that this may be the more common form of plant regeneration *in vitro* in *Gramineae*. Somatic embryo induction is influenced by many factors. The main factors are the plant material and the growth regulators auxin and cytokinin, but medium composition (sugars, nitrogen sources and potassium) and the culture environment (pH, light, temperature and the gas phase) can also affect the induction of somatic embryos (Doughall 1981; Hughes 1981; Thorpe 1994) or compact calli.

We have found in preliminary studies (R.E. Leupin, unpublished) that it is possible to induce a compact callus from vetiver. These structures provide the best chance to regenerate plantlets, but of the various approaches we have tested, the compact callus induction rate has been very low thus far. In this chapter we describe the establishment and maintenance of *in vitro* plantlets of vetiver and the influence of the medium composition, various concentrations of sucrose, 2,4-dichlorophenoxy acetic acid (2,4-D), 6-benzylaminopurine (BA) and α-naphthalene acetic acid (NAA) on the induction of compact calli.

MATERIAL AND METHODS

Plant material

The *Vetiveria zizanioides* from Java was from our stocks. The plants were grown and maintained in a green-house at 25°C and a light / dark cycle of 16 / 8 hours.

Media

For all *in vitro* experiments, MS (Murashige and Skoog 1962), N6 (Chu *et al.* 1975) or modified MS nutrient media supplemented with various concentrations of growth regulators, sucrose (1 - 10 %) and 0.65 % agar (Agar Agar Powder Type S1000; B & V s.r.l. Parma) were used. The pH of the medium was adjusted to 5.8 with KOH or HCl before autoclaving.

The modified MS medium contained 1/2 MS macronutrients, 1/2 MS micro-nutrients, 0.5 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} pyridoxine-HCl, 0.5 mg l^{-1} nicotinic acid, 100 mg l^{-1} myo-inositol, 40 mg l^{-1} NaFe(III)EDTA, 200 mg l^{-1} glycine and 100 mg l^{-1} citric acid.

Establishment and maintenance of *in vitro* plantlets

Plants were cut in separate shoots. Soil was washed off and the length of the shoots was reduced to 5 cm. The cuttings were dipped for 30 sec in 70 % ethanol (v/v), stirred for 20 min in 4 % $Ca(ClO)_2$ with a droplet of Tween 20, rinsed in sterile water and then the outermost leaf was removed. These steps were repeated three times, but the sterilization step with $Ca(ClO)_2$ was reduced each time. The second and third time the cuttings were sterilized for 15 minutes in 2.5 % $Ca(ClO)_2$ and 10 minutes in 1 % $Ca(ClO)_2$, respectively. Following the sterilization, the cuttings were put on modified MS medium supplemented with 25 g l^{-1} sucrose, 0.65 % agar, 1 mg l^{-1} kinetin and 0.1 mg l^{-1} indole acetic acid (VRM8) and cultivated for 1 week in the dark (23°C). Then the cuttings were transferred to the light (23°C with 12 hours photoperiod).

For propagation of the plantlets, small shoots were cultured on modified MS medium containing BA (1 mg l^{-1}), gibberellic acid (0.1 mg l^{-1}), indolebutyric acid (0.1 mg l^{-1}) and 25 g l^{-1} sucrose (vetiver propagation medium, VPM). After the propagation, the shoots were cultured on modified MS medium supplemented with 25 g l^{-1} sucrose, 0.65 % agar, without any growth regulator (VRM0) or on VRM8, on which the shoots produced roots and grew bigger. The plantlets were transferred every 6 - 8 weeks to fresh medium. All *in vitro* plantlets were cultured at 23°C with a 12 hours photoperiod.

Induction of callus

As the used *Vetiveria zizanioides* from Java does not flower, the explants most often used for callus induction (immature embryos, young inflorescences and anthers) are not available. Therefore the so-called crown (Vietmeyer and Ruskin 1993) and the basal leaves of the *in vitro* plantlets were used for callus induction. For that purpose, plantlets grown on VRM0 or VRM8 were cut in slices (≤ 1 mm) starting at the root part of the crown. As long as crown tissue was still available, these slices were defined as crown slices, the following slices as leaf slices (Figure 2.2a - e). Since the boundary between the last crown slice and the first leaf slice was not clearly defined, the results obtained with the leaf slices showed some variations.

These crown- (5 - 7 slices / plantlet) or leaf-slices (2 - 3 slices / plantlet) were placed on Petri dishes with modified MS, MS or N6 nutrient medium containing various concentrations of 2,4-D (0.4 - 10 mg l^{-1}), α-naphthalene acetic acid (NAA, 0 or 0.1 mg l^{-1}), BA (0 - 2 mg l^{-1}) and sucrose (1 - 100 g l^{-1}). All cultures were incubated in the dark at 23° - 25°C.

Histology

For light microscopy, the samples were fixed in a formaldehyde (35 %) - acetic acid - alcohol - water (10:5:50:35) mix for at least 24 hours under vacuum. Fixed tissues were dehydrated in a *t*-butanol series (Johansen 1940) and embedded in paraffin. Sections were cut at 10 µm and stained with safranine/fastgreen or toluidine blue (Gerlach 1984).

RESULTS

Establishment and maintenance of *in vitro* plantlets

To obtain *in vitro* cultures, the plants from the greenhouse needed to be disinfected and propagated. The infection rate was high, but with the propagation of the plantlets we were able to obtain enough plant material for the experiments.

After disinfection of the plant material, it took some months until the shoots started to propagate. The small plantlets have a propagation rate of about 5 - 10 within one month on VPM. No roots developed during the first two months on VPM (Figure 2.1b).

Shoots from VPM medium were transferred to VRM8 or VRM0. On these media the shoots produced roots and began to grow. After 6 - 18 months, depending on the size on VPM, the plantlets were big enough so that they could be cut in slices and used for callus induction (Figure 2.1c).

Within the past 4 years, during propagation of vetiver plantlets, several variations appeared; we found a white-green chimera, a green-light green chimera, a bunch with white dwarf plantlets and several plantlets which had decussate leaves instead of alternate leaves (Figure 2.1d).

The plantlets from the three first variations showed the same phenotype after propagation, while the variant with decussate leaves yielded normal plantlets with alternate leaves after propagation.

Induction of callus

On callus induction medium containing 2,4-D, calli grew on the plant slices after a short time in culture. These calli were soft and watery or covered with a gelatinous mass (Figure 2.2f). After about 4 weeks an additional callus type was observed. This callus was compact and contained organized structures (Figure 2.2f, g). After 6 weeks in culture, the number of slices with compact callus still increased, whereas the other calli did not (data not shown).

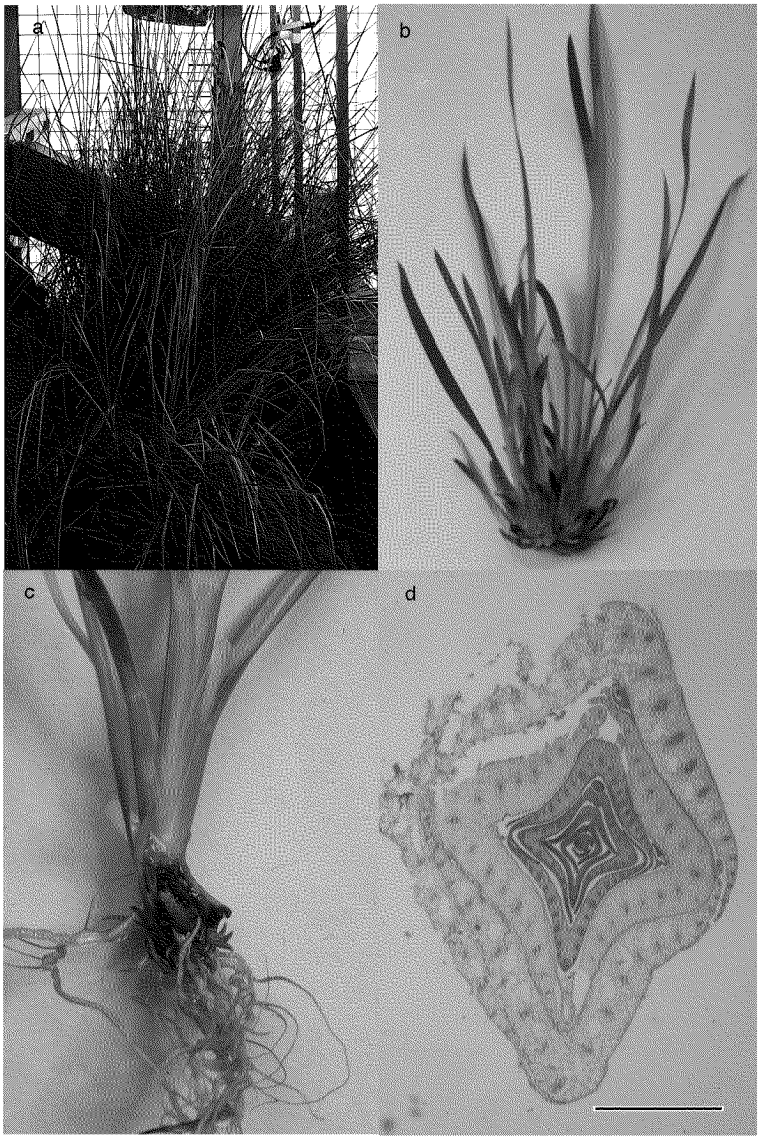

Figure 2.1. Establishment of *in vitro* plantlets of *Vetiveria zizanioides*. (a) *Vetiveria zizanioides* in a green-house; (b) Vetiver plantlets from the propagation medium (VPM); (c) Vetiver plantlets grown on VRM8; (d) Variation appeared during propagation on VPM: vertical section through a vetiver plantlet with decussated leaves (bar = 1 mm)

From preliminary studies we knew that these compact calli provide the best material to regenerate plantlets. As the induction rate of these compact calli was low we tested the influence of different media, growth regulators and sucrose concentrations on compact callus induction.

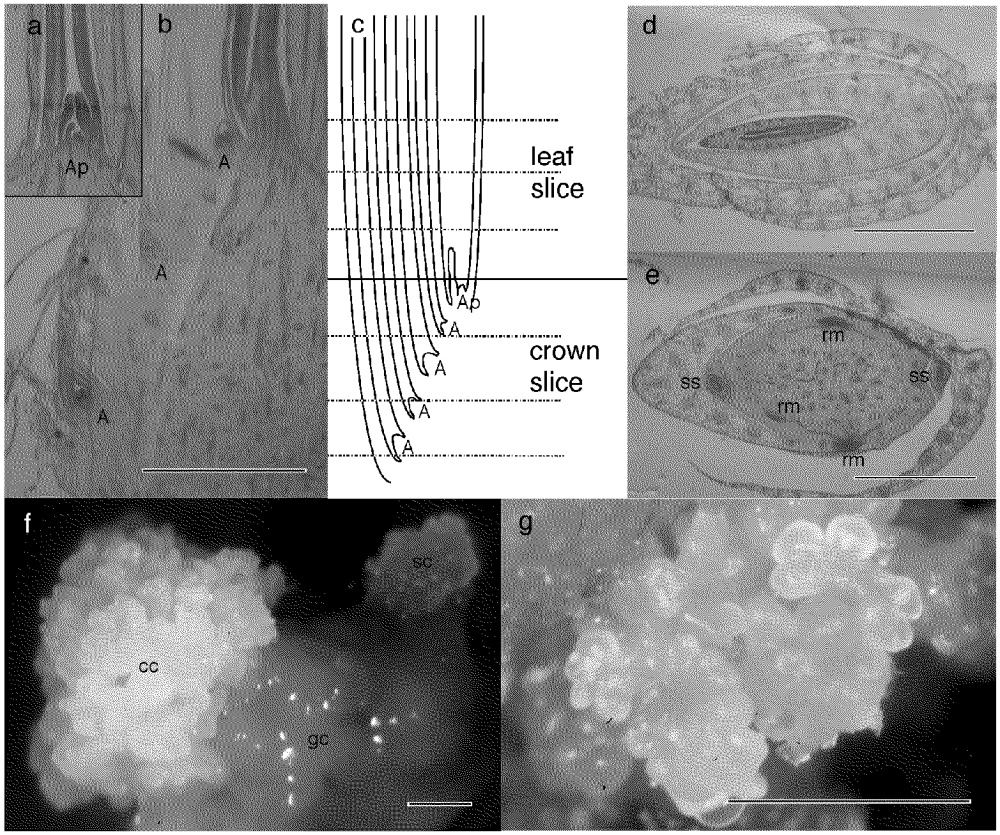

Figure 2.2. Induction of callus from crown and leaf slices of *Vetiveria zizanioides*. (a, b) Longitudinal section through a vetiver bud; (c) Schematic diagram of a longitudinal section through a vetiver bud; (d) Vertical section through vetiver leaves; (e) Vertical section through a vetiver crown; (f, g) Crown slice with callus and compact callus; bar = 1 mm.
A: axillary buds, Ap: apical meristem, rm: root meristem, ss: side shoot, sc: soft and watery callus, gc: callus covered with a gelatinous layer, cc: compact callus

Effect of the medium composition on compact callus induction

To find the optimal 2,4-D concentration to induce compact calli, crown slices were cultured on modified MS medium with 25 g l^{-1} sucrose and various concentrations of 2,4-D (0.4 - 10 mg l^{-1}). Calli were formed at all concentrations tested (80 - 100 % slices with callus), but the higher the 2,4-D concentration, the more calli were covered with a gelatinous layer (Figure 2.1d) and at concentrations higher than 1 mg l^{-1} 2,4-D no compact calli were induced. A subsequent experiment with 2,4-D concentrations between 0.4 and 1 mg l^{-1} showed that 0.5 mg l^{-1} 2,4-D was most suitable for inducing compact calli (Figure 2.3).

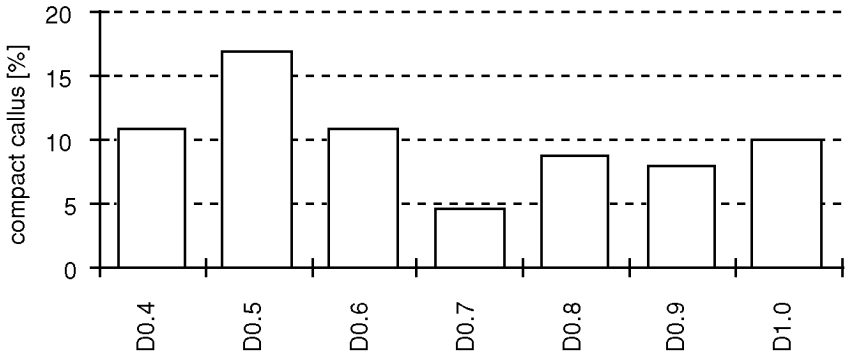

Figure 2.3. Effect of 2,4-dichlorophenoxy acetic acid concentrations on compact callus induction. Plantlets grown on VRM8 were cut in slices (120 crown slices / medium) and cultured on modified MS medium supplemented with 25 g l^{-1} sucrose and 2,4-D concentrations between 0.4 - 1.0 mg l^{-1} (D0.4 - D 1.0). The percentage of crown slices with compact calli was determined after 8 weeks in the dark (23°C)

To compare different basal media, crown slices were cultured for 8 weeks on modified MS, MS or N6 media with 25 g l^{-1} sucrose and 0.5 mg l^{-1} 2,4-D. About 200 crown slices were tested on each medium. Compact calli were formed only on the MS and the modified MS medium and compact callus formation was about the same on both media (2 - 2.4 %). No compact callus was induced on N6 medium.

To test the influence of other growth regulators on compact callus induction, crown and leaf slices were cultured for 8 weeks on modified MS medium containing 2,4-D (0.5 mg l^{-1}), NAA (0 or 0.1 mg l^{-1}), BA (0 - 2 mg l^{-1}) and 50 g l^{-1} sucrose. The percentage of leaf and crown slices with callus decreased with increasing BA concentration. Whereas with 2,4-D alone no compact calli could be found on leaf slices, addition of BA resulted in the same percentage of slices with compact calli for both leaf and crown slices. The percentage of slices with compact calli increased with BA concentration up to 0.5 mg l^{-1}. At 2 mg l^{-1} BA, there was equal or less compact callus induction than with 0.5 mg l^{-1} BA (Figure 2.4). The addition of 0.1 mg l^{-1} NAA seemed to improve the compact callus

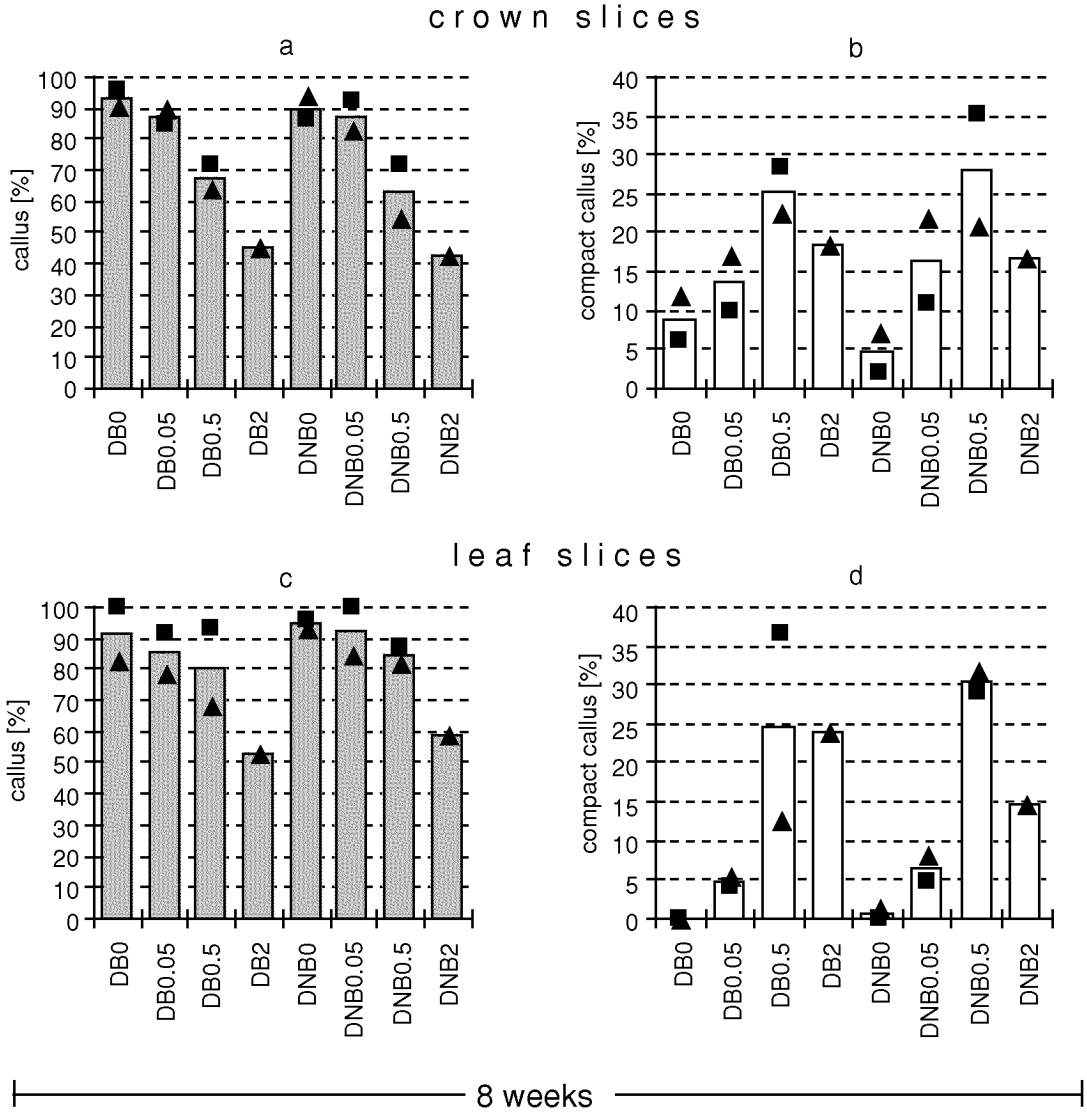

Figure 2.4. Effect of various 6-benzylaminopurine concentrations and the addition of α-naphthalene acetic acid on the induction of callus or compact callus. Plantlets grown on VRM8 were cut in slices (5 crown - and 3 leaf slices / plantlet) and cultured on modified MS medium supplemented with 0.5 mg l^{-1} 2,4-D, different BA concentrations (0/ 0.005/ 0.5/ 2 mg l^{-1}), without or with 0.1 mg l^{-1} NAA and 50 g l^{-1} sucrose (DB or DNB 0/ 0.05/ 0.5/ 2). The percentage of crown (a, b) or leaf slices (c, d) with callus (a, c) or compact callus (b, d) was determined after 8 weeks in the dark (23°C)

■ : experiment 1 (95-100 crown and 21-30 leaf slices / medium), without DB2 and DNB2

▲ : experiment 2 (114-124 crown and 72-75 leaf slices / medium)

▒ : slices with callus (average of experiments 1 and 2)

□ : slices with compact callus (average of experiments 1 and 2)

induction on DB0.5 (Figure 2.4), but as the averages of DNB0.5 and DB0.5 were within the experimental error for these two media, NAA was omitted from the callus induction medium.

To test the influence of the sucrose concentration on callus and compact callus induction, crown and leaf slices were cultured for 8 weeks on modified MS medium supplemented with 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA and various concentrations of sucrose (10 - 100 g l^{-1}). The sucrose concentration did not have much effect on callus induction on crown slices, but the higher sucrose concentrations (50 - 100 g l^{-1}) did have a negative effect on the callus induction on leaf slices (Figure 2.6). The further the used leaf slices were from the apical meristem the more the callus induction was inhibited by high sucrose concentrations (data not shown).

The sucrose concentration had an effect on the induction of the callus type. For crown slices the optimal sucrose concentration for the induction of compact calli was 75 g l^{-1} sucrose, for leaf slices it was between 75 and 100 g l^{-1} sucrose (Figures 2.5, 2.6). DB75 not only induced more slices with compact calli, but also led to bigger compact calli after 8 weeks than seen for induction on DB10.

Effect of the starting material on compact callus induction

To test the influence of the medium on which the plants grew before the compact callus induction experiments, crown and leaf slices from plantlets grown on VRM8 or VRM0 were cultured for 8 weeks on modified MS medium containing 0.5 mg l^{-1} 2,4-D, 0 - 0.5 mg l^{-1} BA, 0 - 0.1 mg l^{-1} NAA and 50 - 100 g l^{-1} sucrose. The plantlets grown on VRM0 formed more calli and more of these were compact calli than did plantlets grown on VRM8 (Figure 2.6).

For the callus induction experiments crown and leaf slices were used. The crown slices contained root and shoot meristems, whereas leaf slices contained neither (Figure 2.2d, e).

Compact calli could be found on all crown slices independent of the origin of these slices within the crown (Figure 2.2c), although in some experiments an increase of the percentage of slices with compact calli was detectable for crown

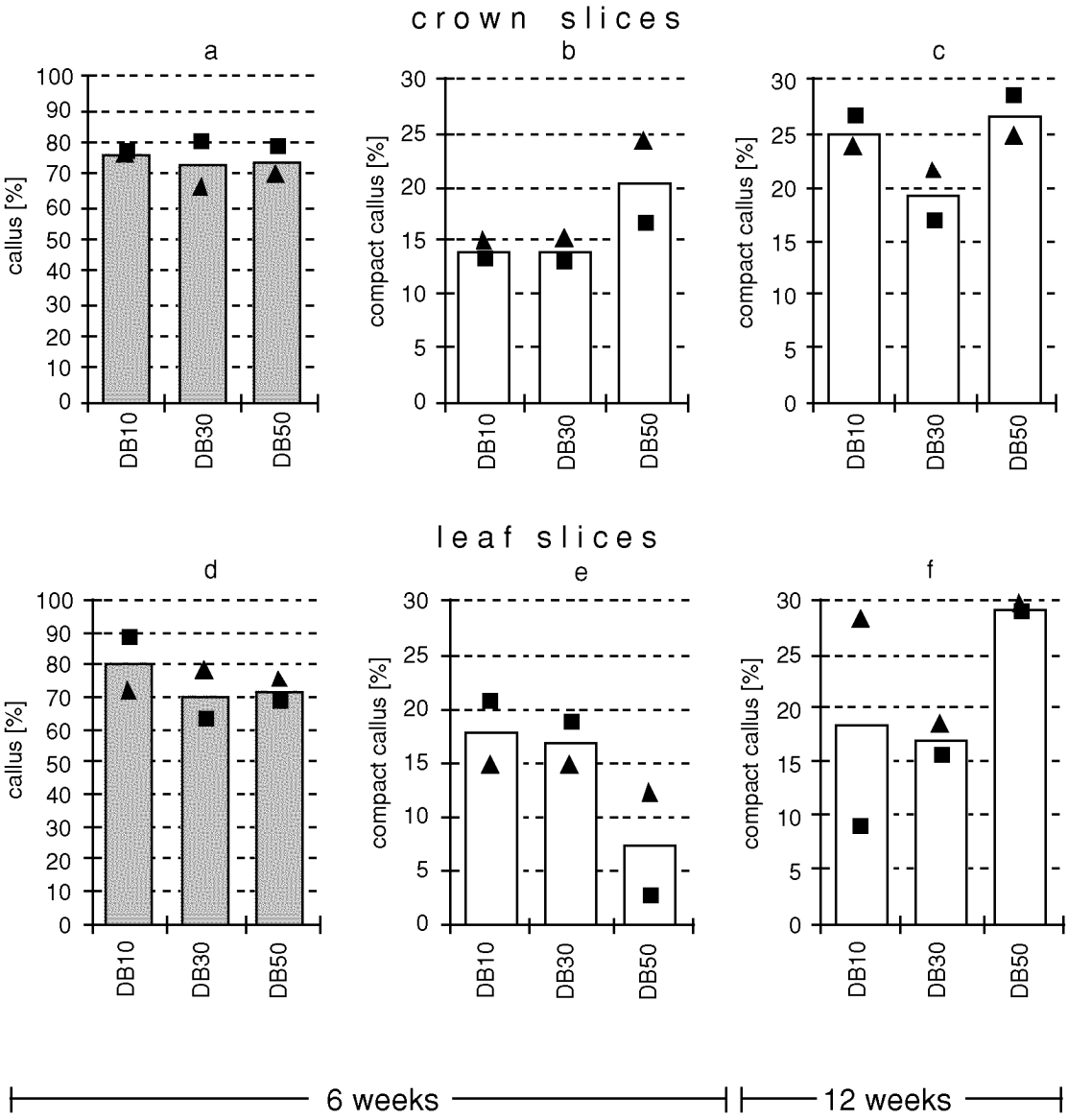

Figure 2.5. Effect of the sucrose concentration on compact callus induction. Plantlets grown on VRM0 were cut in slices (6 crown - and 2 leaf slices / plantlet) and cultured on DB0.5 media supplemented with different concentrations of sucrose (10 / 30 / 50 g l^{-1} = DB10 / DB30 / DB50). The percentage of crown (a, b, c) or leaf slices (d, e, f) with callus (a, d) and compact callus (b, c, e, f) was determined after about 6 (a, b, d, e) and 12 weeks (c, f) in the dark (23°C).

■ : experiment 1 (101-106 crown and 32-38 leaf slices / medium)

▲ : experiment 2 (75-82 crown and 225 leaf slices / medium)

▒ : slices with callus (average of experiments 1 and 2)

□ : slices with compact callus (average of experiments 1 and 2)

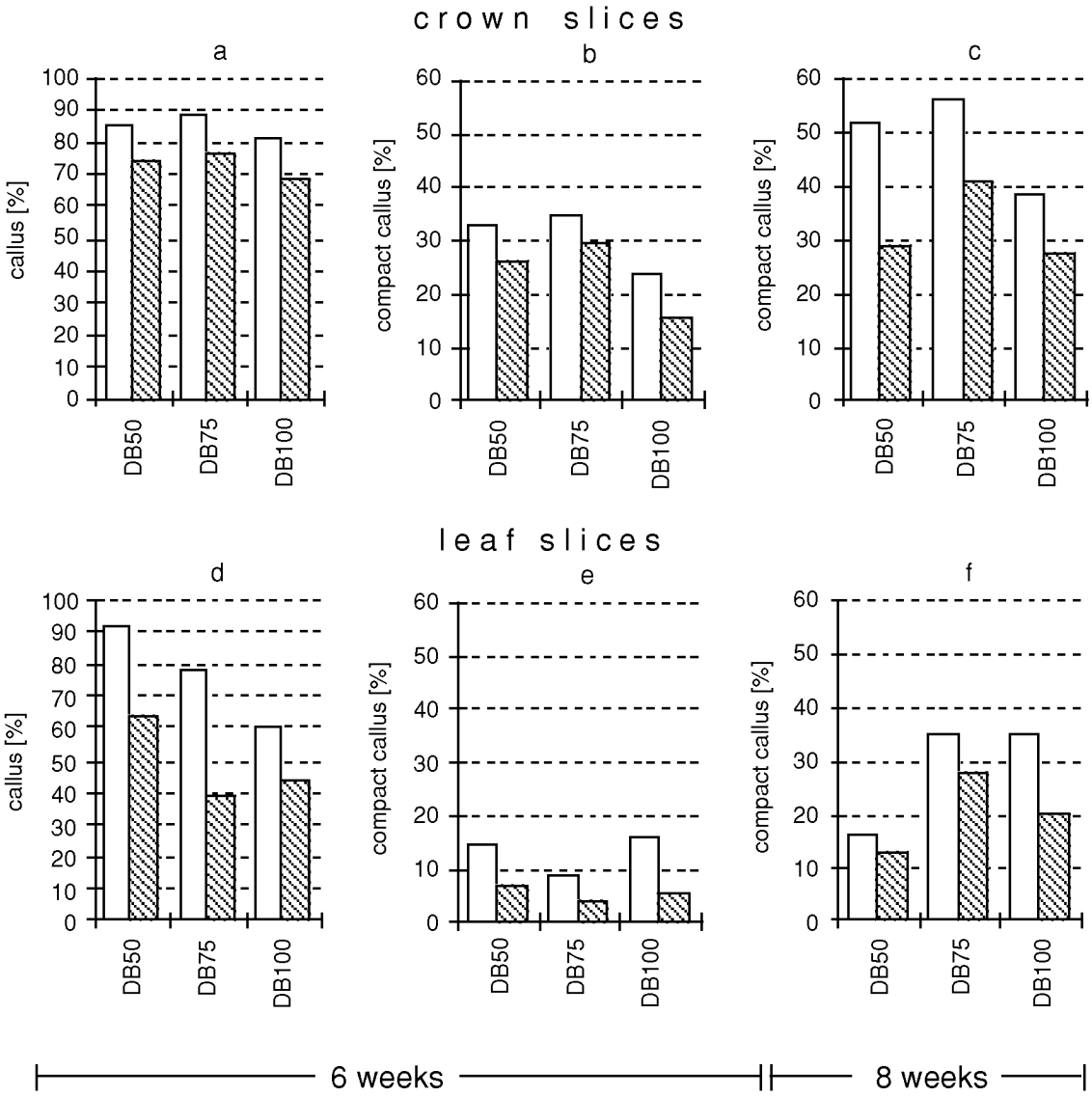

Figure 2.6. Effect of plant growth medium and the sucrose concentration on the compact callus induction. Plantlets grown on VRM0 or VRM8 media were cut in slices (6 crown- and 3 leaf slices / medium) resulting in 125 crown and 75 leaf slices for VRM0 and 115 crown and 69 leaf slices for VRM8. The slices were cultured on modified MS medium supplemented with 0.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA with different concentrations of sucrose (50 / 75 / 100 g l^{-1} = DB50 / DB75 / DB100). The percentage of crown (a, b, c) or leaf slices (d, e, f) with callus (a, d) and compact calli (b, c, e, f) was analyzed after 6 (a, b, d, e) and 8 weeks (c, f) in the dark (23°C).
□: plantlets from VRM0 (125 crown and 75 leaf slices / medium)
▨: plantlets from VRM8 (115 crown and 69 leaf slices / medium)

slices which originated in the region close to the apical meristem (data not shown). No compact calli were induced on leaf slices cultured on medium with 0.5 mg l^{-1} 2,4-D. Leaf slices could be induced to form compact calli by addition of BA (Figure 2.4). The number of leaf slices with compact calli decreased with increasing distance from the apical meristem.

DISCUSSION

In this chapter we showed that it is possible to increase compact callus formation in *Vetiveria zizanioides.*

Although the reproducibility of compact callus induction was poor, with variations up to 20 %, and it was difficult to compare results obtained in experiments done at different times, the general tendency was clear. One reason for this variation in the compact callus induction is the plant itself. Vasil and Vasil (1994) wrote that the physiological condition and developmental stage of the explants were critical in obtaining a desirable response, and the plantlets used for our different experiments had neither the same age nor the same size. Due to the different growth regulators in the medium, the plantlets used as starting material for callus induction might be in different developmental stages, which could explain why slices from plantlets grown on VRM8 or VRM0 did not result in the same callus induction.

We have observed in our experiments with *in vitro* vetiver plantlets that 0.5 mg l^{-1} 2,4-D was sufficient to induce compact calli. Higher concentrations of 2,4-D did not enhance the induction of compact calli, whereas for other *Gramineae* 2,4-D concentrations up to 10 mg l^{-1} or even higher have been used (Flick *et al.* 1983). Often the only plant growth regulator added to the medium to induce callus or somatic embryos is the auxin 2,4-D (Vasil and Vasil 1994). Schenk and Hildebrandt (1972) described that for wheat, barley, rice and bromegrass, kinetin inhibited callus induction and growth, whereas for other *Gramineae*, addition of low levels of kinetin or BA to 2,4-D containing medium supported induction of embryogenic callus (Mathur *et al.* 1988; Sreenath and Jagadishchandra 1991). We found as well that the addition of 0.5 mg l^{-1} BA to

the callus induction medium was beneficial for compact callus induction (Figure 2.4).

Sucrose enhanced compact callus induction; a finding similar to that of Lu *et al.* (1982, 1983), who found that immature embryos of maize formed more embryogenic callus on MS medium with 0.5 mg l^{-1} 2,4-D and 120 g l^{-1} sucrose than with 60 g l^{-1} sucrose. An explanation for this effect could be that osmotic stress enhances somatic embryogenesis (Litz 1986; Thorpe 1994).

We conclude, based on our experiments, that the best medium for compact callus induction is a modified MS medium with 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA and 75 g l^{-1} sucrose. With this medium we have been able to improve compact callus induction on crown slices from 0 - 20 % to 20 - 50 %. It is also possible to induce compact calli on leaf slices, and with this the stage is now set for the regeneration of plantlets, induction of suspension cultures, and regeneration of plantlets from suspension cultures.

ACKNOWLEDGEMENTS

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Chapter 3: Compact Callus Induction and Plant Regeneration of a non-flowering Vetiver (*Vetiveria zizanioides*) from Java

Ruth E. Leupin, Marianne Leupin, Charles Ehret, Karl H. Erismann and Bernard Witholt

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ABSTRACT

Crown and leaf slices of *in vitro* plantlets of a non-flowering *Vetiveria zizanioides* from Java were used to induce compact calli and to regenerate plantlets. The influence of sucrose concentrations (10 or 75 g l^{-1}), cultivation in light or dark and cultivation time on callus induction medium (6 or 12 weeks), on the induction of compact callus and the subsequent regeneration of plantlets was studied. Up to 75 % of crown slices cultured on modified Murashige and Skoog medium supplemented with 0.5 mg l^{-1} 2,4-dichlorophenoxy acetic acid, 0.5 mg l^{-1} 6-benzylaminopurine and 75 g l^{-1} sucrose developed compact callus. For subsequent regeneration of plantlets, callus induction in the light for 6 weeks on the callus induction medium containing 10 g l^{-1} sucrose, and subsequent transfer to the regeneration medium, was the best procedure, regenerating plantlets on around 60 % of the crown or leaf slices, with up to 100 plantlets per slice.

We have compared the efficiency of the above mentioned procedure with several other methods to regenerate plantlets. Our findings indicate that the procedure developed in this study was best in regenerating plantlets for the used vetiver variant.

INTRODUCTION

The tropical grass *Vetiveria zizanioides* belongs to the subfamily of *Panicoideae*, which includes maize, sorghum, sugarcane and lemongrass (Vietmeyer and Ruskin 1993). The vetiver plant contains in its root an essential oil, the vetiver oil, which is used as a component for perfumes, scenting soaps and as a fixative to prevent the evaporation of more volatile oils (Vietmeyer and Ruskin 1993). Because a completely synthetic vetiver oil cannot be manufactured at a realistic price (Vietmeyer and Ruskin 1993), vetiver variants with more oil or with a different oil composition are of interest. New variants could be obtained by traditional breeding (Gupta *et al.* 1983; Lal *et al.* 1998; Sethi 1982; Sethi and Gupta 1980) or, since not all vetiver plants flower and the germination

rate of the seed is low (Vietmeyer and Ruskin 1993) by regeneration of plantlets via tissue cultures, which are putative somaclonal variants.

Several groups have regenerated plantlets of vetiver from inflorescence tissues (Keshavachandran and Khader 1997; NaNakorn *et al.* 1998; Sreenath *et al.* 1994), from leaf material (Mathur *et al.* 1989; Mucciarelli *et al.* 1993; Sreenath *et al.* 1994) and from the mesocotyl part of young seedlings (George and Subramanian 1999). Except George and Subramanian (1999), all of these workers used *in vivo* plant material to induce calli. Although the *in vivo* material is disinfected directly before the callus induction and subsequent regeneration, losses due to infection still do occur (Sreenath *et al.* 1994). Furthermore, the plant surface is often damaged by the sterilization techniques, which also influences the experiment. To prevent such problems, we used *in vitro* plantlets as starting material. The disinfected shoots were first propagated *in vitro* and infected plantlets were removed before callus induction.

In this study we describe the optimization of compact callus induction and subsequent regeneration of plantlets from crown and leaf slices of *in vitro* plantlets of a non-flowering vetiver. We have also compared the efficiency of the optimized procedure with those of Sreenath *et al.* (1994), Mathur *et al.* (1989) and Mucciarelli *et al.* (1993).

MATERIAL AND METHODS

Plant material

The *Vetiveria zizanioides* from Java was from our stocks. The plants were grown and maintained in a green-house at 25°C and a light / dark cycle of 16 / 8 hours.

Media

For all *in vitro* experiments Murashige & Skoog (MS) (Murashige and Skoog 1962) or modified Murashige & Skoog (modified MS) nutrient media supplemented with various concentrations of growth regulators, sucrose (1 - 10 %)

Table 3.1. Media used for callus induction and for plant regeneration of vetiver.

Medium [1]		Basal medium	Growth regulators [2]				Additional compounds	Sucrose	References
			2,4-D	IAA	Kin	BA			
			[µM]				[mg l^{-1}]	[g l^{-1}]	
Ind.	DB10	modified MS	2.26	-	-	2.22	-	10	
	DB75	modified MS	2.26	-	-	2.22	-	75	
Reg.	D0.1B1(25)	modified MS	0.45	-	-	4.44	-	25	
	D0.1B1(75)	modified MS	0.45	-	-	4.44	-	75	this study
Ind.	Ma1	MS	4.52	-	1.16	-	ascorbic acid (40)	30	
Reg.	Ma2	MS	-	5.71	2.32	-	ascorbic acid (40)	30	(Mathur et al. 1989)
Ind.	Mu1	MS	9.05	5.71	4.65	-	casein hydrolysate (100)	30	
Reg.	Mu2	MS	-	5.71	0.46	-	casein hydrolysate (100)	30	(Mucciarelli et al. 1993)
Ind.	Sr1	MS	4.52	-	4.65 [3]	-	-	30	
Reg.	Sr2(30)	MS	-	-	-	-	-	30	
	Sr2(100)	MS	-	-	-	-	-	100	(Sreenath et al. 1994)

[1] *Ind.: callus induction medium; Reg.: plantlet regeneration medium*
[2] *2,4-D: 2,4-dichlorophenoxy acetic acid; IAA: indole acetic acid; Kin: kinetin; BA: 6-benzylaminopurine*
[3] *for this experiment kinetin was used. Sreenath et al. (1994) also used 4.44 µM BA*

and 0.65 % agar (Agar Agar Powder Type S1000; B & V s.r.l. Parma) were used. The pH of the medium was adjusted with KOH or HCl to 5.8 before autoclaving.

Modified MS medium contains 1/2 MS macronutrients, 1/2 MS micronutrients, 0.5 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} pyridoxine-HCl, 0.5 mg l^{-1} nicotinic acid, 100 mg l^{-1} myo-inositol, 40 mg l^{-1} NaFe(III)EDTA, 200 mg l^{-1} glycine and 100 mg l^{-1} citric acid.

Establishment and maintenance of *in vitro* plantlets

In vitro plantlets were disinfected as previously described (Chapter 2). For propagation, plantlets were cultured on modified MS medium with the growth regulators 6-benzylaminopurine (BA; 1 mg l^{-1}), gibberellic acid (0.1 mg l^{-1}) and indolebutyric acid (0.1 mg l^{-1}), and 25 g l^{-1} sucrose (VPM). After the propagation, the shoots were cultured on modified MS medium supplemented with 25 g l^{-1} sucrose, 0.65 % agar, without any growth regulator (VRM0), on which the shoots produced roots and grew bigger. The plantlets were transferred every 6 - 8 weeks to fresh medium. All *in vitro* plantlets were cultured at 23°C with a 12 hours photoperiod.

Callus and compact callus induction

To induce calli and compact calli, *in vitro* plantlets grown on VRM0 were cut in slices (≤ 1 mm) (Chapter 2). These crown slices (5 - 8 slices / plantlet) or leaf slices (2 - 3 slices / plantlet) were placed on different callus induction media (result and discussion section and Table 3.1) and maintained at 23°C either under 12 h daily illumination or in the dark.

Plant regeneration

After 2, 6, 8 or 12 weeks on induction medium, crown and leaf slices were transferred to the different regeneration media (Table 3.1). Data were collected after 6, 8, 12, 16, 18 or 24 weeks of culture in the light (Figure 3.1).

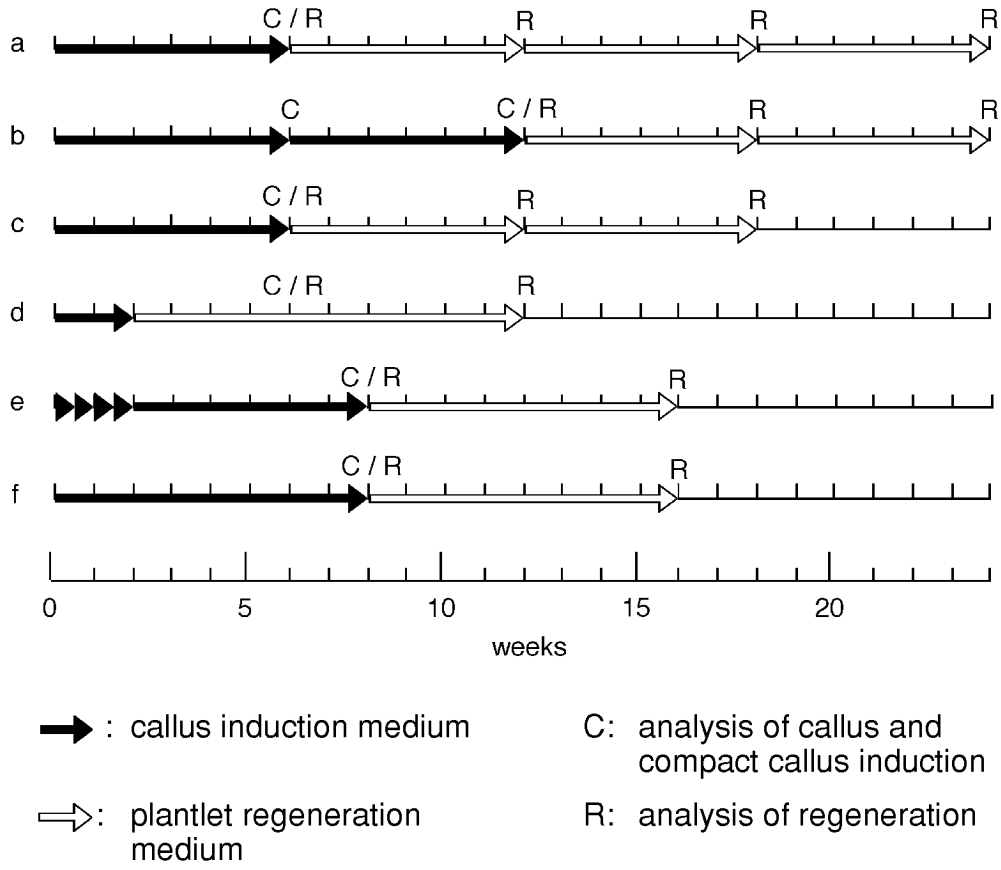

Figure 3.1. Time sequence of the different callus induction and plantlet regeneration experiments. The following time schedules were used: a, b: to test the influence of the sucrose concentration in the callus induction medium (DB10, DB75) and cultivation in light or in dark during callus induction on the compact callus induction and the subsequent shoot regeneration; c - f: to compare the different procedures for callus induction and shoot regeneration; c: time schedule for DB10/D0.1B1(25) or DB75/D0.1B1(25); d: time schedule from Mucciarelli *et al.* (1993); e: time schedule from Mathur *et al.* (1989); f: time schedule from Sreenath *et al.* (1994)

Histology

For light microscopy, the samples were fixed in a formaldehyde (35 %)-acetic acid-alcohol-water (10:5:50:35) mix for at least 24 hours under vacuum. Fixed tissues were dehydrated in a *t*-butanol series (Johansen 1940) and embedded in Paraffin. Sections were cut at 10 µm and stained with safranine / fastgreen or toluidine blue (Gerlach 1984).

RESULTS AND DISCUSSION

Influence of sucrose concentrations and cultivation in light or dark on compact callus induction and on subsequent plantlet regeneration

Based on our earlier experience with vetiver callus induction (Chapter 2), we have used modified MS medium containing 0.5 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg l^{-1} 6-benzylaminopurine (BA) for induction of compact calli on crown and leaf slices of *in vitro* plantlets. This DB medium was supplemented with 10 or 75 g l^{-1} sucrose (DB10 / DB75). The plant slices were maintained at 23°C either under 12 h daily illumination (DB10(light) / DB75(light)) or in the dark (DB10(dark) / DB75(dark)). Crown and leaf slices were prepared from about 35 *in vitro* plantlets (4 - 6 crown slices / plant and 3 leaf slices / plant). After 6 weeks in culture, half of the crown and leaf slices were transferred to regeneration medium (Figure 3.1a). The remaining slices were cultivated for another 6 weeks on the induction media (Figure 3.1b) and then also transferred to the regeneration medium. This experiment was repeated three times.

Callus and compact callus induction

After a short time in culture, soft and gelatinous calli grew on the plant slices. Callus induction on crown slices exceeded 75 %, and on leaf slices it varied between 40 % and 97 %. There was no further callus induction after 6 weeks. Callus induction on crown slices was better in the light, while on leaf slices more calli were induced in the dark (data not shown).

After about 4 weeks in culture, compact calli containing organized structures were observed on plant slices (Figure 3.2a). This compact callus type resembled embryogenic callus, described as nodular, hard, yellow, and opaque (Sreenath and Jagadishchandra 1991) or as compact, highly organized, slow growing and pale white to light yellow in color (Vasil and Vasil 1994). Most compact calli were induced on crown slices cultured in the light on callus induction medium with 75 g l^{-1} sucrose. For leaf slices the effects of the sugar

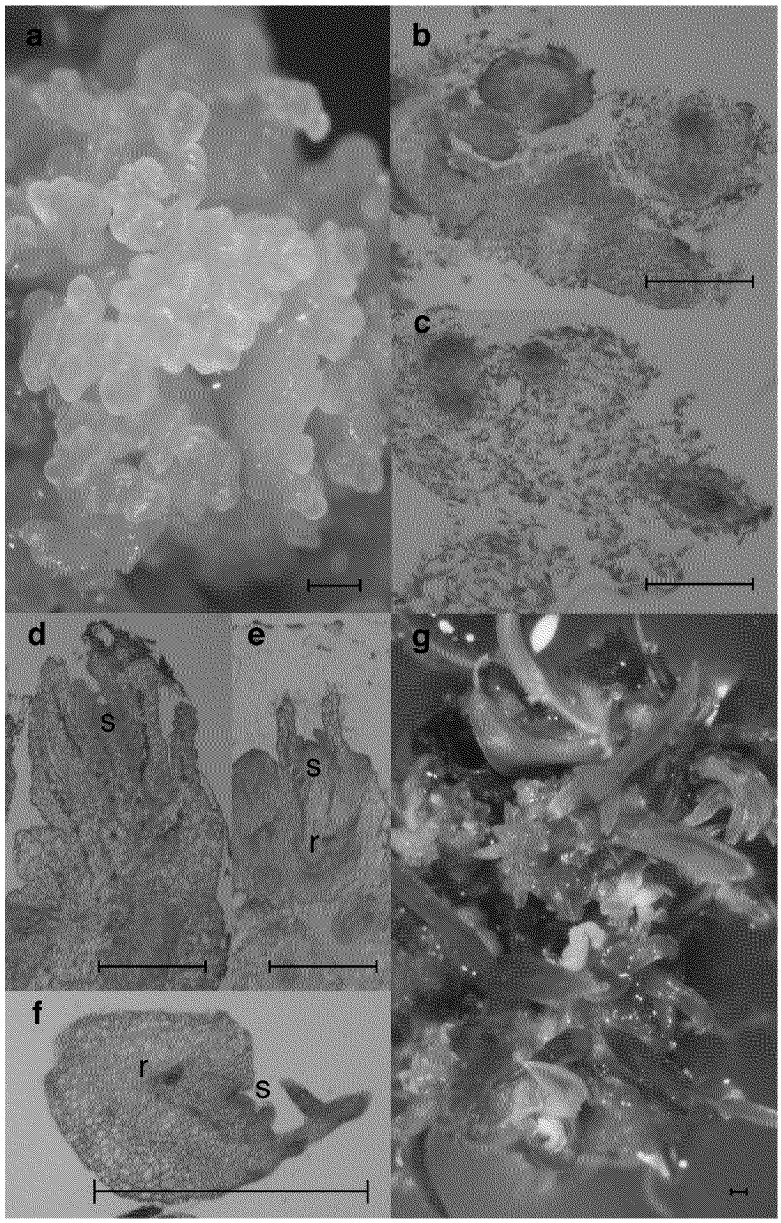

Figure 3.2. From compact callus to regenerated plantlets. (a) Compact callus; (b, c) Sections through compact callus from DB75 with bipolar structures; (d - f) Sections through compact callus from DB10, (d) shoot regeneration, (e, f) somatic embryos with shoot (s) and root (r) meristems; (g) Callus with regenerated plantlets; bar: 0.5 mm

concentration and light were less pronounced (Figure 3.3a, b). Although on some slices compact callus was seen after 6 weeks, after 12 weeks the compact callus was not detectable any more. The number of leaf and crown slices with compact calli still increased after 6 weeks, except on DB10(light) (Figure 3.3a, b). On DB75, not only did the percentage of slices with compact calli increase, but even the amount of compact callus per crown slice increased and some crown slices were completely covered with compact calli (results not shown).

To compare the morphological characteristics of calli induced on DB10 or DB75, calli were sectioned and examined with light microscopy. Bipolar compact structures were found on the sections from DB75 (Figure 3.2b, c). Sections from DB10 contained shoots and somatic embryos in addition to compact bipolar structures (Figure 3.2d - f).

Plant regeneration

The first signs of regeneration were already seen after 12 weeks on callus induction media. In the light, calli of crown and leaf slices showed green spots and on DB10 even regenerated plantlets, whereas in the dark plantlets regenerated on DB10 only on a few leaf slices (Figure 3.3c, d). After transferring the plant slices to regeneration medium, the best regeneration was achieved for crown slices induced on DB10 in the light and for leaf slices induced on DB10 both in the light and in the dark (Figure 3.3c, d). The results of Figure 3.3 show that the influence of light during callus induction on subsequent plantlet regeneration depended as much on the plant material used as on the sugar concentration in the callus induction medium.

The regeneration rate after 12 weeks decreased for crown and leaf slices, except for calli induced on DB10(light) where plantlets began to regenerate on the callus induction medium (Figure 3.3c, d). George and Subramanian (1999) reported that their vetiver callus maintained the morphogenic potential on medium containing polyvinyl pyrrolidone and casein hydrolysate. Therefore these two compounds should be tested in the future.

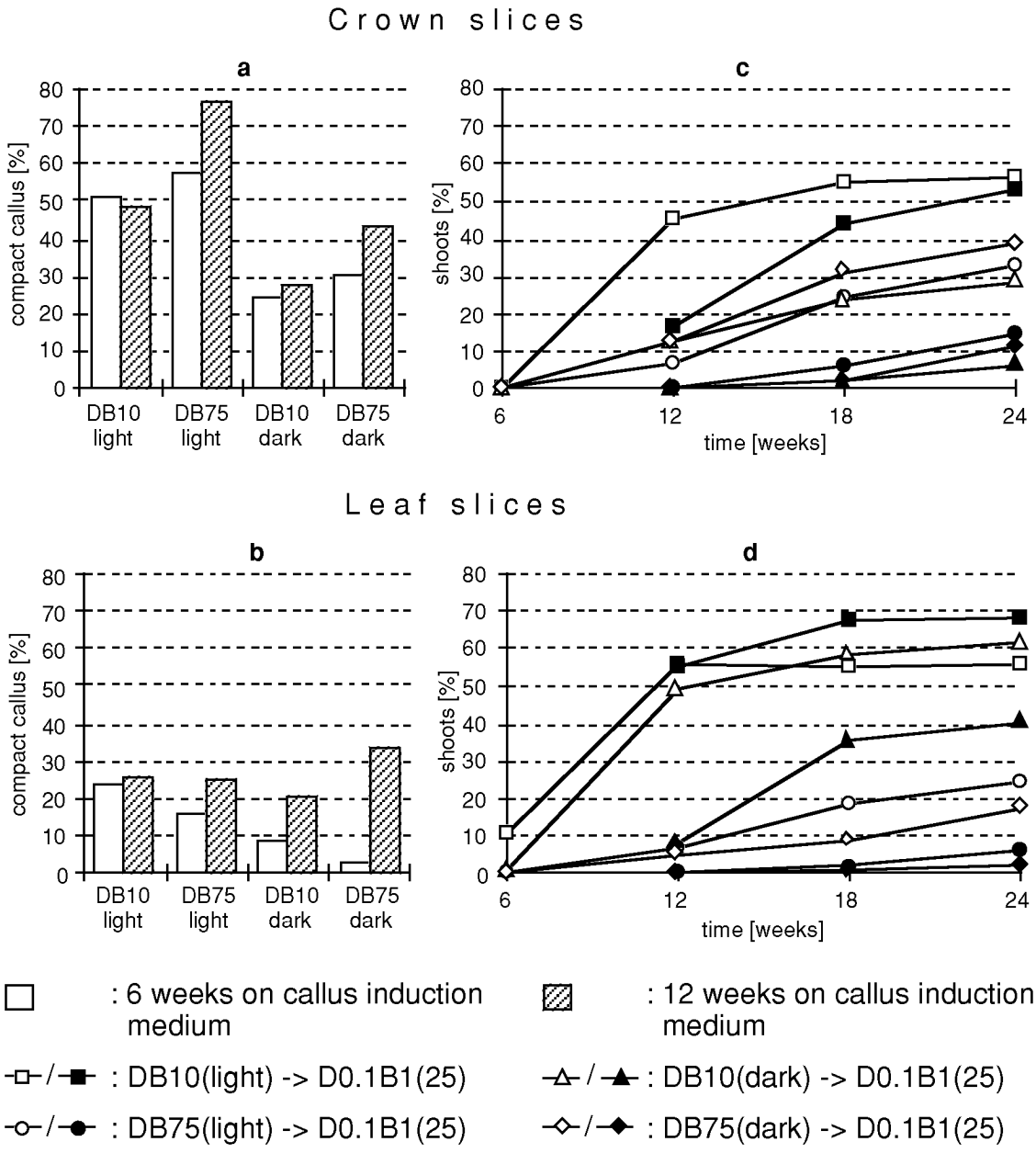

Figure 3.3. Effect of sucrose concentrations and cultivation in light or dark on callus and compact callus induction and subsequent plantlet regeneration. Crown (a, c) and leaf (b, d) slices were cultured on DB10 or DB75 in the light or in the dark at 23°C. The percentage of slices with compact callus was determined after 6 and 12 weeks (a, b). After 6 (open symbols) or 12 (filled symbols) weeks the slices were transferred to regeneration medium D0.1B1(25). The percentage of slices with shoots was determined after 6, 12, 18 and 24 weeks (c, d).

Influence of addition of α-naphthalene acetic acid to the callus induction medium

Earlier callus induction experiments on DB medium containing additional 0.1 mg l^{-1} α-naphthalene acetic acid (NAA) (DNB medium) resulted in slightly higher percentage of slices with compact callus, but the values were still within the error of the repetitions (Chapter 2). Therefore, the influence of NAA addition to the callus induction media on compact callus induction and subsequent plantlet regeneration was tested.

Crown and leaf slices of 33 plantlets were put on DB10, DB75, DNB10 and DNB75 and cultured at 23°C with a photoperiod of 12 hours. After 6 weeks the slices were transferred to the regeneration medium D0.1B1(25) (Figure 3.1c).

The changes found by adding NAA to the callus induction medium were too small to be significant. On leaf slices, NAA had no or a slight positive effect, whereas for crown slices, there was no or even a slightly negative effect (Figure 3.4).

Influence of starting material on plantlet regeneration

Although the same treatment was used for the compact callus induction on DB10 or DB75 in the light and subsequent regeneration, only half the percentage of crown slices with regenerated plantlets were obtained during the experiment with the additional NAA (Figure 3.4) compared to the light/dark comparison experiment (Figure 3.3). Vasil and Vasil (1994, 1986) reported that the growth conditions of the donor plants as well as the physiological condition and the developmental stage of the explants are critical in obtaining a desirable response. The *in vitro* vetiver plantlets used in this study were not always in the same physiological state. This seems to have a larger effect on the crown slices than on the leaf slices, since the regeneration of plantlets from leaf slices as explant was about the same in both experiments (Figure 3.3, 3.4). As crown slices contain, beside leaf tissue, many other cell types like root and shoot meristems, this could be a reason for the differences obtained with crown and leaf slices as explant for compact callus induction and subsequent plantlet regeneration.

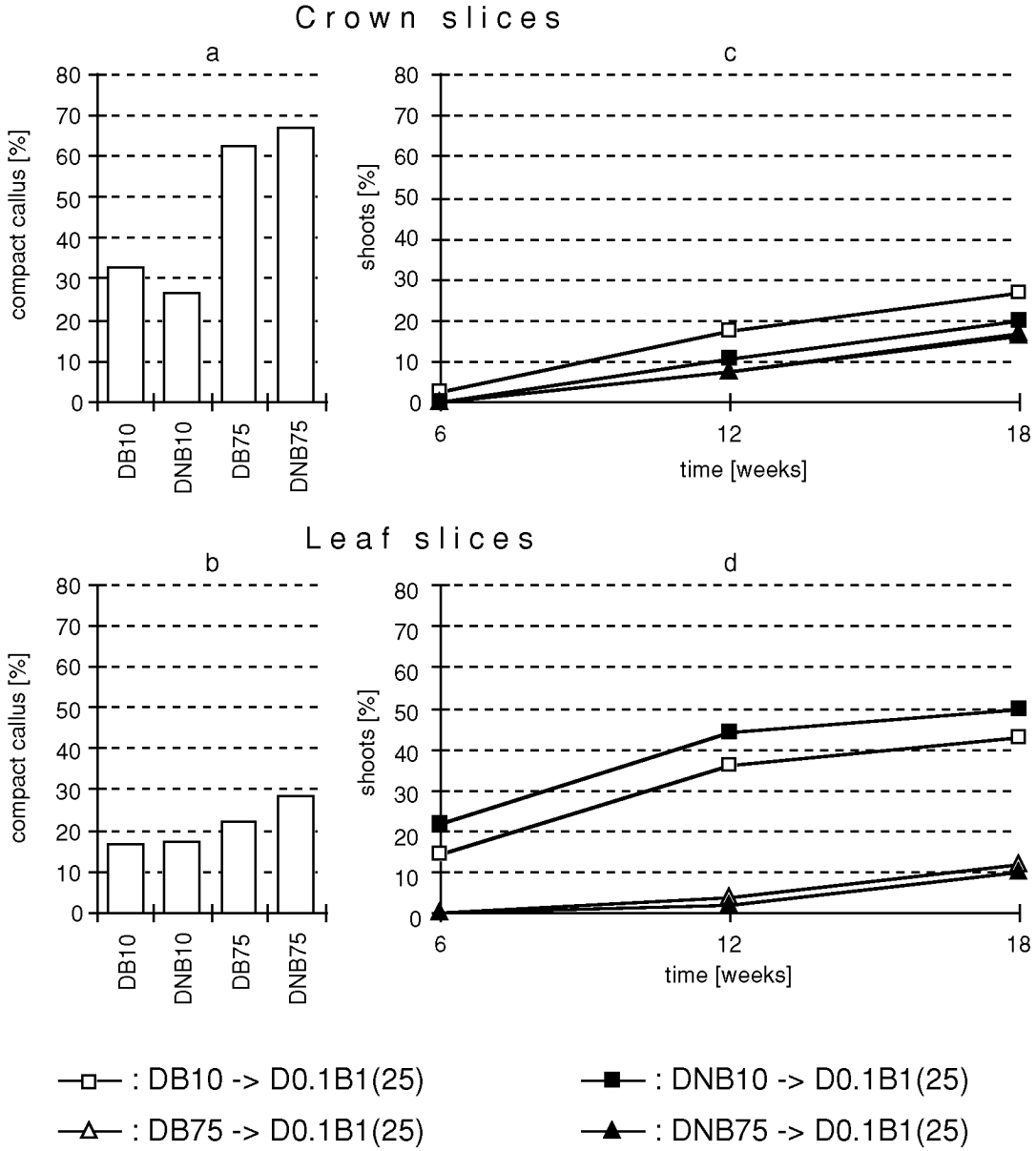

Figure 3.4. Effect of additional α-naphthalene acetic acid to the callus induction media on compact callus induction and subsequent plantlet regeneration. Crown (a, c) and leaf (b, d) slices were cultured on DB10 or DB75 without or with additional 0.1 mg l^{-1} α-naphthalene acetic acid (DNB10, DNB75) in the light at 23°C. The percentage of slices with compact callus was determined after 6 weeks (a, b). After 6 weeks the slices were transferred to regeneration medium D0.1B1(25). The percentage of slices with shoots was determined after 6, 12 and 18 weeks (c, d).

Plantlet regeneration from leaf slices

For leaf slices, not all plantlets regenerated from compact calli, since only 20 % of the slices developed compact calli, while plantlets were regenerated on up to 70 % of the slices. Thus, either shoots regenerated from compact callus before these were detectable, or they regenerated from another callus type or directly from leaf cells. To test this, leaf slices were cultured in the light on DB10, on D0.1B1(25) or on DB10 followed after three weeks by D0.1B1(25). On 12 % of the slices, plantlets regenerated directly on the callus induction medium DB10, whereas after subsequent cultivation on D0.1B1(25) 23 % of the slices regenerated plantlets. Since on D0.1B1(25), plantlets were observed on only 4 % of the slices, which was most probably due to carry over of meristems, a callus induction step is necessary to regenerate plantlets from leaf slices.

Plantlet regeneration from crown slices

Despite a higher rate of compact callus induction on DB75 (Figure 3.3), regeneration of plantlets from crown slices with compact callus was more successful after induction on DB10 (90 %) than after induction on DB75 (20 %). This could be explained either by a regeneration block for the compact calli from DB75 and precocious germination of compact calli from DB10 or by induction of an additional callus type with improved regeneration ability on DB10 in the light.

With respect to the first option, the compact callus obtained on DB75 had the appearance of embryogenic callus described by Sreenath and Jagadishchandra (1991). Lu *et al.* (1983, 1984) reported for immature embryos of rye and maize as starting material, that higher sucrose concentrations (6 or 12 % instead of 3 % sucrose) resulted in an increase of the amount of embryogenic callus, as was observed in our experiments on compact callus induction. Sreenath (1991, 1994) reported that at low levels of sucrose (3 %), somatic embryos germinate before they attain the typical grass embryo morphology, and that this precocious germination could be suppressed by using higher sucrose concentrations. Thus, the higher regeneration obtained after induction on DB10 in the light may be due to precocious germination of shoots. Looking at the microscopical sections of the DB75 calli, the bipolar structures do not look like typical grass

embryos. Therefore, the best combination of chemical and physical stimuli to obtain embryos and regenerate more plantlets must still be found.

Since Sreenath *et al.* (1994) obtained very good plant regeneration with 100 g l^{-1} sucrose in their regeneration medium, more sucrose was added to our regeneration medium. After prior induction of crown and leaf slices for 6 weeks on DB75 in the light, the slices were transferred to regeneration medium containing either 25 g l^{-1} or 75 g l^{-1} sucrose. On the regeneration medium with 75 g l^{-1} sucrose, fewer crown and leaf slices regenerated plantlets (results not shown). Therefore, the higher sucrose concentration did not improve the regeneration and other stimuli have to be found to regenerate plantlets.

With respect to the second option, Vasil and Vasil (1994) described in maize two callus types of which type I is compact, slow growing and rapidly loses regeneration ability, whereas type II is soft, friable, fast growing, highly regenerative and maintains its competence for regeneration for a long time. Tomes (1985) found that the formation and stabilization of a type II callus was strongly inhibited by high sucrose concentration in the culture medium. If DB75 induces mainly callus of type I, while DB10 induces mainly a callus similar to type II, this could explain why DB75 calli rapidly lose the ability to regenerate. However, this does not explain why calli from DB10(dark) did not regenerate better than calli from DB75 (Figure 3.3); light also appears to be an important factor in addition to low sucrose concentrations for a subsequent high regeneration efficiency from crown slices. It is not clear whether calli from DB10(light) retained the regeneration ability up to 12 weeks or whether the regeneration efficiency was so high because the shoots begin to regenerate while still on the callus induction medium. This should be tested by comparing the compact calli from DB10 and DB75 in more detail and by trying to regenerate plantlets after longer times on induction medium.

What procedure might be used to obtain more shoot regeneration from compact calli induced on DB75? One approach would be to test different combinations of chemical and physical stimuli to improve regeneration. Increasing the sucrose concentration in the regeneration medium as described by Sreenath *et al.* (1994) did not improve the regeneration from DB75 induced calli. Another possibility is to prevent the compact callus from reaching the

regeneration block or to reduce the rapid loss of regeneration ability. Tomes (1985) has reported that higher sucrose concentrations increased the initial frequency of response, but inhibited further maintenance of embryogenic callus in maize. Vasil *et al.* (1984) were able to improve subsequent culture characteristics by again reducing the sucrose concentration. The advantages of a high percentage of compact callus induction on DB75 and the high regeneration efficiency after induction on DB10(light) might be combined by first inducing compact calli for 1 - 6 weeks on DB75(light) followed by a transfer to DB10(light) to maximize the subsequent regeneration potential, then followed by a final transfer to regeneration medium.

Optimized procedures to induce compact callus and to regenerate plantlets

The optimized procedure to regenerate plantlets is to cut the *in vitro* plantlets grown on VRM0 in leaf and crown slices, to culture the slices for 6 weeks on DB10 in the light and to regenerate plantlets by subsequently transferring the slices to D0.1B1(25). As the regeneration medium D0.1B1 contains 1 mg l^{-1} BA, which already showed a stimulating effect on the propagation, it was not possible to determine how many plantlets had regenerated on one slice and how many had simply propagated. However, after prior induction on DB10(light), it was possible to obtain up to 100 plantlets from one slice.

To obtain a lot of compact calli, the optimized procedure is to cut the *in vitro* plantlets grown on VRM0 in crown slices and induce compact calli on DB75 in the light. Supposing that the compact calli from DB75 are able to regenerate more plantlets given the right stimuli, this medium should not be ignored.

Comparison of different *in vitro* regeneration methods

After optimization, our procedure to regenerate plantlets was compared with several methods described previously (Mathur *et al.* 1989; Mucciarelli *et al.* 1993; Sreenath *et al.* 1994). Crown and leaf slices were cultured on different callus induction media (Table 3.1) at 23°C with a photoperiod of 12 hours. After 2, 6 or 8 weeks on induction medium, crown and leaf slices were transferred to

Table 3.2. Comparison of different procedures for compact callus induction and plantlet regeneration.

a) percentage of slices with callus or compact callus

				Crown slices			Leaf slices		
Medium [1]		Time	Time	Slices	Callus	Compact callus	Slices	Callus	Compact callus
Ind.	-> Reg.	schedule [2]	[weeks]	[no]	[%]	[%]	[no]	[%]	[%]
DB10	-> D0.1B1(25)	c	6	214	95	33	102	73	17
DB75	-> D0.1B1(25)	c	6	211	97	63	99	49	22
Mu1	-> Mu2	d	6	220	74	0	98	97	6
Ma1	-> Ma2	e	8	198	93	1	98	94	4
Sr1	-> Sr2(30)	f	8	199	90	1	102	81	6
Sr1	-> Sr2(100)	f	8	208	90	1	99	84	2

b) percentage of slices with regenerated shoots

			Crown slices						Leaf slices					
Medium [1]		Time	Slices	Shoots [%] after [3]					Slices	Shoots [%] after				
Ind.	-> Reg.	schedule [2]	[no]	6w	8w	12w	16w	18w	[no]	6w	8w	12w	16w	18w
DB10	-> D0.1B1(25)	c	214	3	- [4]	17	-	27	102	15	-	36	-	73
DB75	-> D0.1B1(25)	c	211	0	-	8	-	17	99	0	-	4	-	12
Mu1	-> Mu2	d	220	0	-	0	-	-	98	0	-	0	-	-
Ma1	-> Ma2	e	198	-	0	-	0	-	98	-	0	-	0	-
Sr1	-> Sr2(30)	f	199	-	0	-	0	-	102	-	0	-	0	-
Sr1	-> Sr2(100)	f	208	-	0	-	0	-	99	-	0	-	0	-

[1] *Ind.: callus induction medium (see Table 1); Reg.: plantlet regeneration medium (see Table 1)*
[2] *Method see Figure 1*
[3] *w: weeks*
[4] *- : not analyzed*

the different regeneration media (Table 3.1), on which they were cultured for another 8 or 12 weeks (Figure 3.1c - f).

Starting with *in vitro* vetiver plantlets from Java, DB10 and DB75 callus induction media and subsequent regeneration on D0.1B1(25) were more effective in regenerating plantlets than other methods tested (Table 3.2). Constabel and Shyluk (1994) have already pointed out that published culture procedures can not always be reproduced successfully because too many biological factors such as genotype, physiological condition of the source material and differences in culture conditions may interfere. In this study, a non-flowering vetiver variant from Java was used, while Mucciarelli *et al.* (1993) used a plant from Somalia, Sreenath *et al.* (1994) described regeneration from inflorescence and Mathur *et al.* (1989) used a wild cultivar of vetiver and therefore most probably a flowering vetiver variant. Another factor which might influence the results is that we used *in vitro* plantlets as starting material, whereas the previous reports were based on plant material from *in vivo* plants which had to be disinfected prior to the regeneration experiments. Holme and Petersen (1996) reported a difference in the formation of embryogenic callus between leaf explants from *in vitro*-grown shoots and from greenhouse-grown plants.

It would be interesting to test the combinations DB10/D0.1B1(25) and DB75/D0.1B1(25) with *in vitro* crown and leaf slices from different variants of vetiver, to determine whether the differences between the results of Mathur *et al.* (1989), Mucciarelli *et al.* (1993) and Sreenath *et al.* (1994) and those from this study are due to the vetiver variant or to the *in vitro* explants.

CONCLUSIONS

In summary, two interesting methods are now available for compact callus induction and regeneration of plantlets from a non-flowering vetiver variant from Java by using crown and leaf slices from *in vitro* vetiver plantlets as starting material. First, the combination of callus induction on DB10(light) and regeneration on D0.1B1(25) allows regeneration of plantlets from crown and

leaf slices within a short time (12 - 18 weeks). Second, DB75 efficiently induces compact callus on many crown slices. Although thus far the regeneration efficiency has been low, the compact callus from DB75 was successfully used to induce liquid cultures (Mucciarelli and Leupin, in press).

The procedures for callus induction and subsequent plant regeneration described in this study provide a solid basis to regenerate plantlets which might be somaclonal variants with more essential oil or with an altered oil composition. If insufficient variation is found, further variations could be obtained by inducing mutations with irradiation or mutagenic chemicals. Since it takes 15 to 22 months until vetiver contains a sufficient amount of the complete vetiver oil (Roth and Kornmann 1997; Weiss 1997a), we did not yet test whether oil variants can be found among the regenerated plantlets.

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Chapter 4: Liquid Culture Induction and Plantlet Regeneration of Vetiver (*Vetiveria zizanioides*)

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ABSTRACT

To induce liquid cultures and subsequently to regenerate plantlets of *Vetiveria zizanioides* , the influence of the callus induction medium (different sucrose concentrations) and the composition of the liquid medium (different basal media and different growth regulators) on the liquid culture induction were studied. Three types of cultures were obtained: a mucilaginous type, cultures with loose calli and cultures containing compact cell clumps. Since the cultures containing compact clumps looked most promising to regenerate plantlets, we tried to increase the number of these cultures. The induction depended mainly on the starting callus and therefore on the callus induction medium whereas the changes of the liquid culture medium itself did not increase the induction of liquid cultures with compact clumps. From two of these liquid cultures with compact clumps we were able to regenerate a few plantlets.

INTRODUCTION

Vetiveria zizanioides is a tropical grass. A major reason why this plant is of interest is that the roots contain an essential oil which consists of more than 150 sesquiterpenoids (Akhila *et al.* 1981). The vetiver oil is used as a component for perfumes, scenting soaps and as a fixative to prevent the evaporation of more volatile oils (Vietmeyer and Ruskin 1993).

Since not all vetiver plants flower and the germination rate of the seed is low, it is difficult to produce variants via traditional sexual breeding. An alternative is to produce variants in tissue cultures.

In previous experiments it was shown that it is possible to regenerate plantlets via callus on solid medium. As the occurrence of somaclonal variations increases with the duration of the disorganized phase and the extent of the disorganization (Karp 1994), regeneration from a suspension culture would also be of interest. Vasil and Vasil (1986) found that much more variability is found in cell cultures than in regenerated plants, and that during regeneration of shoot meristems, some degree of selection is imposed with the result that only a fraction of the variability present in the cell cultures is actually recovered in the regenerated plants. If the variation rate is too low, mutations can still be induced

by irradiation or mutagenic chemicals. Due to the fact that with shaking, the cell clumps are smaller than on solid medium and often fall apart, the generation of chimera plants should be reduced.

In this study, we describe the influence of starting callus from different callus induction media, as well as the influence of different liquid media on the establishment of liquid cultures and on subsequent regeneration of plantlets from these liquid cultures.

PLANT MATERIAL

As starting material for the *in vitro* cultures non-flowering Vetiver plants from Java were used. The plants were cut in separate shoots and disinfected with calcium hypochloride ($Ca(ClO)_2$). The disinfected cuttings were put on modified MS medium (Murashige and Skoog 1962) supplemented with 25 g l^{-1} sucrose and 0.65 % agar (VRM0). Modified MS medium contains 1/2 MS macro-nutrients, 1/2 MS micronutrients, 0.5 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} pyridoxine-HCl, 0.5 mg l^{-1} nicotinic acid, 100 mg l^{-1} myo-inositol, 40 mg l^{-1} NaFe(III)EDTA, 200 mg l^{-1} glycine and 100 mg l^{-1} citric acid. For propagation of the plantlets, they were cultured on solid modified MS medium containing 25 g l^{-1} sucrose and the growth regulators 6-benzylaminopurine (1 mg l^{-1} BA), gibberellic acid (0.1 mg l^{-1}) and indolebutyric acid (0.1 mg l^{-1}).

CALLUS INDUCTION

To obtain calli for liquid culture induction, the *in vitro* plantlets grown on VRM0 (for establishment and maintenance of *in vitro* plantlets see chapter 2) were cut in leaf and crown slices and cultured on different callus induction media. The induction media were composed of modified MS medium supplemented with 2,4-dichlorophenoxy acetic acid (0.5 mg l^{-1} 2,4-D), benzylamino-purine (0 or 0.5 mg l^{-1} BA), sucrose (1 - 10 %) and 0.65 % agar. The pH of the medium was adjusted with KOH or HCl to 5.8 before autoclaving. The cultures were maintained at 23°C either under 12 h daily illumination or in the dark.

Calli were formed on crown and leaf slices within 6 weeks on callus induction medium. Three types of calli could be distinguished. The gelatinous and the soft

calli types were observed first, after 2 - 4 weeks, whereas a third calli type with compact structures (Figure 4.1a) appeared only after about 4 - 8 weeks in culture. These calli with compact structures were subcultured for another 1 - 9 months, after which they were used to induce liquid cultures.

ESTABLISHMENT AND MAINTENANCE OF LIQUID CULTURES

The calli were transferred after 3 - 5 months on callus induction medium to deep Petri dishes, (5 cm diameter) containing 10 ml of liquid medium (Table 4.1). The cultures were kept at 23°C in the dark on a rotatory shaker at 70 rpm and were subcultured every second week by replacing the old medium with fresh medium. As soon as the cultures were dense enough, they were transferred to 6 cm culture vessels (Greiner 967161, 190 ml, Greiner GmbH, Nürtingen, FRG) containing 15 ml liquid medium.

As was the case for the callus induction, three different culture types were distinguished in the liquid cultures: mucilaginous and thick cultures; cultures with loose, soft calli; and cultures containing compact cell clumps (Figure 4.1b, c). In mucilaginous cultures the medium became viscous and embedded the loose calli. Since we were interested in adequately growing liquid cultures, these cultures were discarded when they remained mucilaginous after another month of subculturing. Other cultures remained liquid but the calli were soft and failed to separate in smaller pieces. It took a long time until cultures started to form compact cell clumps. Once formed, cultures with compact clumps grew faster than the other cultures, with a doubling time of the fresh weight of about 1 to 2 months (data not shown).

Plant slices with compact calli from various callus induction media were used as source to induce liquid cultures in different media (Table 4.1). When calli were induced on callus induction media with 0.5 mg l^{-1} 2,4-D and 10, 25, 50 or 75 g l^{-1} sucrose, no liquid cultures with compact structures were subsequently established (data not shown). Calli with compact structures were induced on more slices by adding 0.5 mg l^{-1} BA to the callus induction medium and increasing the sucrose concentration (Chapter 2). Therefore, more compact calli for liquid culture induction are available. The changes of the callus induction medium also affected the induction of liquid cultures with compact clumps.

Table 4.1. Composition of the liquid media

Basal medium [mg l^{-1}]:

	modified AA	modified N6
Macronutrients	AA	N6
Micronutrients	MS	MS
NaFe(III)EDTA	29	37
Thiamine-HCl (Vit B_1)	10	0.1
Pyridoxine-HCl (Vit B_6)	1	0.5
Nicotinic acid	1	0.5
myo-Inositol	100	100
Sucrose	20'000	30'000
Sorbitol	25'000	10'000
L-Glutamine[1]	876	200
L-Asparagine · H_2O [1]	300	113
L-Arginine [1]	174	-
Glycine [1]	7.5	-
L-Proline [1]	-	500

Liquid media [mg l^{-1}]:

	AAF	mN6	mN6+B	mN60.5D	mN60.5D0.5B	mN60.1D
Basal medium	modified AA	modified N6				
2,4-D	1	1	1	0.5	0.5	0.1
BA	-	-	0.5	-	0.5	-
pH	5.8	5.8	5.8	5.8	5.8	5.8

AA : Müller and Grafe 1978
N6 : Chu *et al.* 1975
MS : Murashige and Skoog 1962
2,4-D : 2,4-dichlorophenoxy acetic acid
BA : 6-benzylaminopurine
[1] Amino acids were filter sterilized and added to the autoclaved medium

Compact calli from callus induction medium supplemented with 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA (DB) and low concentrations of sucrose (10 or 30 g l^{-1} / DB10 or DB30) did not give rise to any liquid cultures with compact clumps. On the other hand, if plant slices with compact calli, induced on callus medium supplemented with 0.5 mg l^{-1} BA and 50, 75 or 100 g l^{-1} sucrose (DB50, DB75 or DB100), were used as source to establish liquid cultures, liquid cultures with compact clumps were produced (Table 4.2). It was observed that, by increasing the sucrose concentration of the callus induction medium, not only on more slices compact calli were induced, but there were also more compact structures per callus. Per plant slice, the amount of compact callus thus increased, compared to the amount of soft callus. This change of the ratio between compact and soft callus may explain the observed difference in establishment of liquid culture with compact clumps.

The difference in the induction of liquid cultures with the modified N6 or the modified AA basal medium was minor. However, since somewhat more cultures with compact clumps were induced on mN6 than on AAF, we selected the modified N6 basal medium for further experiments (Table 4.2).

Table 4.2. Establishment of liquid cultures

callus induction		development of the cultures on liquid medium					
medium [1]	time [months]	medium [2]	cultures [numbers]	induction time [months]	appearance of the cultures compact [%]	loose [%]	mucilaginous [%]
DB10	4	mN6	10	10	0	20	80
DB30	4	mN6	8	10	0	0	100
DB50	4	mN6	11	10	18	0	82
DB75	3-5	mN6	124	6-14	11	13	76
DB100	8-10	mN6	24	7-8	13	-[3]	-[3]
DB75	4-5	AAF	38	8-14	5	11	84
DB75	3-5	mN6+B	62	8-14	10	14	76
DB75	3	mN60.1D	13	6	0	100	0
DB75	3	mN60.5D	25	9	8	-[3]	-[3]
DB75	3	mN60.5D0.5B	25	9	0	-[3]	-[3]

[1]: DBx: modified MS medium supplemented with 0.5 g l^{-1} 2,4-dichlorophenoxy acetic acid, 0.5 mg l^{-1} benzylaminopurine and x g l^{-1} sucrose
[2]: composition of the media see Table 1
[3]: no data

As the growth regulator BA in the callus induction medium had a beneficial effect on the induction of calli with compact structures (Chapter 2) and on the subsequent establishment of liquid cultures, 0.5 mg l^{-1} BA was added to the liquid culture medium. Unfortunately, BA did not have the same beneficial effects in liquid medium; it influenced neither the induction of compact clumps nor the induction of the other liquid culture types (Table 4.2). Accordingly, BA was not further added to the liquid culture medium.

In earlier experiments with callus induction media, higher 2,4-D concentrations induced more gelatinous calli (Chapter 2). Therefore, to reduce the number of the mucilaginous liquid cultures and maybe obtain more cultures with compact clumps, the 2,4-D concentration was lowered from 1 to 0.5 or even to 0.1 mg l^{-1}. This resulted in fewer mucilaginous cultures, but the percentage of liquid cultures with compact clumps did not increase (Table 4.2). The addition of 0.5 mg l^{-1} 2,4-D to the liquid culture medium had no effect on the percentage of cultures with compact clumps, but with 0.1 mg l^{-1} 2,4-D in the liquid culture medium, none were induced and the cultures turned brown. The reduction of

the 2,4-D concentration in the medium also had another effect: in some cultures root-like structures were found (Figure 4.1d). Some of these structures even produced something like side-roots. Histological observation showed that these structures were not roots, but callus growing in a root-like fashion (Figure 4.1e). In time some of these structures reverted back to callus clumps. Addition of 0.5 mg l^{-1} BA to the mN60.5D medium resulted in more cultures with root-like structures and no compact clumps (Table 4.2).

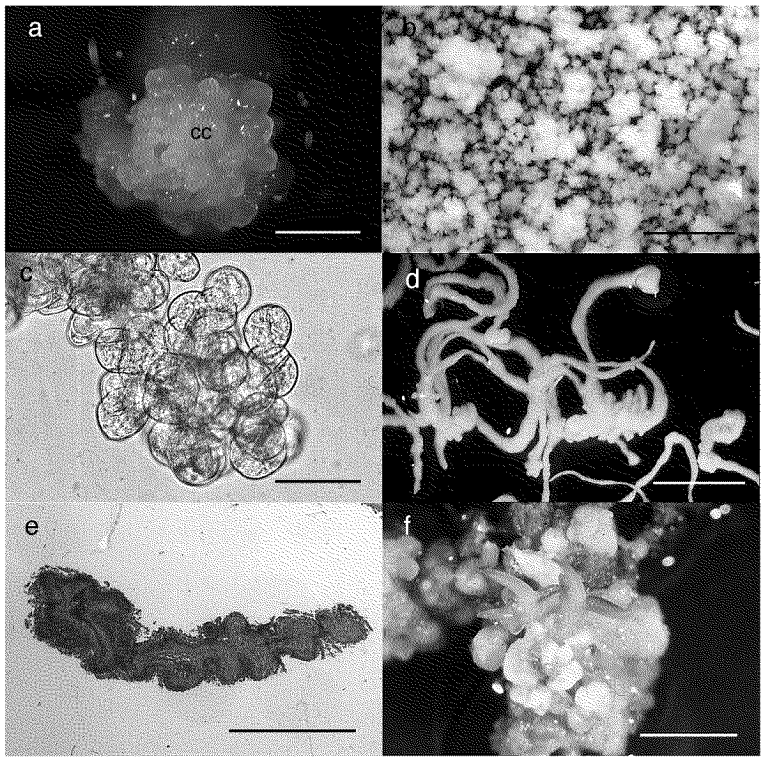

Figure 4.1. Liquid culture induction and plantlet regeneration of *Vetiveria zizanioides*. (a) Compact callus (cc) was used as starting material for liquid culture induction (bar = 2 mm); (b, c) Liquid cultures with compact clumps after about 1 year on mN6 (b: bar = 5 mm, c: bar = 50 µm); (d) Root-like structures from mN60.5D0.5B (bar = 5 mm); (e) Section of a root-like structure from mN60.5D0.5B (bar = 2 mm); (f) Regenerated plantlets from the liquid culture on mN60.5D (bar = 3 mm).

PLANT REGENERATION FROM LIQUID CULTURES

For the regeneration experiments compact clumps from the liquid cultures were transferred to solid callus induction media DB75 or DB10 (solid modified MS medium with 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BA and 75 or 10 g l^{-1} sucrose). The resulting calli were subsequently transferred to regeneration media to test the ability of the culture to regenerate plantlets. For regeneration the 2,4-D concentration was either reduced to 0.1 mg l^{-1} and the BA concentration was increased to 1 mg l^{-1} (D0.1B1), or 2,4-D was omitted and 0.5 mg l^{-1} BA (B0.5) or 1 mg l^{-1} kinetin and 0.1 mg l^{-1} indole acetic acid (VRM8) were added. For all three regeneration media the modified MS medium was supplemented with 25 g l^{-1} sucrose and 0.65 % agar.

Some of the clumps became brown, others remained white and grew as fine granular callus, a few produced bigger compact structures which became green, and from two cultures we were able to regenerate a few plantlets (Figure 4.1f). Both cultures regenerated on DB75 callus induction medium followed by D0.1B1 regeneration medium. The two calli which regenerated plantlets came from two different cultures. One callus developed from a 9 months old mN6 liquid culture, the other from a 6 months old mN60.5D liquid culture. At this stage these cultures were a mix between the original calli, loose calli and compact clumps. Afterwards no more plantlets were regenerated from these cultures, thus it is not clear whether the plantlets were regenerated from the liquid culture or simply represented carry-over primordia from the original material.

It took 6 - 14 months before compact structures developed in liquid cultures and even longer until enough compact clumps were available for regeneration experiments, so that only a few regeneration experiments could be done. At a later stage, when enough compact clumps were available in the liquid cultures, they no longer regenerated. To regenerate more plantlets from the liquid cultures, faster methods to obtain liquid cultures or methods to prolong the regeneration potential of liquid cultures must be found.

Several factors influence the production of liquid cultures: the starting material, the composition of the liquid medium, the treatment of the cultures

and environmental conditions (temperature, shaking speed, cultivation in dark / light, ...).

The starting material seems to be an important factor, as in the experiments it was shown that the calli induced on different callus induction media had an effect on the liquid culture induction. The changes of the liquid media (mN6 or AAF) or different concentrations of growth regulators (1 or 0.5 mg l^{-1} 2,4-D and 0 or 0.5 mg l^{-1} BA) did not show any effect on the induction of liquid cultures with compact clumps. The first step in the improvement of the liquid culture is to improve the starting callus. One possibility is to change the ratio between soft and compact callus in the starting material by tearing the calli into small pieces and discarding any soft callus present. Another possibility is to further improve the callus induction medium to obtain better callus types with which the liquid culture can be established faster.

One problem in our process was that the callus clumps used for inoculation remained more or less intact and did not split up. Patnaik *et al.* (1997) were able with palmarosa to establish suspension cultures with cell aggregates by sieving and resuspending the culture in fresh medium. This method also has the advantage that carry-over primordia from the original material will be removed in time.

As soon as the starting material is improved and the subculture method is optimized it might be worth to make changes to the liquid medium composition (growth regulator, sucrose or sorbitol concentrations, additional compounds,...) and the environmental conditions.

In this study we showed that the induction of liquid vetiver cultures containing compact clumps is possible and we obtained some regenerated plantlets from these cultures. However, before the establishment of liquid cultures and subsequent plant regeneration provide a useful method to efficiently regenerate plantlets which might be somaclonal variants, additional optimization is necessary.

ACKNOWLEDGEMENTS

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Chapter 5: Comparing analysis methods for detecting quantitative and qualitative changes in the vetiver oil composition

Ruth E. Leupin, Charles Ehret, Karl H. Erismann and Bernard Witholt

ABSTRACT

Several methods to assess quantitative and qualitative changes of large numbers of small scale samples of vetiver extracts were compared: via olfaction, inhibition of microbial growth and analysis by thin layer chromatography (TLC) and gas chromatography (GC). By smelling, it is possible to detect if oil is present, though the results are subjective and only approximate. Vetiver oil efficiently inhibits growth of three actinomycetes and reduces red color production of one actinomycete, but the amount necessary to obtain inhibition is high and it is not known which compound inhibits the growth or the red color production. Analysis by TLC or GC has the advantage that these methods not only detect whether the sample contains oil, but also separate components and therefore make it possible to detect qualitative changes. In contrast to GC, several samples can be analyzed simultaneously by TLC and non-volatile compounds can be detected. The detection limit for TLC is 5 - 10 µg vetiver oil, which is high, and the analysis provides limited resolution with only about 15 spots for more than 300 components. Nevertheless, it is possible to detect changes in oil composition. GC also fails to resolve the oil in single components, but as the separation is better, the amount of oil and changes of the oil composition can be determined more exactly. For a GC analysis only about 0.5 µg oil is necessary. The analysis time is 90 minutes per GC run. Since GC analysis allows higher sensitivity and resolution than TLC, but the latter enables analysis of far more samples in parallel, TLC is preferred for a preliminary analysis of many samples, whereas GC analysis provides more detailed information for smaller sets of selected samples.

INTRODUCTION

The tropical grass *Vetiveria zizanioides* belongs to the subfamily of *Panicoideae*, which includes maize, sorghum, sugarcane and lemongrass (Vietmeyer and Ruskin 1993). One reason why vetiver is cultivated, is that the roots contain an essential oil, consisting of more than 300 sesquiterpenoids (de Guzman and Oyen 1999), which is used as a component for perfumes, scenting

soaps and as a fixative to prevent the evaporation of more volatile oils (Vietmeyer and Ruskin 1993). Because a completely synthetic vetiver oil can not be manufactured at a realistic price (Vietmeyer and Ruskin 1993), vetiver variants with more oil or with a different oil composition are of interest. New variants could be obtained by traditional breeding (Gupta *et al.* 1983; Lal *et al.* 1998; Sethi 1982; Sethi and Gupta 1980) or, since not all vetiver plants flower and the germination rate of the seed is low (Vietmeyer and Ruskin 1993) by regeneration of plantlets via tissue cultures (George and Subramanian 1999; Keshavachandran and Khader 1997; Leupin *et al.* 2000; Mathur *et al.* 1989; Mucciarelli *et al.* 1993; NaNakorn *et al.* 1998; Sreenath *et al.* 1994).

To find oil variants, all plantlets regenerated via tissue culture have to be tested for increased oil content or altered oil composition. Plants require 15 to 22 months to produce the complete vetiver oil (Roth and Kornmann 1997; Weiss 1997a). Starting from *in vitro* plantlets, it will take even longer before all regenerated plantlets can be screened since they have first to be established in soil and grow larger. Clearly, an earlier pre-screening would be of interest. However, without being able to induce the oil in an earlier stage, this will not be possible. Consequently, the oil induction experiments and the screening of all regenerated plantlets involve large numbers of small scale extractions and analyses. For oil induction experiments, a fast screening method to test whether the oil is induced is needed, whereas for pre-screening of the regenerated plantlets and minimization of the extraction a miniaturized analysis method which detects qualitative and quantitative changes with small amounts of essential oil has to be found. Different methods like gas chromatography (GC), high pressure liquid chromatography (HPLC), column chromatography (CC) and thin layer chromatography (TLC) were described for the analysis of essential oils (Banthorpe 1991; Croteau and Ronald 1983; Harborne 1984b; Kubeczka 1985).

In this study we tested and compared olfactive detection, bacterial inhibition, TLC and GC to detect quantitative and qualitative differences for sets of small samples.

MATERIAL AND METHODS

Distillate, extract and vetiver oils

To test the different analysis methods, mainly vetiver oil Bourbon (Givaudan-Roure), and distillates and solvent extracts of vetiver roots from Java were used. In addition, different commercially available vetiver oils were also analyzed (Table 5.1).

Table 5.1. Different commercially available vetiver oils

	origin	company
vetiver oil, Bourbon	- [1]	Givaudan-Roure, Dübendorf, Switzerland
vetiver oil	El Salvador	PRIMAVERA Life, Sulzberg, Germany
vetiver oil, Bourbon	West India	ELEXISIS, Graz, Austria
vetiver oil	La Reunion	Seidenberg Collection, Hagenbuch, Switzerland
vetiver oil	-	MIGROS, imp. Germany
Peti's vetiver oil	-	Duftschloss zum Wolkenstein, Eschenz, Switzerland
Peti's vetiver fragrance	-	Duftschloss zum Wolkenstein, Eschenz, Switzerland
artificial vetiver oil	-	neoLab, Labor Spezialprodukte, Heidelberg, Germany

[1] : -, not indicated

Distillate: Dry vetiver roots from Java were distilled with phosphate buffer (0.5M, pH 8). The oil was extracted from the distilled water with methyl *tert*-butyl ether (MTBE) (see Chapter 6).

Solvent extract: Dry vetiver roots from Java were extracted overnight with solvents (hexane , MTBE, ethyl acetate or ethanol) at room temperature (see Chapter 6).

Inhibition of bacteria

Strains and medium

To test the inhibitory effect of vetiver oil Bourbon (Givaudan-Roure) on bacteria, *Staphylococcus aureus* Cowan I (ATCC12598) and 3 actinomycete strains were used. *S. aureus* was grown on LB medium (Sambrook *et al.* 1989) (1 liter contains 10 g Bacto trypton, 5 g yeast extract, 5 g NaCl and 15 g agar) and the three actinomycetes were cultured on M65 Streptomyces medium (DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) (1 liter contains 4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g $CaCO_3$, with or without 15 g agar, pH 7.2)

Filter paper disc diffusion method

To test the influence of vetiver oil on bacterial growth, a filter paper disc diffusion method was used (Vincent and Vincent 1944). The spores of the actinomycetes were transferred from solid plates to liquid medium by shaking the plate with glass beads (ø 2-3 mm) and resuspending the spores adhering on the glass beads in water. These resuspended spores of the actinomycetes and pre-cultures of *S. aureus* were diluted ($\approx 10^8$, $\approx 10^6$, $\approx 10^4$ colony forming units (cfu) per Petri dish) and layered in soft agar on Petri dishes (5 cm diameter) containing LB (*S. aureus*) or M65 medium (actinomycetes).

15 µl of the vetiver oil, pure or diluted in dimethyl sulphoxide (DMSO) was added to filter paper discs (6.5 mm diameter), resulting in total amounts of around 15, 7.5, 0.8, 0.08, 0.008 or 0 mg vetiver oil per paper disc. Each filter paper was transferred to a separate Petri dish. The Petri dishes were incubated at 37°C and the inhibition zones were measured after 1 - 6 days.

Influence of the vetiver oil concentration on the red color production of the actinomycete 3 in liquid culture

Spores of the actinomycete 3 were transferred to M65 liquid medium. To 1 ml of the culture, 10 µl diluted vetiver oil was added and the tubes were

incubated at 37°C in a rotation wheel.

After incubation for 2 - 3 days at 37°C, the cultures were harvested by centrifugation (5 min at 14'000 rpm) and the supernatant was measured at OD 538 nm.

Thin layer chromatography (TLC)

Extracts were separated on silica gel plates (Silica gel 60 F_{254}, MERCK), using the solvents hexane, isooctane, isopropanol, diethyl ether, acetonitrile, methanol, methylene chloride, chloroform, toluene or the solvent combinations chloroform-methanol, ethyl acetate-hexane, petroleum ether-diethyl ether-acetic acid.

To detect the different compounds, UV (254 nm), iodine vapor, and different staining solutions like anisaldehyde-acetic acid-sulfuric acid (2 ml : 40 µl : 20 µl), vanillin-sulfuric acid (1 - 5 g vanillin per 100 ml sulfuric acid), phosphomolybdic acid and 2,6-dichlorophenol-indophenol (0.1 % in ethanol) were tested (Cosicia 1984; Gibbons and Gray 1998; Merck 1970). To stain, the plates were sprayed with a staining solution and, after drying, heated until the color spots were visible. If not mentioned otherwise, UV, iodine vapor and anisaldehyde-acetic acid-sulfuric acid staining were used consecutively to detect the vetiver oil.

As spots often overlapped, it was not possible to determine the midpoint of the spot to calculate the Rf value. Therefore, we describe spots with zones (Rf*(start)-Rf*(end)).

$$Rf^* = \frac{\text{distance of zone (start or end) from origin}}{\text{solvent front distance from origin }^{1}}$$

[1]: when hexane and chloroform were used as consecutive solvents, the running front of chloroform was used as reference.

Due to the different amounts of oil loaded and slightly different running conditions, Rf* values varied up to 10 % within the experiments.

Gas chromatography

A Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector and a DB-WAX column (25 m x 0.32 mm, 0.25 µm film thickness, J & W Scientific, FISONS) was employed. Hydrogen was used as carrier gas at a rate of 2 ml min^{-1}. The samples were injected in the splitless mode. Injector and detector temperatures were maintained at 200°C and 300°C, respectively. The column oven temperature was programmed for 80°C (after 4 min) to 220°C at 2.5°C min^{-1} and the final temperature was held for 20 min. Peak areas and retention times were measured by electronic integration (Hewlett Packard 3390A integrator).

Before injecting in the GC, internal standard was added to all samples resulting in around 0.01 - 0.05 µg dibutyl phthalate per injection.

Fractionating of the sample (vetiver oil or extract) in hydrocarbons, acids and remaining components (alcohols, ketones,...)

To obtain hydrocarbons, a hexane extract of vetiver roots was loaded on a silica column. The eluent contained the hydrocarbons (Croteau and Ronald 1983; Kubeczka 1985) whereas the rest stayed on the silica column.

The acids were separated by extracting a hexane extract with base (1M KOH) (Kubeczka 1985; Shibamoto and Nishimura 1982). The KOH phase was acidified with concentrated HCl and extracted with hexane.

Scrape-off experiment

A hexane extract, after extraction of the acid compounds with 1M KOH, was separated by TLC with hexane and chloroform. On both sides of the TLC plate, a strip was cut off and stained. The rest of the plate was, with the help of these two lanes, divided in zones, starting at the origin (sample 1) up to a zone between the hexane and chloroform running fronts (sample 34). The silica of each of these samples was scraped off, eluted with MTBE, and the eluates were analyzed by GC and TLC.

RESULTS AND DISCUSSION

In this chapter, we describe experiments to optimize analysis methods for small samples containing less than 1 mg vetiver oil. This was necessary to enable rapid analysis and screening of large numbers of small plant samples, providing information about the quantity and the quality of the vetiver oil.

Description and optimization of analysis methods

Olfactive detection

As the vetiver oil is volatile, the nose can be used to determine whether plant samples contain vetiver oil. No extraction is necessary and detection of the oil is easy and fast. In a small experiment, the variation in sensitivity within a test panel of 12 persons was determined. 5 µg vetiver oil was still detected by all test persons. However, fewer persons detected lower amounts of oil and 0.005 µg vetiver oil was detected by only 2 persons (Table 5.2). Results obtained by nose are subjective and depend on the person and on his / her state of health (Gardner and Bartelett 1999). For an untrained person, it is difficult to determine the exact amount or even to recognize the compounds of the oil. Therefore, this analysis method is useful only to indicate whether a sample contains oil. The test is non-destructive and the sample can then be analyzed by other analysis methods.

Bacterial inhibition by vetiver oil

Vetiver oil has antibacterial and antifungal properties (Chaumont and Bardey 1989; Dikshit and Husain 1984; Gangrade *et al.* 1991; Gangrade *et al.* 1990; Hammer *et al.* 1999; Maruzzella and Sicurella 1960). This fact can be used to develop an analysis method to screen plant material or extracts for the presence of vetiver oil.

Table 5.2. Comparison of different analysis methods for vetiver oil

	olfactive detection [a]	inhibition of bacteria [b]	TLC	GC
amount per analysis [µg]	0.005 - 5	5 - 80 [c]	5 - 10	0.5
simultaneous samples	1	many	≈ 15 / plate (10 cm wide)	1
time needed	≈ 1 min + break before next sample	3 - 5 days (growth and analysis)	2 h (separation and staining)	1.5 h (run) + data analysis
detection of compounds:				
- volatile	+ [a]	[d]	+	+
- non-volatile	- [a]	[d]	+	-
oil changes detectable:				
- quantitative	rough [a]	[d]	rough [e]	+
- qualitative	rough [a]	[d]	rough [e]	+
reproducibility	[a]	low	+	+

[a] : depends on the testing person
[b] : test organism: actinomycete strain 3 (Erismann, unpublished)
[c] : Filter paper disc diffusion method: 80 µg vetiver oil per filter
Inhibition of red color production in liquid cultures: 5 - 50 µg vetiver oil per ml culture
[d] : not known which of the oil compounds inhibit the growth or color production
[e] : several compounds at same spot, therefore small changes are not detectable

Filter paper disc diffusion plate method

The inhibitory effect of different amounts of vetiver oil was tested with the filter paper disc diffusion plate method (Vincent and Vincent 1944). *Staphylococcus aureus*, which is susceptible to vetiver oil (Gangrade *et al.* 1990; Hammer *et al.* 1999), and three pre-selected actinomycetes (Erismann, unpublished data) were chosen to test and to optimize the usefulness of this screening method.

For *S. aureus*, 0.8 mg vetiver oil was the lowest amount of vetiver oil which still slightly inhibited bacterial growth (inhibition zone: ø 7.5 mm) (Table 5.3).

For the actinomycetes 1 and 2 the inhibition zone was measured after 1 day in culture. For actinomycete 3 the analysis was only possible after 2 days. The optimal cell number was around 10^6 cfu per Petri dish. With a higher inoculum,

the filter paper was rapidly overgrown, and with a lower inoculum, the bacteria did not grow well.

We observed that the inhibition zone around the filter paper containing 7.5 mg vetiver oil was larger than that of about 15 mg (non-diluted oil), and that DMSO itself did not inhibit the growth. Apparently, the addition of DMSO to the vetiver oil intensified the inhibitory effect. The lowest oil concentration that still inhibited growth was about 0.08 mg for all three actinomycetes (Table 5.3).

Vetiver oil did not only inhibit the growth of the tested actinomycetes, it also influenced the spore formation of actinomycetes 1 and 2. With 15 and 7.5 mg vetiver oil a spore-less zone was observed after 3 days incubation (Table 5.3). For actinomycete 3, vetiver oil also inhibited the production and excretion of red color to the medium. After 6 days in culture a lighter red zone was still observed with 0.08 mg vetiver oil (Table 5.3).

Table 5.3. Antibacterial activity of vetiver oil and its dilutions tested by filter paper disc diffusion method

	ø inhibition zone [mm] [a]						
vetiver oil [mg]	*S. aureus* overnight	Actinomycete 1		Actinomycete 2		Actinomycete 3	
		1 day	3 days	1 day	3 days	2 days	6 days
≈15 [b]	11	13	11 (13) [d]	11	10	30	28
7.5	10	15.5	12 (14)	16	12 (13)	34	28
0.8	7.5	13.5	8	12.5	7	26	8 [20] [e]
0.08	ni [c]	9.5	ni	9	ni	16	ni [10]
0.008	ni	ni	ni	ni	ni	ni	ni
DMSO	ni	ni	ni	ni	ni	ni	ni

[a] : filter paper ø 6.5 mm
[b] : 15 µl not-diluted vetiver oil
[c] : no inhibition
[d] : ( ) zone with no spores
[e] : [ ] zone with less red color

Correlation of red color production by actinomycete 3 and vetiver oil content in the liquid medium

Since vetiver oil inhibits the red color production by actinomycete 3, we used liquid cultures to measure this inhibition spectrophotometrically. As the analysis method should also be suitable for solvent extracts of vetiver, the effects of hexane, ethyl acetate and MTBE on red color formation were also tested.

In liquid culture, DMSO alone inhibited the red color production. This made it difficult to determine whether the inhibition by vetiver samples was due to vetiver oil or due to DMSO. Since MTBE, hexane and ethyl acetate were not inhibitory, the vetiver oil was dissolved in MTBE for further tests.

After 3 days in culture, no growth was observed with 0.5 mg vetiver oil per ml culture. With 0.05 mg little growth was observed and the medium was light red. It depended on the experiment, whether lower concentrations could still be detected: in some experiments 0.005 mg per ml culture were detectable, whereas in others 0.025 mg did not show any inhibitory effect (results not shown). Therefore the detection limit is between 0.005 and 0.05 mg vetiver oil (Table 5.2). The lack of reproducibility could be explained by the difficulty to inoculate constant numbers of spores and by the observation that on solid medium the phenotype of some colonies changed with time: some remained dark red whereas others did not.

With these experiments, it was shown that growth of the actinomycetes and red color production were inhibited by vetiver oil. However, the amount of oil needed for inhibition of red color production was still high and it is not known which components inhibit the bacteria and whether perhaps other compounds in the plant extract are inhibitors. Summarizing, it is difficult to determine the presence, the amount or the composition of the vetiver oil from the inhibition of bacterial growth or color production. Therefore, at least with the tested strains, this method is not useful to determine quantitative or qualitative changes of the vetiver oil.

Thin layer chromatography (TLC)

TLC is a simple and fast analysis method and several samples can be analyzed simultaneously on one TLC plate.

Staining

As the vetiver oil itself is colorless and as only a few components are detectable by UV (vetivones (Andersen 1970)), a staining method had to be chosen. Several detection methods for terpenoids have been described (Cosicia 1984; Croteau and Ronald 1983; Gibbons and Gray 1998; Merck 1970). However, staining methods are seldom very specific. They rarely detect compounds solely of the given class and often will not detect every single compound (VanMiddlesworth and Cannell 1998). Therefore, different detection methods like anisaldehyde-acetic acid-sulfuric acid, vanillin-sulfuric acid or phosphomolybdic acid staining for terpenoids, 2,6-dichlorophenol-indophenol staining for organic acids, UV(254 nm) and iodine vapor were tested.

After anisaldehyde-acetic acid-sulfuric acid staining, pink, dark and light violet, yellow and brown spots appeared. After vanillin-sulfuric acid staining pink and brown spots were visible and phosphomolybdic acid staining resulted in green-black spots on a yellow background (results not shown). The three staining methods resulted in similar spot patterns. Due to the bigger color range of the spots, the anisaldehyde-acetic acid-sulfuric acid staining was chosen for further use. With 2,6-dichlorophenol-indophenol, the lowest smear of the solvent extract could be identified as acidic compounds (result not shown). The detection by UV and by iodine vapor resulted in only a few spots (Figure 5.1a, b). Since these two detection methods did not influence the staining with anisaldehyde-acetic acid-sulfuric acid and give some additional information, they were used further in combination with the anisaldehyde-acetic acid-sulfuric acid staining (Figure 5.1).

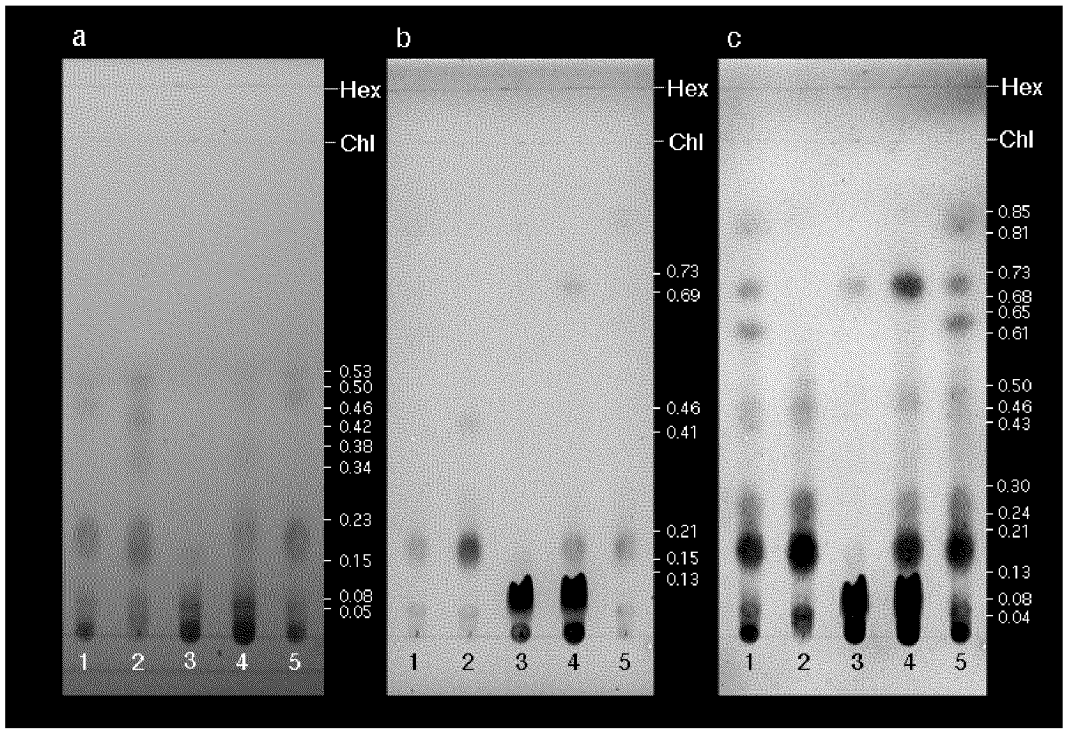

Figure 5.1. Thin layer chromatogram of different vetiver oil extracts. Vetiver oil Bourbon (Givaudan-Roure) (lanes 1 and 5), a distillate of vetiver roots from Java (lane 2), a MTBE extract of the distilled roots (lane 3) and a MTBE extract of vetiver roots from Java (lane 4) were separated on a silica plate with hexane (Hex) and chloroform (Chl) as consecutive solvents. The spots were visualized with UV (254 nm) (a), iodine (b) and stained with anisaldehyde-acetic acid-sulfuric acid (c). The Rf* zones of the spots are indicated on the right side of the plates.

Influence of the mobile phase on the separation

TLC separates the compounds on silica plates according to their relative polarities (Gibbons and Gray 1998). As vetiver oil contains about 300 compounds ranging from polar acids to apolar hydrocarbons, it is not possible to separate all compounds within one run. To obtain maximum information in one run, a series of mobile phases, varying from polar to apolar, were tested.

With an apolar solvent like hexane or isooctane, the major part of the oil remained at the origin and the hydrocarbons run up (Croteau and Ronald 1983). With polar solvents like methanol, diethyl ether, ethyl acetate, acetonitrile and 1-butanol, the major part of the oil was found in the upper half of the plate. The best separation over the whole length of the plate was found with chloroform,

methylene chloride and a combination of hexane-ethyl acetate (7:1) (data not shown). There was tailing of the acid spot with all solvents. By adding acetic acid to the mobile phase (petroleum ether-diethyl ether-acetic acid, 8:2:1), the tailing of the acid could be avoided (Gibbons and Gray 1998). As a consequence, the acid components run with the other oil compounds and disturbed the analysis more than the tailing spot did (data not shown). Therefore, no acetic acid was added to the mobile phase. As the overlapping of the acid spot with the darkest oil spot was smaller after development with chloroform than with methylene chloride or hexane-ethyl acetate, chloroform was used as the mobile phase for TLC in further experiments. However, when hexane and chloroform were used as consecutive solvents to separate vetiver oil, the highest spot could be separated in two spots (Rf*: 0.68 - 0.73, 0.81 - 0.85). Therefore this combination was used in further experiments (Figure 5.1).

Detection limit

To determine the lowest amount of vetiver oil still detectable by TLC, different amounts of a vetiver oil Bourbon (Givaudan-Roure) (0.7/ 1.7/ 3.4/ 5.1/ 6.8/ 8.5 / 10.2/ 11.9/ 13.6/ 15.3/ 17 µg) were developed on a TLC plate (Figure 5.2). With 3.4 µg vetiver oil, faint spots were still detectable by UV. With iodine, 6.8 µg oil was necessary to detect all spots stained with iodine. After staining with anisaldehyde-acetic acid-sulfuric acid, the darkest spot (Rf* 0.13 - 0.21) was faintly visible with 0.7 µg vetiver oil, whereas all spots were faintly visible with 5 µg and from about 10 µg on, all spots were clearly detectable (Figure 5.2, Table 5.2). From 0.7 to 5 µg the increased spot intensity was clearly detectable, but with higher amounts the differences were no longer obvious (Figure 5.2). To determine the amount of oil in an extract, different concentrations of the control have to be added on the same TLC plate since the color intensity varied between individual plates. In conclusion, TLC is useful for a rough analysis of many samples, to determine whether oil is present and approximately how much.

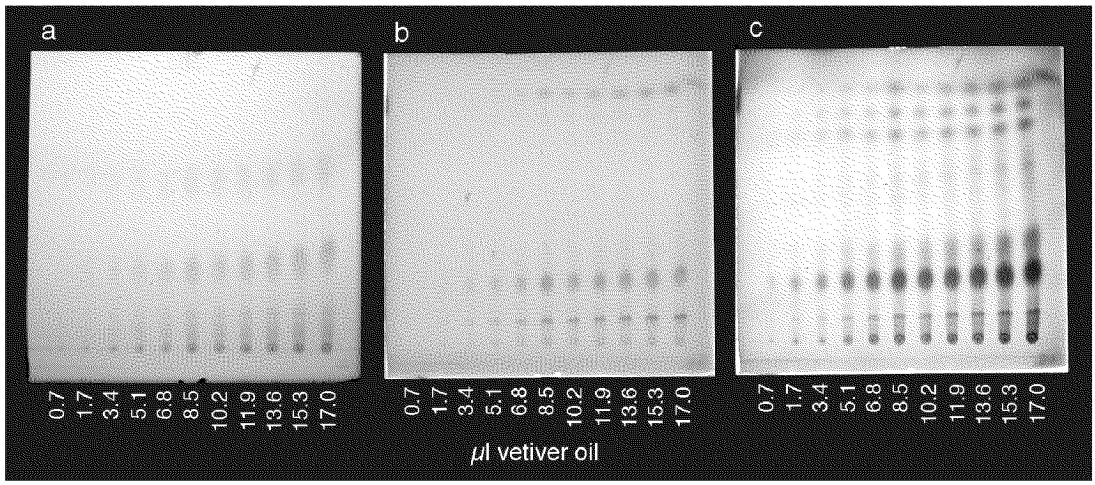

Figure 5.2. Detection limit of vetiver oil by TLC. Different amounts of vetiver oil Bourbon (Givaudan-Roure) were separated on a silica plate with hexane and chloroform as consecutive solvents. The spots were visualized with UV (254 nm) (a), iodine (b) and stained with anisaldehyde-acetic acid-sulfuric acid (c).

Gas chromatography (GC)

Gas chromatography (GC) has been the classical tool for analysis and isolation of the lower, more volatile terpenoids (Banthorpe 1991). With the temperature program used, a complete GC run required 90 min per sample (Table 5.2). The separation was not complete, but by using more shallow temperature gradients, the runs took longer and the separation did not improve. Changes in amount and composition should nevertheless be detectable. To obtain a reasonable chromatogram which shows minor components without overloading the major components, about 0.5 µg vetiver oil should be injected (Table 5.2).

One major problem with GC is that non-volatile compounds cause base line shifting and increased noise. Samples containing non-volatile compounds must therefore be cleaned (column chromatography, TLC, etc.) (Croteau and Ronald 1983) or cleaning runs must be carried out between sample injections.

To compare extract chromatograms, an internal standard must be added. The internal standard should not be too volatile, so that it does not evaporate during concentration of the sample, nor should it be soluble in water (i.e., in the rest water after distillation). Additionally, the internal standard peak should not overlap with sample peaks. At the same time, for TLC the internal standard spot

should run among the oil spots, so that it can be scraped off together with the oil. Of the tested chemicals, dibutyl phthalate and methyl vanillate (4-hydroxy-3-methoxy-benzoic acid-methylester) met these conditions, except that methyl vanillate gave a bright spot on the TLC chromatogram with UV, which disturbed the analysis of the oil. Therefore dibutyl phthalate was generally used as internal standard. If a second internal standard was needed, methyl vanillate was used as well.

GC chromatograms were subdivided in three segments for analysis. Segment A contained mainly hydrocarbons, segment C contained mainly acidic components and segment B contained the remaining components (alcohols, ketones,..) (Figure 5.3). To analyze changes in more detail, segments A and B were subdivided in peak groups (Aa, Ba - Bf).

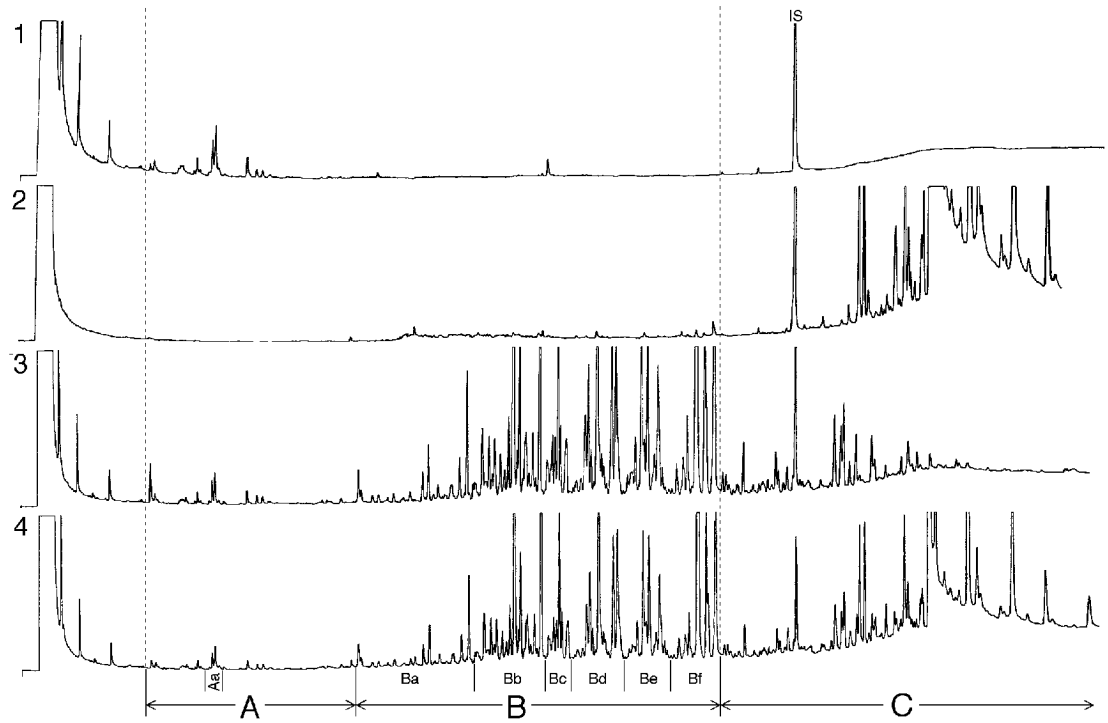

Figure 5.3. Subdivision of a gas chromatogram of vetiver oil. A hexane extract of vetiver roots (4) was fractionated in a hydrocarbon fraction (1), an acid fraction (2) and a hexane extract without acid fraction (3). For the analysis of the data the chromatogram was divided in segment A containing mainly hydrocarbons, segment C containing mainly acids, and segment B containing the remaining components (i.e. alcohols, ketones,...). The segments A and B were subdivided in peak groups (Aa, Ba - Bf). IS: internal standard

Comparison between TLC and GC

Analysis by TLC and GC has the advantage that these methods fractionate as well as detect oil in the test samples. TLC separated the vetiver oil in several spots, while GC resulted in more than 150 peaks. With these two methods it should be possible to determine quantitative and qualitative changes in the oil composition.

Comparison of the separation methods

TLC separates the vetiver oil components according to their polarities (Gibbons and Gray 1998), whereas GC separates them according to their polarities and volatilities (Harborne 1984a). To obtain a correlation between TLC spots and GC peaks, different fractions were analyzed by GC and by TLC. First the vetiver oil was fractionated in hydrocarbons, acids and remaining components (i.e. alcohols, ketones,...) (Figure 5.3). Later, to obtain a more exact correlation between TLC and GC, a hexane extract without acidic compounds was separated on a TLC plate. Scraped off samples were analyzed with both methods (Figure 5.4).

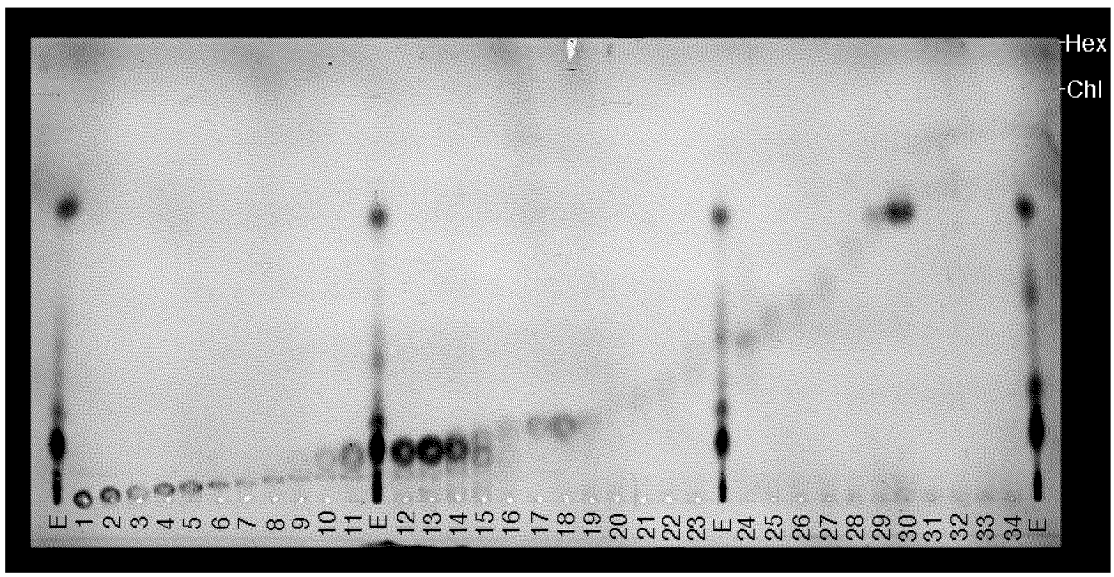

Figure 5.4.A Correlation of thin layer chromatography spots and gas chromatography peaks.

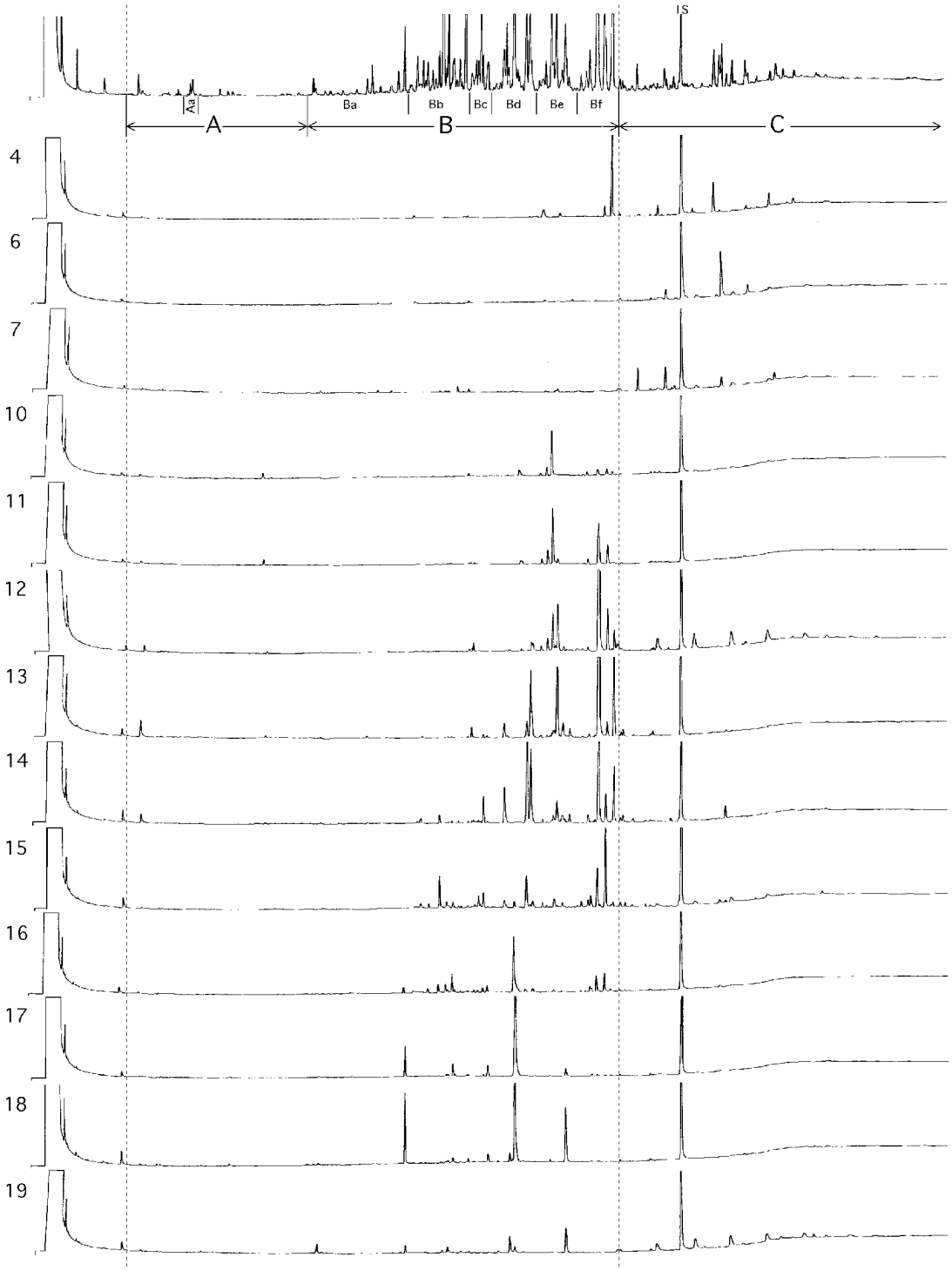

Figure 5.4.B. Correlation of thin layer chromatography spots and gas chromatography peaks.

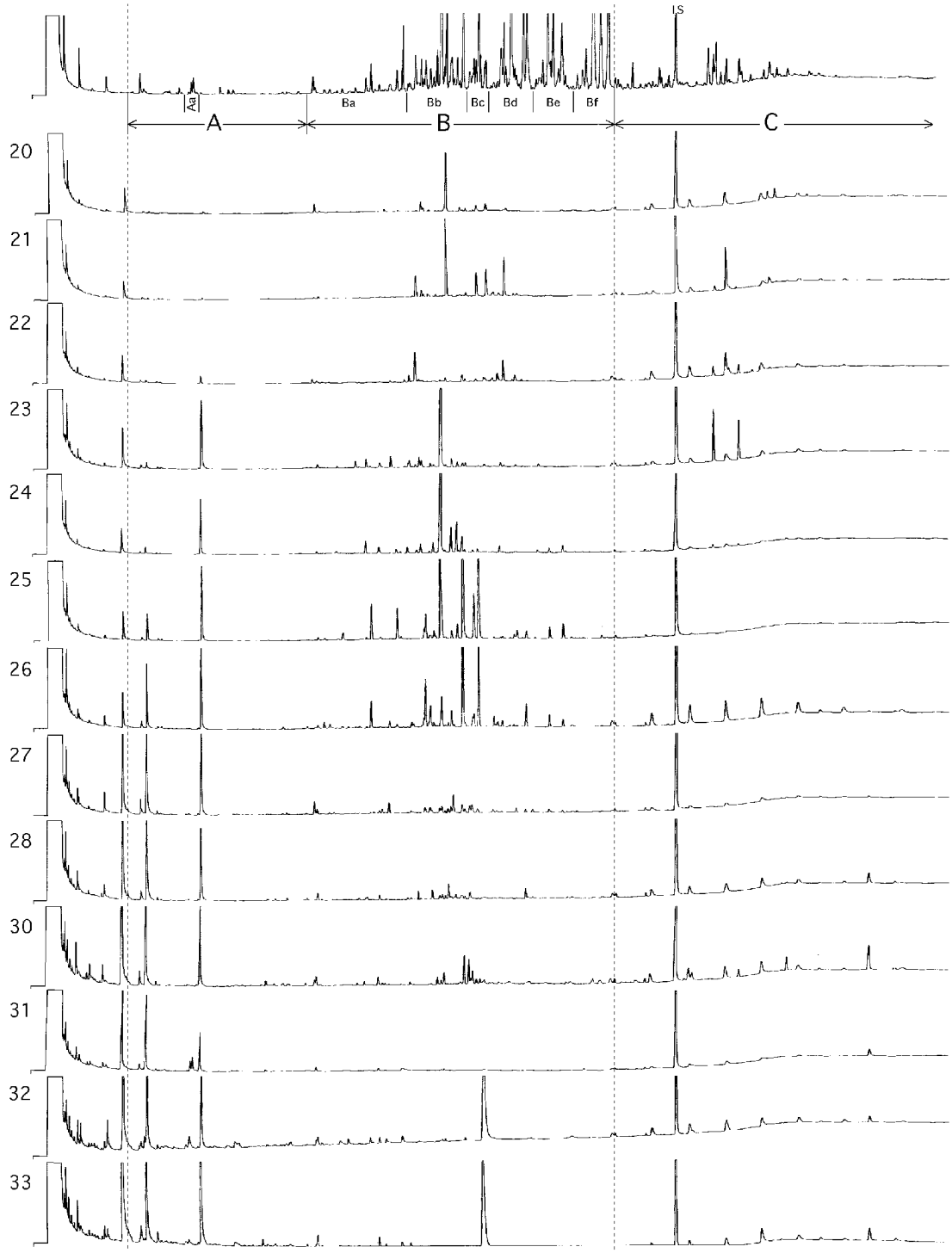

Figure 5.4.B. (continued)

Figure 5.4. Correlation of thin layer chromatography spots and gas chromatography peaks. A hexane extract of vetiver roots without the acid fraction (see Figure 5.3(3)) was separated on a TLC plate and the plate was divided in 34 zones (samples 1 - 34). The re-eluted samples were analyzed by TLC (panel A) and GC (panel B). Of the GC chromatograms only the samples containing peaks are presented. E: vetiver oil; IS: internal standard

The separation in hydrocarbons, acids and remaining components was similar for both methods: the hydrocarbons (segment A, Rf* 0.81 - 0.85) were followed by the alcohols, ketones, ... (segment B, Rf* 0.13 - 0.81) and finally the acids (segment C, Rf* 0 - 0.13) (Figure 5.1, 5.3). However, the subdivision of the GC chromatogram in peak groups did not correlate with the TLC spots (Figure 5.4). These differences in separation between GC and TLC made it possible to separate some compounds by TLC, that have a different Rf*-value, but the same retention time in the GC analysis. For example, in segment Bf of the GC chromatogram, some bigger peaks contained more than one compound (found in samples 4 and 12, Figure 5.4 panel B). The same was found with TLC: scraped off samples showed spots with the same Rf* zone, but they did not contain the same composition or ratio of compounds by GC (i.e. sample 10-14). Therefore, we concluded that either not all vetiver components are stained by anisaldehyde or some are masked by more strongly staining compounds.

Comparison of detection methods

Another difference between TLC and GC was the detection method. GC detects volatile compounds, whereas on TLC plates all compounds which are visualized by UV, iodine, anisaldehyde or other staining methods, can be detected. Since anisaldehyde-acetic acid-sulfuric acid stains many other compounds like sugars, phenols and steroids besides terpenoids (Gibbons and Gray 1998), some scraped off samples (i.e. samples 1 - 3) of TLC spots showed no GC peaks (data not shown).

A violet spot (Rf* 0.67 - 0.77), that could be observed by TLC, was separated during scraping off in two samples (29 and 30). Sample 30 showed some peaks in the GC analysis, but sample 29 did not. Therefore, it can be concluded that the compounds giving rise to the violet color in sample 29 are either not volatile or not detectable with the GC method.

Detection of qualitative changes

The scrape-off experiment showed that the separation of the oil is not complete with either TLC or GC. This is especially so for TLC, where a few

spots are detectable while the oil contains more than 300 components. To see whether these two analysis methods are sufficient to detect qualitative changes, different commercially available vetiver oils as well as a distillate and a hexane extract of our roots from Java were analyzed. Since the vetiver oil composition depends on the type and origin of the plant material (Dethier *et al.* 1997), the harvesting time, the treatment of the roots and the distillation conditions (Anonymous 1976), differences in oil compositions were expected.

With both methods differences were observed (Figure 5.5). The hexane extract was the only sample showing an acid spot (Rf* 0 - 0.13) and many peaks in segment C. In our distillate, fewer acids were found. This was expected, since by distilling with a phosphate buffer (pH 8) fewer acids were extracted (Chapter 6). The other oil samples had a few peaks in this segment, but no acid spots were recognizable on TLC.

In the hexane extract and the distillate of roots from Java, the highest spot (Rf* 0.81 - 0.9) was not detectable. Moreover, fever segment A hydrocarbon peaks were found in the GC chromatograms. The two extracts contained only the Aa peaks whereas the other vetiver oils contained also other segment A peaks. The intensity of the hydrocarbon spot correlated well with the area and the amount of the peaks in segment A. For example vetiver oil Bourbon (Givaudan-Roure), Bourbon (Elixisis) and from El Salvador (PRIMAVERA Live) resulted in a fainter TLC spot, showing fewer segment A GC peaks, whereas the “artificial vetiver oil” resulted in a strong TLC spot, containing also a large number of segment A peaks (Figure 5.5).

Except for the “artificial vetiver oil”, all oils contained components running between Rf* 0.15 and 0.30 on TLC. In the “artificial vetiver oil”, these spots were completely absent and no segment B peaks were seen in the GC chromatogram. TLC of Peti's vetiver fragrance showed one TLC spot and GC showed peaks in segment Ba - Bc, but the main TLC spots and the corresponding GC peaks in segments Bd - Bf were absent. Within the Rf* zone 0.5 - 0.8, the TLC pattern of the oils varied, but without scraping off the TLC spots within this zone and analyzing each of these by GC, it is not possible to find the correlation between the TLC spot pattern and changes within the GC chromatogram.

With both methods it was possible to detect changes in oil composition, with TLC only roughly and with GC more exactly. When changes of the oil composition have to be determined more precisely, a combination of different chromatographic methods (Wolf 1996) or a high-resolution chromatographic separation (i.e. gas chromatography-tandem mass spectrometry or comprehensive gas chromatography (GCxGC)) (Cazaussus *et al.* 1988; Marriott *et al.* 2000; Sellier *et al.* 1991) of the crude oil will be necessary. However, to compare miniaturized extraction methods and to pre-screen regenerated *in vitro* plantlets, a one-step analysis that generates significant information is highly desirable. Therefore, TLC and GC are useful analysis methods for this purpose.

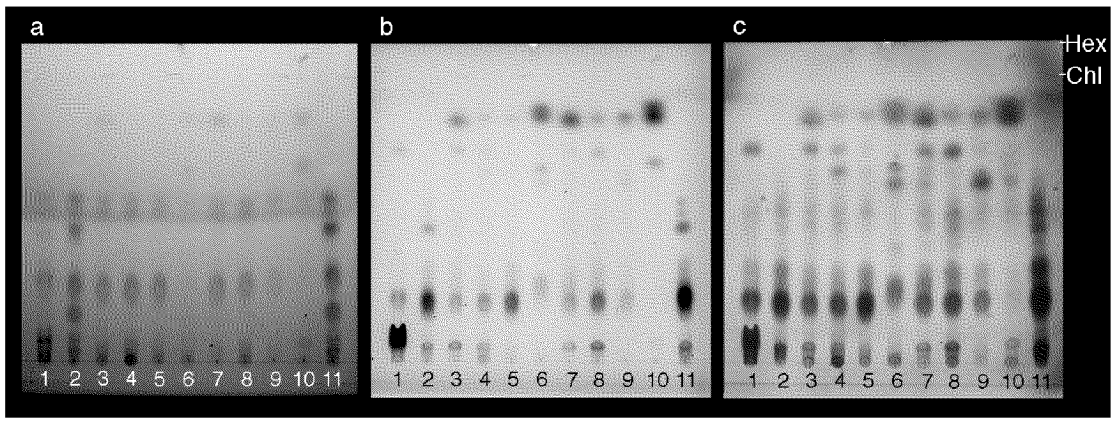

Figure 5.5.A

Figure 5.5. Thin layer chromatography and gas chromatography of commercially available vetiver oils. Different commercially available vetiver oils (samples 3 - 10) as well as a distillate (samples 2 and 11) and a hexane extract (sample 1) of our roots from Java were separated by TLC (panel A) and GC (panel B).
Panel A: TLC was carried out on a silica plate with hexane (hex) and chloroform (chl) as consecutive solvents. The spots were visualized with UV (254 nm) (a), iodine (b) and stained with anisaldehyde-acetic acid-sulfuric acid (c). Panel B: GC analysis. IS: internal standard
sample 1: hexane extract of roots from Java
sample 2: distillate of roots from Java
sample 3: vetiver oil from La Reunion (Seidenberg Collection)
sample 4: vetiver oil Bourbon (Givaudan-Roure)
sample 5: vetiver oil Bourbon from West India (ELEXISIS)
sample 6: Peti's vetiver fragrance (Duftschloss zum Wolkenstein)
sample 7: Peti's vetiver oil (Duftschloss zum Wolkenstein)
sample 8: vetiver oil from El Salvador (PRIMAVERA Life)

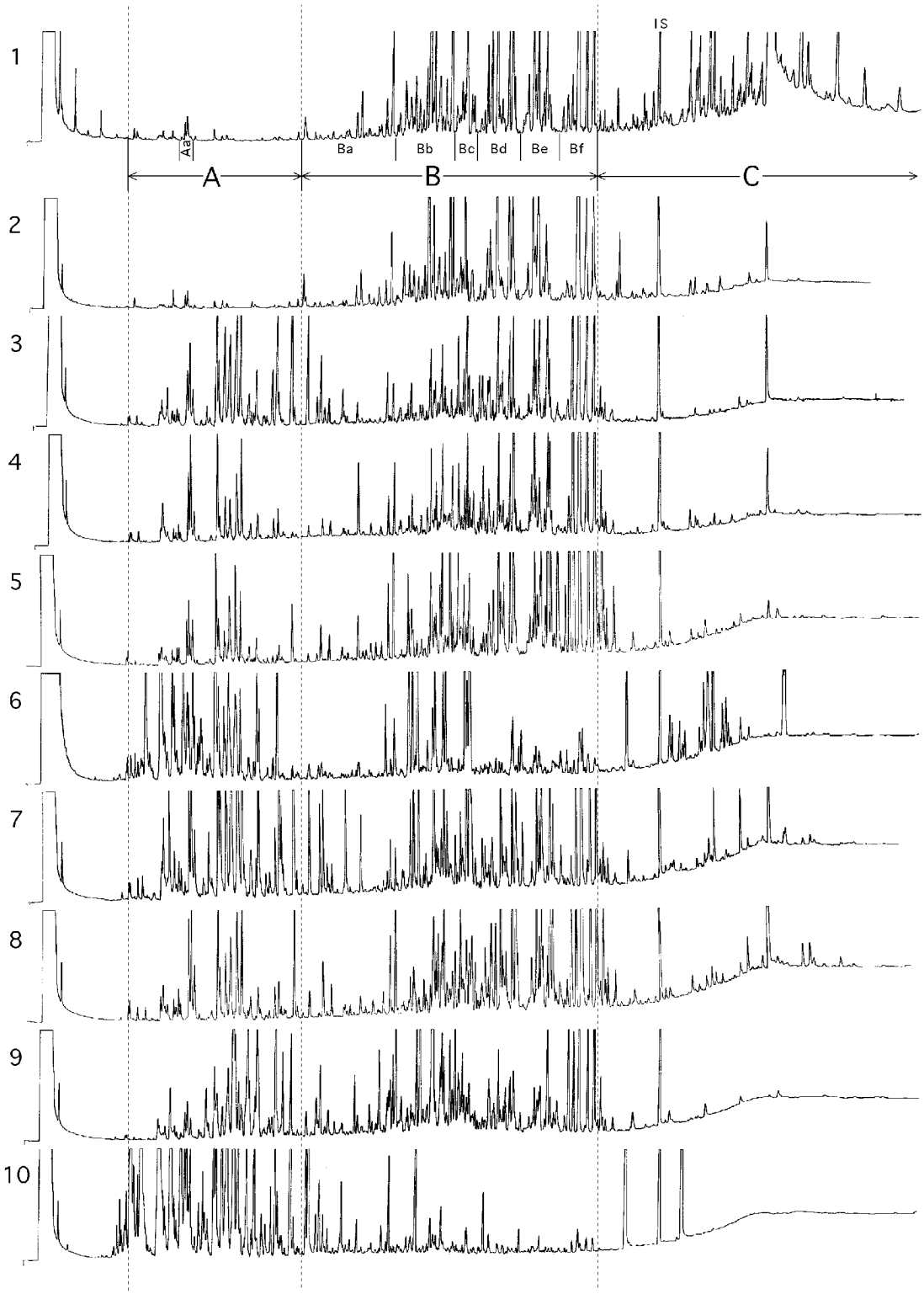

Figure 5.5.B.

Figure 5.5. (continued)
sample 9: vetiver oil (MIGROS)
sample 10: “artificial vetiver oil” (neoLab, Labor Spezialprodukte)
sample 11: only on TLC plates: 1.5 times the amount of sample 2 loaded

Artifact production

During preparation for TLC the vetiver oil components are exposed to air and oxygen sensitive compounds could therefore be decomposed. This could be the reason why some scraped off samples showed additional spots on TLC and additional peaks in the GC chromatograms (Figure 5.4, i.e. samples 23 - 34). Moreover, during the evaporation of the solvent, volatile hydrocarbons may be lost (Kubeczka 1985).

Similarly, the hot injection port of the GC may introduce several artifacts like isomerization, dehydration and polymerization (Croteau and Ronald 1983). Such artifacts can influence the identification of the separate compounds.

Comparison of the different analysis methods

To optimize the extraction of the vetiver roots, TLC is useful for an initial analysis to detect non-volatile compounds and to roughly determine the amount of oil in individual samples, to estimate the amount of sample necessary for GC. For the more exact analysis, GC should be used.

For the oil induction experiments, the nose provides a very useful and rapid first analysis (Table 5.2), but for yield determination and identification of the induced compounds, TLC or GC should be used. Also here, TLC is useful to detect non-volatile compounds and to estimate the amount and the composition of the oil, but GC is necessary as a final and definitive method.

Finally for the pre-screening of the plantlets only a small amount of oil is available. GC is best suited for this purpose.

ACKNOWLEDGEMENTS

The Vetiver roots were provided by Mr. Heini Lang, Jakarta. This work was supported by Givaudan-Roure Forschung AG Dübendorf, Switzerland and by the Swiss Federal Office for Economic Policy, project no. 2561.1 of the Commission for Technology and Innovation.

Chapter 6: Comparison of Vetiver oil extraction by water distillation and solvent extraction

Ruth E. Leupin, Charles Ehret, Karl H. Erismann and Bernard Witholt

ABSTRACT

The essential oil of *Vetiveria zizanioides* was extracted by water distillation and solvent extraction, to optimize the methods and to compare the resulting extracts.

With water distillation, only volatile compounds are extracted from the roots, however due to the necessary cooling system, only a few samples can be distilled simultaneously and the process is time consuming. By using 0.5 M phosphate buffer at pH 8 and by rinsing the cooler with solvent, the time necessary for the distillation of 6 g dry roots was reduced from an initial 3 days (3 litre distilled water) to about 5 hours (100 - 300 ml distilled water).

With solvent extraction at room temperature, many samples can be extracted in parallel. However, non-volatile compounds are also extracted from the root material.

Analysis by gas chromatography showed that similar amounts of the volatile components were extracted with hexane, methyl *tert*-butyl ether, ethyl acetate and ethanol. Hexane extracted less of the non-volatile compounds than did the other solvents and was selected as preferred extractant. Although the hexane extract contained less of the alcohols and hydrocarbons and more acidic and non-volatile compounds than the phosphate buffer distillate, the gas chromatograms of the distillate and the hexane extract were comparable. Thus, we concluded that hexane extraction of small plant root or tissue samples permits an adequate initial analysis of the oil composition.

INTRODUCTION

Vetiver oil is an essential oil from the roots of the tropical grass *Vetiveria zizanioides*. The vetiver plant is harvested after 15 - 22 months and the oil is extracted from the roots by steam distillation (Roth and Kornmann 1997; Weiss 1997a). Depending on the vetiver variant, the yield varies between 0.1-3.3 % oil (Akhila *et al.* 1981; Anonymous 1976; Roth and Kornmann 1997). It contains more than 300 bicyclic and tricyclic sesquiterpenoids (hydrocarbons, alcohols,

ketones, esters, aldehydes and carboxylic acids) (Akhila *et al.* 1981; de Guzman and Oyen 1999; Roth and Kornmann 1997; Vietmeyer and Ruskin 1993).

Several methods have been described to extract essential oils from plant material, including steam or water distillation, solvent extraction, enfleurage, expression, supercritical carbon dioxide extraction and microwave extraction (Craveiro *et al.* 1989; Roth and Kornmann 1997; Weiss 1997b). The composition of the resulting oils can vary significantly (Boutekedjiret *et al.* 1997; Pino *et al.* 1996; Scheffer 1996; Simándi *et al.* 1999). Depending on the plant material and the use of the oil, different extraction methods are in use. In industry, vetiver oil is produced by steam distillation, although other methods like solvent extraction (Anonymous 1976; Hegnauer 1986; Naves 1974; Weiss 1997a) and supercritical fluid extraction have also been used (Blatt and Ciola 1991; Weiss 1997a).

As the vetiver oil is a valuable raw material in perfumery, new vetiver variants with a higher essential oil yield or a different odor tonality (another ratio of the different components) are of interest. New variants have been obtained by traditional breeding (Gupta *et al.* 1983; Lal *et al.* 1998; Sethi 1982; Sethi and Gupta 1980). Since several vetiver cultivars do not flower, there have also been attempts to produce somaclonal variants by regenerating plantlets via tissue culture (Chapter 3) (George and Subramanian 1999; Keshavachandran and Khader 1997; Leupin *et al.* 2000; Mathur *et al.* 1989; Mucciarelli *et al.* 1993; NaNakorn *et al.* 1998; Sreenath *et al.* 1994).

It takes at least 15 - 22 months until such plantlets contain the complete vetiver oil, and it would therefore be advantageous to assay regenerated plantlets earlier for changes in oil composition to eliminate poor oil producers and thus reduce the space necessary to cultivate only the most promising plants. Therefore, a suitable method to extract oil from a large number of small samples has to be developed.

In this chapter, water distillation was optimized to reduce the distillation time, different solvents were compared for extraction at room temperature and finally, the optimized distillation and solvent extraction methods were compared.

MATERIAL AND METHODS

Plant material

The dry vetiver roots were provided by Mr. Heini Lang, Jakarta. For the extraction experiments, the roots were cut in 5 mm pieces. Fresh roots were harvested from the *Vetiveria zizanioides* from Java. The plants from our stock were grown outside during summer and in a greenhouse at 15°C in winter.

Distillation

Dry or fresh vetiver roots (5 - 6 g) were extracted by water distillation in an oil bath at 140°C (Figure 6.1). To examine the influence of the pH on the distillation, water (H_2O) and phosphate buffer (P-buffer, 0.05 M and 0.5 M) at different pH (4.6, 7, 8, 9) were used to distill the essential oil. After every fraction of 100 ml distilled water, another 100 ml pre-warmed water was added to the roots. Depending on the experiment, up to 30 fractions of 100 ml distilled water were collected. Since the distillation was stopped overnight, longer distillation experiments were divided in batches of at most 10 fraction per day. The 100 ml fractions and the residual water or P-buffer, which remained after distillation, were extracted with 10 ml methyl *tert*-butyl ether (MTBE) (sample 1 - x, sample R). To extract all acids, the residual water or buffer was acidified with concentrated HCl and extracted a second time with 10 ml MTBE (sample R acid). The distilled roots were extracted overnight in 50 ml MTBE at room temperature (sample roots). All samples were analyzed by GC.

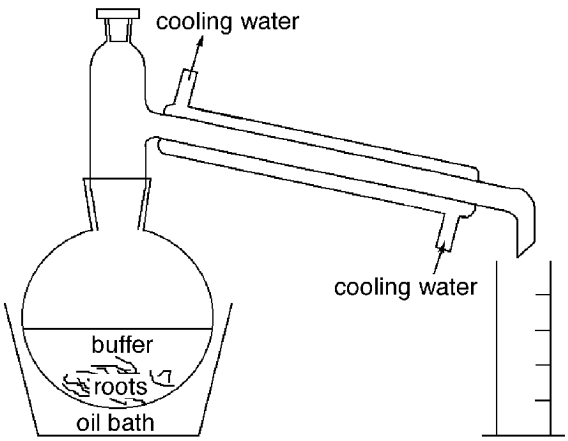

Figure 6.1. Distillation apparatus used in this study to distill vetiver roots.

Solvent extraction

5 g vetiver roots, pre-wetted with 10 ml water or dry, were extracted with 50 ml of different solvents (hexane, ethyl acetate, MTBE, ethanol). The samples were stirred overnight at room temperature. After removing the solvent (extract 1), new solvent (50 ml) was added. After another 24 hours stirring, the extract was again removed (extract 2). This was repeated once or twice more (extract 3, extract 4).

Comparison of distillation and solvent extraction

Hexane extract: 10 g dry roots pre-wetted with 20 ml water were extracted 3 times overnight at room temperature with 100 ml hexane each time (hexane extract 1, 2, 3).

Distillation: Dry vetiver roots (5 g), hexane extracts (50 ml of each hexane extract 1, 2 and 3) and the extracted roots were distilled in 0.5 M P-buffer (pH 8). The hexane from the hexane extract was distilled off first (sample hexane). After every 100 ml distilled water fraction, the distillation was stopped and the cooler was rinsed twice with 10 ml MTBE (sample 1a /1b). The 100 ml distilled water fraction was extracted three times with 10 ml MTBE (sample 1, 1', 1"). After three fractions of 100 ml distilled water (sample 1, 2, 3), the distillation was stopped. All samples were analyzed by GC.

Gas chromatography

A Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector (FID) and a DB-WAX column (25 m x 0.32 mm, 0.25 µm film thickness) was employed. Hydrogen was used as carrier gas at a rate of 2 ml min^{-1}. The samples were injected in the splitless mode. Injector and detector temperature were maintained at 200°C and 300°C, respectively. The column oven temperature was programmed for 80°C (after 4 min) to 220°C at 2.5°C min^{-1} and the final temperature was held for 20 min. Peak areas and retention times were measured by electronic integration (Hewlett Packard 3390A integrator).

Before injecting in the GC, internal standard (dibutyl phthalate) was added to all samples. To compare the composition of the extracts, the GC chromatogram (Figure 6.2) was divided in three segments (A, B, C). Segment A contained mainly hydrocarbons, segment C contained mainly the acidic compounds and segment B contained residual compounds (alcohols, ketones,...) (Chapter 5). To detect the effects of the extraction methods on the oil composition in more detail, the segments A and B were subdivided (Aa, Ba - Bf; Figure 6.2). In the following text, X compounds refer to the compounds running within segment X of the GC chromatogram.

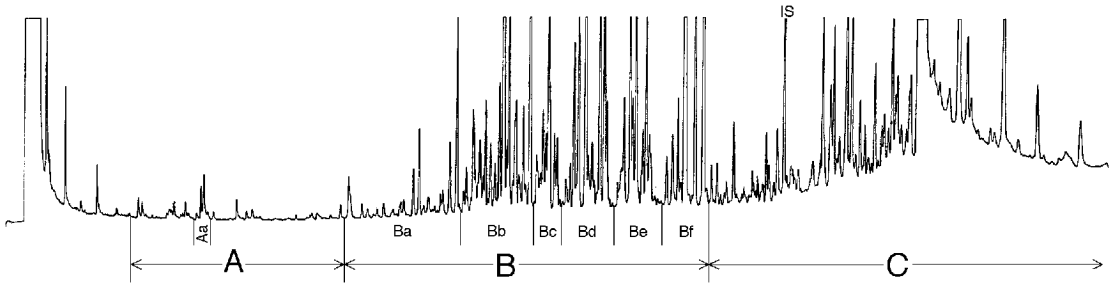

Figure 6.2. Gas chromatogram of vetiver oil on a DB-Wax column. For the analysis of the data the chromatogram was divided in segments A, B and C. Segment A contains mainly hydrocarbons, segment B contains mainly alcohols, aldehydes and ketones and segment C contains mostly acids. The segments A and B were subdivided in Aa and Ba - Bf. IS: internal standard (dibutyl phthalate)

Estimation of the amount of oil based on GC analysis

To determine the amount of any compound Z from a GC chromatogram, the response factor of that compound Z and the internal standard (IS) has to be determined. The FID response is roughly proportional to the number of carbon atoms present in the compounds, however the response is also affected by hetero atoms and various functional groups (Flanagan 1993). Therefore, for each compound the response factor can be different and has to be determined separately.

$$\text{response factor} = \frac{\text{peak area}}{\text{amount}}$$

Using this response factor, it is possible to estimate an unknown amount of compound Z from a GC chromatogram.

$$\text{amount of compound Z [µg]} = \frac{\text{peak area Z}}{\text{peak area IS}} \cdot \frac{\text{response factor IS}}{\text{response factor Z}} \cdot \text{amount IS}$$

As many vetiver oil components are not isolated and the structure not elucidated, it is not possible to determine the response factor for each single component. Since vetiver oil components are expected to be all sesquiterpenoids, they should have C15 and exceptionally C14. The main differences are the number and the position of the functional groups. To simplify the calculation, we assume that all vetiver oil components have the same response as dibutyl phthatate ($C_{16}H_{22}O_4$). This results in:

$$\text{amount of compound Z [µg]} = \frac{\text{peak area Z}}{\text{peak area IS}} \cdot 1 \cdot \text{amount IS}$$

The amount of oil in each segment X was estimated relative to 1 µl internal standard containing 0.025 µg dibutyl phthalate by summing up all approximate amounts of the compounds within segment X.

$$\text{material in segment X [µg]} = \frac{\sum \text{peak areas in segment X}}{\text{peak area IS}} \cdot 0.025\ \text{µg}$$

It has to be noted that the calculated amount is only an approximate amount of oil, based on the assumption described above. Depending on the commercial vetiver oil tested (see chapter 5), the calculated amount was up to 1.5 times of the amount used (results not shown).

Dry weight of the extracts

The solvent of the solvent extracts and of the distilled samples was removed by distillation and by evaporation with nitrogen. The glass vials with extract were kept for several days (typically 3 - 7) in a desiccator to remove traces of solvent or water before the dry weight was determined.

RESULTS AND DISCUSSION

In this study, we describe experiments to optimize the oil extraction by water distillation and by solvent extraction. Water distillation has the advantage that it extracts only volatile compounds, but it is time consuming and laborious (Weiss 1997a): only a few samples can be distilled simultaneously due to the limitation of the necessary cooling system. Solvent extraction at room temperature is a useful option, as no cooling is necessary and many samples can be extracted at the same time. Extracts can be injected directly in the GC for analysis (Chapter 5). One problem of solvent extraction however is that not only volatile compounds but also non-volatile compounds are extracted, which may give rise to problems during subsequent GC analysis (Scheffer 1996).

Distillation

Comparison of water and phosphate buffer distillation

Essential oils are mainly extracted by steam distillation or water distillation. We therefore tested water distillation (H_2O distillation) for three days. During the distillation, the pH of the water dropped to 4.6 (Figure 6.3a). Banthorpe (1991) explained this drop in pH by liberation of the acid material from plant vacuoles during distillation. This drop of pH can, in combination with high temperature, lead to artifacts such as elimination of water, rearrangements of terpenoids or other modifications (Banthorpe 1991). As a remedy, Banthorpe (1991) proposed to conduct steam distillations in the presence of a near neutral buffer. We tested the influence of the pH on the distillation of samples containing phosphate

Figure 6.3. Water distillation of vetiver roots with water or phosphate buffer (0.05M) at different pH. Dry vetiver roots were distilled with water (H_2O, ✱) or 0.05M phosphate buffer (P0.05) at pH 4.6 (O), 7 (◇) and 9 (△) over three days (samples 1 - 10 / 11 - 20 / 21 - 30). To observe pH changes, the pH was measured every day once (a). All samples were analyzed by GC. The yield of material A, B and C was followed during the distillation (b, c, d). Additionally, the dry weights of the samples were determined (e).
app: cooler rinsed with MTBE; R: remaining water or buffer extracted with MTBE; R acid: remaining water or buffer acidified before extraction with MTBE; roots: distilled roots extracted with MTBE

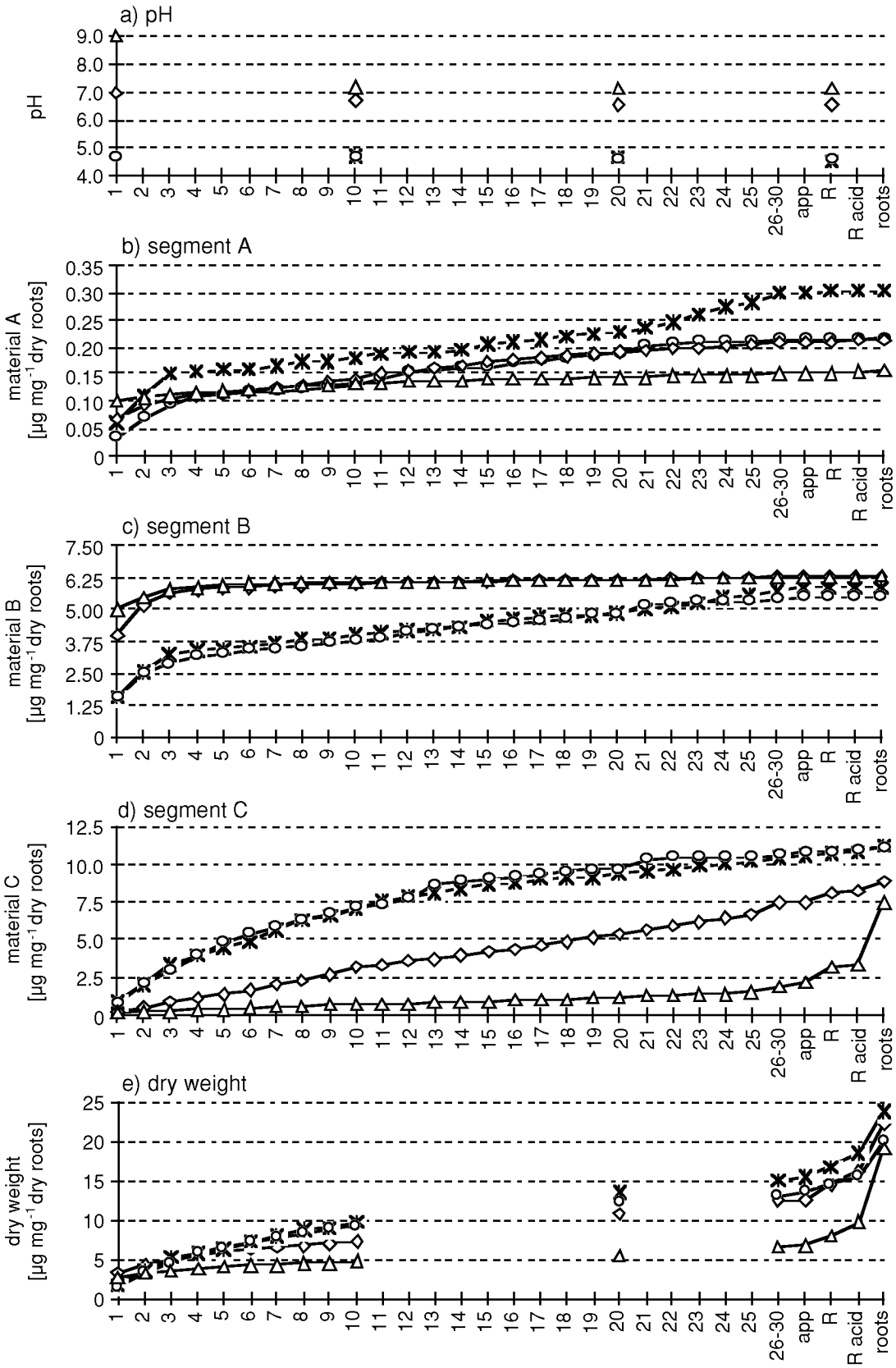
a) pH
pH
9.0
8.0
7.0
6.0
5.0
4.0
b) segment A
material A
[µg mg-1 dry roots]
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
c) segment B
material B
[µg mg-1 dry roots]
7.50
6.25
5.00
3.75
2.50
1.25
0
d) segment C
material C
[µg mg-1 dry roots]
12.5
10.0
7.5
5.0
2.5
0
e) dry weight
dry weight
[µg mg-1 dry roots]
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26-30 app R R acid roots

Figure 6.3.

buffer (P-buffer) at pH 7.0, 8.0 and 9.0. To exclude that observed changes are due to the P-buffer and not to the pH, a P-buffer at pH 4.6 was tested as well. In the first experiments, a 0.05 M P-buffer (P0.05) was used, but the buffering effect was not strong enough and the pH dropped during the distillation (Figure 6.3a). For later experiments a 0.5 M P-buffer (P0.5) was used, which was sufficient to maintain a more constant pH (Figure 6.4a).

We found no differences in extraction behavior for B and C compounds after distillation with H_2O or P0.05/pH4.6. Differences were found only for the material A: H_2O distillation resulted in slightly more hydrocarbons than P0.05/pH4.6 distillation (Figure 6.3). We concluded therefore that observed differences in material B and C are due to the pH and not to the phosphate buffer.

As expected for a distillation with a pH higher than or equal to 7, fewer acidic compounds (segment C) were distilled. Additionally, the B compounds were extracted faster than with H_2O or P0.05/pH4.6 (Figure 6.3). The composition of A compounds was influenced by the pH of the distillation, since distillations with pH lower than 7 or pH 9 resulted in additional peaks which were not present in the other distillates (data not shown). These additional peaks could be due to artifacts, caused by the pH or the long distillation time, or from a selective extraction of some hydrocarbons.

Since after 6 (P0.05/pH9) or 10 (P0.05/pH7) fractions more than 95 % of the material B had already been extracted, subsequent distillations with 0.5 M P-buffer were reduced to 10 fractions (Figure 6.4). Due to the 0.5 M P-buffer the pH during the P0.5/pH7 distillation remained constant and the distillation showed the same behavior as with P-buffers at pH 8 or 9 (Figure 6.4).

Figure 6.4. Water distillation of vetiver roots with phosphate buffer (0.5M) at different pH. Dry vetiver roots were distilled with phosphate buffer (0.5M, P0.5) at pH 7 (◇), 8 (□: 10 samples, ■: 5 samples) and 9 (△, ▲) (samples 1 - 10). The pH of the buffer was measured before and after the distillation (a). All samples were analyzed by GC. The yield of material A, B and C was followed during the distillation (b, c, d). Additionally, the dry weights of the samples were determined (e).
app: cooler rinsed with MTBE; R: remaining water or buffer extracted with MTBE; R acid: remaining water or buffer acidified before extraction with MTBE; roots: distilled roots extracted with MTBE

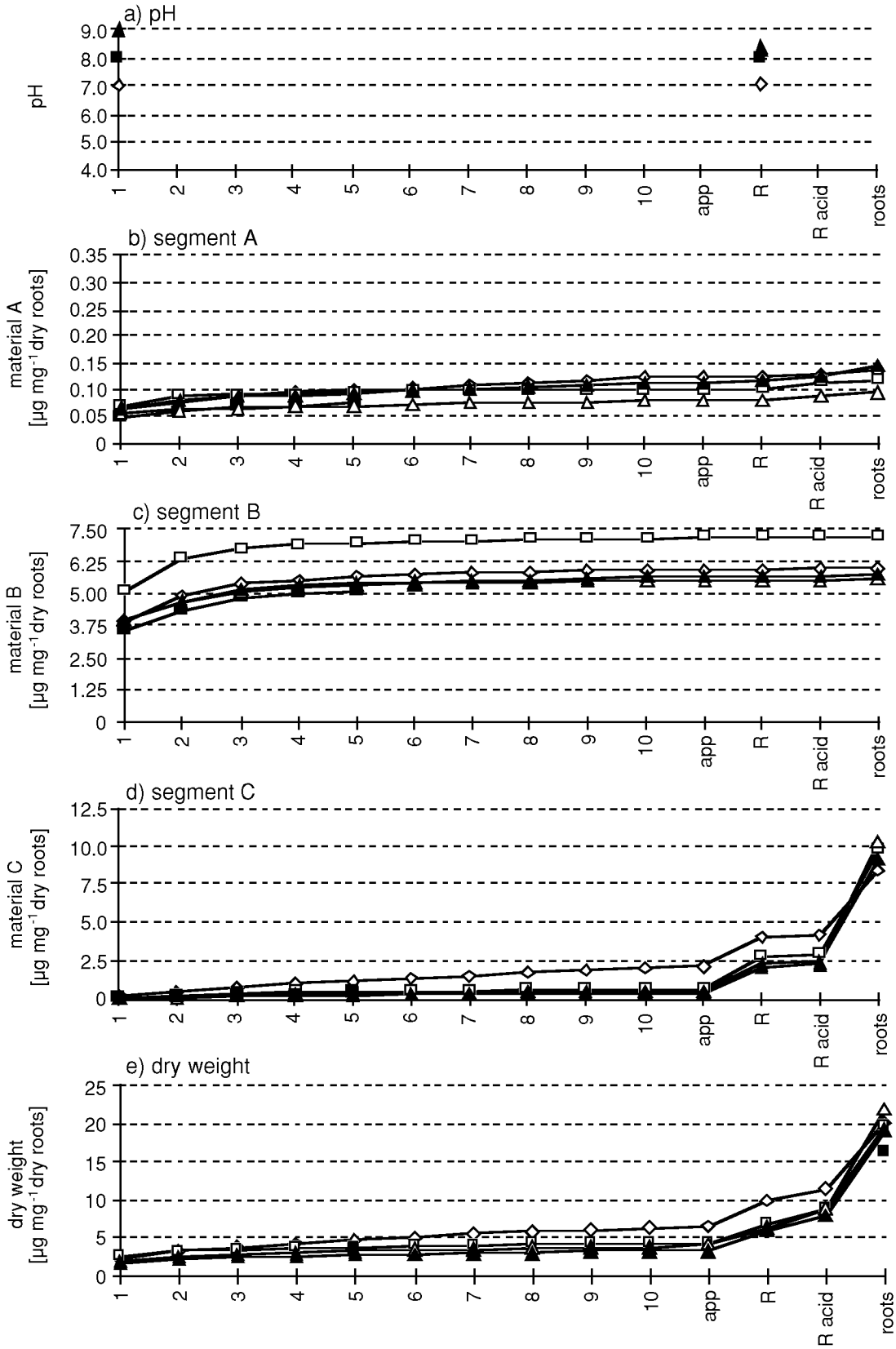
a) pH
pH
9.0
8.0
7.0
6.0
5.0
4.0
b) segment A
material A
[µg mg-1 dry roots]
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
c) segment B
material B
[µg mg-1 dry roots]
7.50
6.25
5.00
3.75
2.50
1.25
0
d) segment C
material C
[µg mg-1 dry roots]
12.5
10.0
7.5
5.0
2.5
0
e) dry weight
dry weight
[µg mg-1 dry roots]
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
app
R
R acid
roots

Figure 6.4.

We tested in more detail whether the pH of the distillation buffer has an effect on the composition of the oil (Table 6.1). Some variations in the fractions Ba - Bf (up to 0.05) were found, but as such variations were also observed for the repetitions of P0.5/pH8 and 9 distillations, they were most probably caused by the plant material and integration errors of the GC and not by the pH of the P-buffer.

Table 6.1. Oil composition of different distillates, as determined by GC

	Ratio of material X normalized to material B [b]								
	H_2O	0.05 M P-buffer			0.5 M P-buffer				
segment [a]		pH 4.6	pH 7	pH 9	pH 7	pH 8 [c]		pH 9 [c]	
A	0.051	0.039	0.033	0.024	0.021	0.014	0.015	0.014	0.020
Aa	0.013	0.010	0.012	0.009	0.008	0.005	0.006	0.006	0.006
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Ba	0.048	0.048	0.033	0.024	0.058	0.060	0.056	0.052	0.054
Bb	0.218	0.223	0.247	0.245	0.264	0.248	0.268	0.222	0.269
Bc	0.064	0.055	0.064	0.091	0.072	0.104	0.074	0.115	0.071
Bd	0.193	0.187	0.178	0.183	0.183	0.182	0.185	0.177	0.178
Be	0.142	0.146	0.143	0.134	0.130	0.129	0.118	0.136	0.139
Bf	0.335	0.336	0.305	0.285	0.294	0.277	0.299	0.301	0.289
C	1.805	1.975	1.177	0.339	0.359	0.093	0.065	0.073	0.085

[a]: Division of the segments see figure 6.2
[b]: To directly compare the oil composition of the different distillates the material A, B, C, Aa and Ba to Bf were normalized to the material B
[c]: two repetitions

Distillation with H_2O, P0.05/pH4.6 and P0.05/pH7 extracted between 12.3 and 14.9 mg vetiver oil per gram roots, whereas P0.05/pH9 and the P0.5 distillations yielded at most 6.3 mg vetiver oil per gram roots (Figure 6.3e, 6.4e). This nicely reflects the observed differences in the material C (Figure 6.3d, 6.4d). The total dry weight including the distilled samples, the extract of the residual water and the root extract was about 20 - 23 mg per gram roots for all distillations (Figure 6.3e, 6.4e). By comparing different commercially available vetiver oils we found that they contain few acidic components (Chapter 5). We concluded therefore that they are not important for the typical vetiver odor. Accordingly, buffered distillation with a pH higher than 7 was chosen for further separations. P0.5/pH8 or P0.5/pH9 showed the same distillation behavior for the A, B and C compounds. To avoid changes of the oil composition due to a too high pH, we chose pH8 for further distillations. Since after 5 distillation

fractions more than 95 % of the total material B was already distilled from the roots, the distillation of dry roots was reduced to 5 fractions (Figure 6.5).

Comparison between dry and fresh root distillation

Since for the analyzes of the oil content, fresh material could also be used, we compared the distillation of fresh and dried roots. Fresh roots from a vetiver plant from Java were harvested. One part was distilled directly (fresh roots) and the other was dried for 7 days at room temperature (dried roots). For these freshly harvested roots, 10 fractions were again collected. The distillation of the fresh harvested roots (fresh or dried) took longer than the previous distillations with dry roots: the distillation of 100 ml water took about 1.5 to 2 hours for each of the first 5 fractions for the fresh harvested roots, whereas for the dry roots only the distillation of the first 100 ml needed up to 2 hours, the following 100 ml fractions requiring only about 0.5 to 1 hour. Therefore, the 10 fractions of the fresh root distillation were distributed over two days, with 5 samples each. It did not only take longer to distill 100 ml water, the oil was also extracted more slowly from the fresh roots (dried and fresh) than from the dry roots (Figure 6.4, 6.5).

Since only minor amounts of oil were obtained by rinsing the cooler after distillation of dry roots, the cooler was initially also not rinsed after distilling fresh roots. However, after distillation of the fresh roots, some oil remained in the cooler, since it smelled strongly like vetiver oil. Therefore, for the experiment with the dried fresh roots, the cooler was again rinsed with 10 ml MTBE after distillation (sample app), yielding about 33 % of the total extracted oil (Figure 6.5). As the oil adhering to the cooler was lost from the fresh root distillation, the dried root and the fresh root distillations could not be compared.

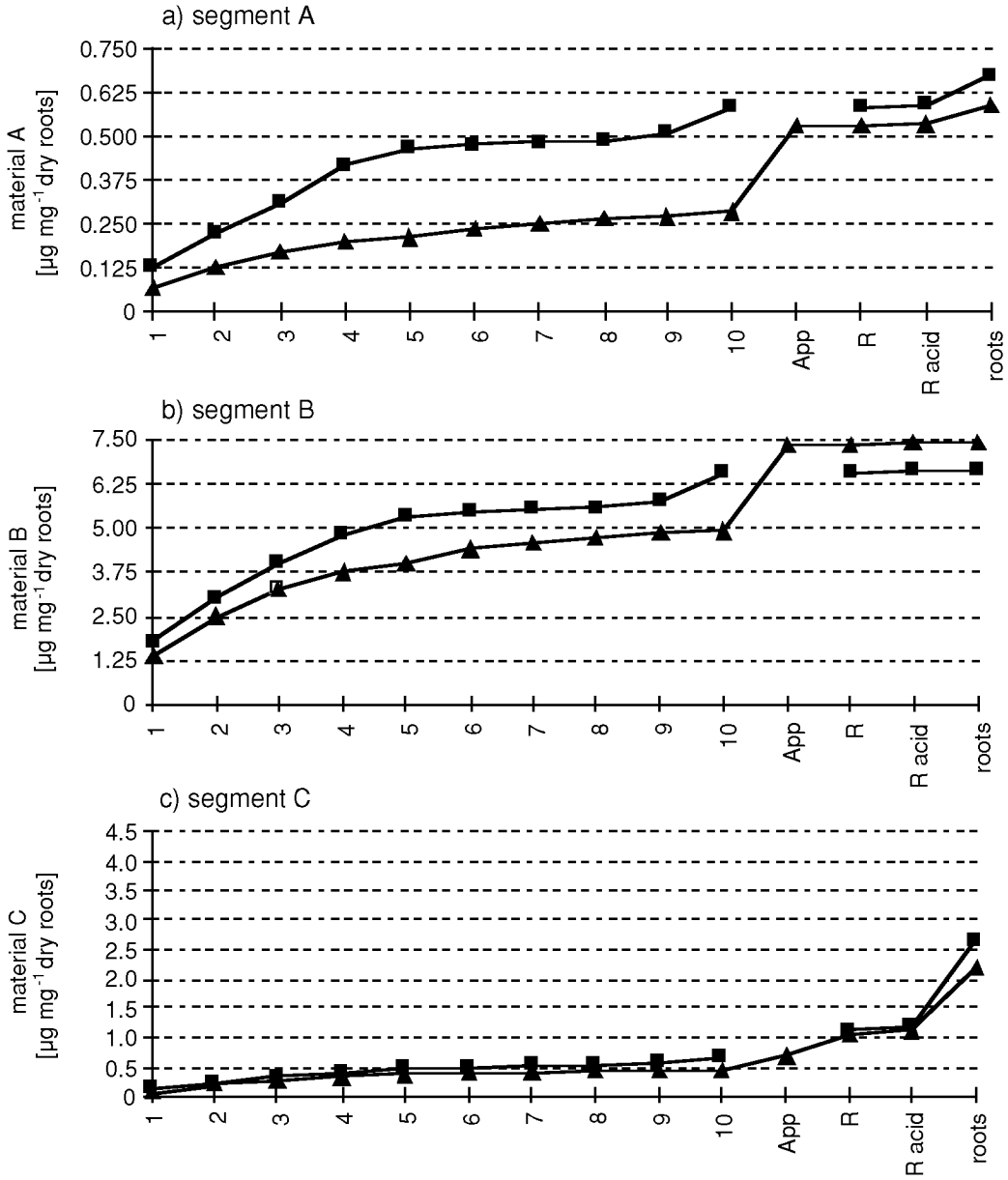

Figure 6.5. Comparison between water distillation of fresh and dried fresh vetiver roots. Fresh (■) and dried fresh vetiver roots (▲) were distilled with phosphate buffer (0.5M, pH8) over two days (samples 1 - 5/ 6 - 10). All samples were analyzed by GC. The yield of material A, B and C were followed during the distillation (a, b, c).
app: cooler rinsed with MTBE; R: remaining water or buffer extracted with MTBE; R acid: remaining water or buffer acidified before extraction with MTBE; roots: distilled roots extracted with MTBE

Influence of glass surface adhesion of vetiver oil on distillation

From the observation with the dried root distillation, we concluded that not all distilled oil remained in the water fraction and a part adhered to the cooler unit. To determine how much oil remained in the 100 ml distilled water and how much adhered to the cooler, the distillation was stopped after removal of every 100 ml water fraction and the cooler was rinsed with 10 ml MTBE (sample xa). For the first two fractions of 100 ml distilled water, up to 60 % of the material A, up to 40 % of the material B and up to 20 % of the material C adhered to the cooler. With the subsequent three distillation steps, less than 10 % of the oil distilled in these fractions adhered to the cooler (Figure 6.6). An additional careful rinsing of the cooler after the five distillation steps resulted in a recovery of about 4 % of the total distilled oil (sample 5b, Figure 6.6).

Since the major part of the oil was distilled in the first few distillation steps, further distillations were reduced to 3 fractions of 100 ml distilled water. To extract the maximal amount of distilled oil, the cooler was rinsed twice with 10 ml MTBE (samples xa and xb) and the distilled water was extracted 3 times with 10 ml MTBE (samples x, x' and x").

Most of the oil components were found in the first extraction of the distilled water and in the first rinsing of the cooler. However, the following two water extraction steps and the second rinsing of the cooler still contained sufficient oil (Figure 6.8) to warrant their collection.

Reduction of distillation steps

For small amounts of roots, it is important to extract the material in as little liquid as possible, else the concentration will be below the detection limit of the GC (Chapter 5). The ratio of the material Ba - Bf in the three distillation steps was similar (data not shown). A reduction in distillation steps would therefore not change the oil composition. By reducing 3 distillation steps to 2 or 1 steps, the oil content would be reduced to 94 % or 80 - 90 % respectively in only 2/3 or 1/3 of the solvent (Figure 6.8).

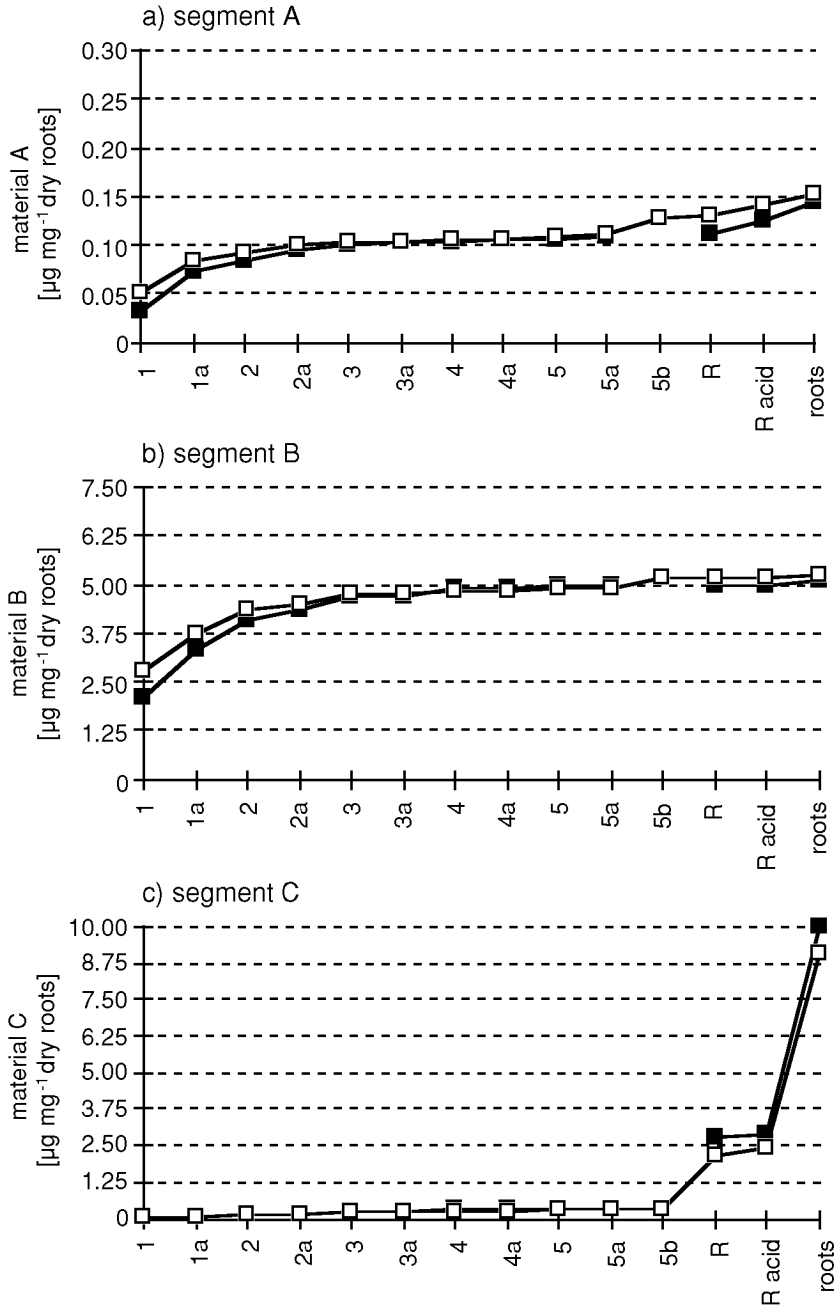

Figure 6.6. Influence of rinsing of the glass material on the amount of vetiver oil extracted by water distillation. Dry vetiver roots were distilled with phosphate buffer (0.5M, pH8) (samples 1 - 5). To obtain all oil distilled within 100 ml water, the cooler was additionally rinsed with MTBE (sample xa). All samples were analyzed by GC. The yield of material A, B and C was followed during the distillation (a, b, c). The distillation was performed twice (□, ■).
5b: the cooler was rinsed a second time with MTBE; R: remaining water or buffer extracted with MTBE; R acid: remaining water or buffer acidified before extraction with MTBE; roots: distilled roots extracted with MTBE

Solvent extraction

Usually, benzene is used to extract vetiver roots (Anonymous 1976; de Guzman and Oyen 1999; Weiss 1997a), but for ecological and health reasons, this solvent was not tested. To find the best solvent to extract the complete vetiver oil from the roots, solvents with different polarity were tested (hexane, MTBE, ethyl acetate and ethanol). Since we had observed that the separation of the solvent and roots after the extraction is easier if the roots were pre-wetted with water, about 20 % (v/v) water was added to the roots before adding the different solvents. To test if this additional water influences the composition of the extract, one batch of roots was extracted with MTBE without adding water (MTBE (dry)). After filtration through two paper filters, the MTBE(dry) extract still contained small particles and had to be filtered through a 0.2 µm filter. The GC analysis showed that the MTBE(dry) extract contained slightly more material A than the MTBE extract with pre-wetted roots, but less material B and C (Figure 6.7). These difference between MTBE(dry) and MTBE extracts could be due to losses during the additional filtration through the 0.2 µm filter or due to a beneficial effect of the water on the extraction of B and C compounds.

Table 6.2. Oil composition of the solvent extracts, as determined by GC

	Ratio of material X normalized to material B [b]				
segment [a]	hexane	MTBE(dry)	MTBE	ethyl acetate	ethanol
A	0.014	0.038	0.023	0.036	0.019
Aa	0.008	0.029	0.015	0.018	0.010
B	1.000	1.000	1.000	1.000	1.000
Ba	0.047	0.054	0.048	0.061	0.065
Bb	0.234	0.223	0.241	0.217	0.180
Bc	0.069	0.068	0.066	0.066	0.056
Bd	0.182	0.193	0.182	0.181	0.208
Be	0.117	0.112	0.109	0.107	0.115
Bf	0.350	0.349	0.355	0.343	0.372
C	2.410	2.543	2.317	2.564	3.713

[a] : Division of the segments see figure 6.2
[b] :To directly compare the oil composition of the different distillates the material A, B, C, Aa and Ba to Bf were normalized to the material B

The solvents hexane, MTBE and ethyl acetate extracted the same material B and C, whereas ethanol extracted less. Ethanol and hexane extracted fewer hydrocarbons (segment A) than the other solvents (Figure 6.7, Table 6.2). For

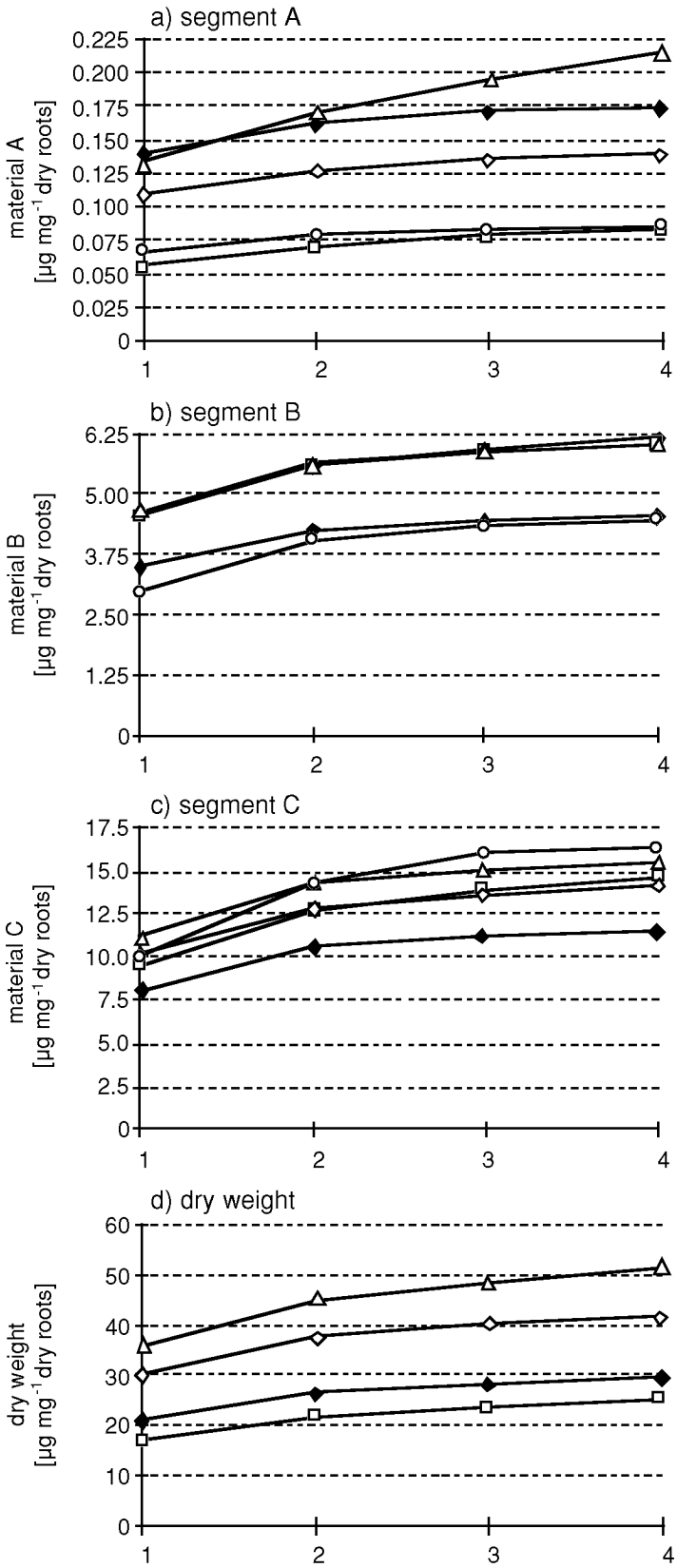

Figure 6.7. Solvent extraction of vetiver roots with different solvents. Before the dry vetiver roots were extracted with different solvents (hexane (□), MTBE (◆, ◇), ethyl acetate (△) and ethanol (○)) the roots were wetted with water (≈20 % v/v solvent), except for MTBE(dry) (◆), to which roots no water was added. The extracts were analyzed by GC. The yield of material A, B and C was followed over the 4 extraction steps (a, b, c). Additionally, the dry weights of the samples was determined (d), except for ethanol, because by evaporating the ethanol-water mix the essential oil would also evaporate.

hexane, MTBE(dry), MTBE and ethyl acetate extracts, the ratio of the material Ba - Bf was similar to that of the distilled oil. For the ethanol extract, the ratio was slightly different (Tables 6.1, 6.2).

Since only the volatile components are detectable by GC analysis, the dry weight of the extract was determined to estimate the amount of the non-volatile in addition to the volatile compounds. The dry weight of the ethanol extract was not determined, as by evaporation of the ethanol-water mix, the essential oil would also evaporate. Since hexane, MTBE and ethyl acetate extracts resulted in the same material B and C, the differences between the dry weight of these solvent extracts must have been due to the differences in non-volatile compounds.

Of the tested solvents, hexane is the best for extracting the complete oil (material A, B and C) and the smallest amount of non-volatile compounds (Figure 6.7). Since non-volatile compounds impair the quality of the GC analyzes, hexane was chosen for further solvent extractions.

Comparison of distillation and solvent extraction

With the previous experiments we optimized the conditions for solvent extraction and for distillation. To determine the amount of non-volatile compounds in a hexane extract, the dry weight of a hexane extract and a distillate were compared. The H_2O distillate contained similar amounts of volatile compounds (material B and C) after 26 fractions as a hexane extract did after 3 extractions. The calculated difference in dry weight between the H_2O distillate and the hexane extract was about 9 mg per gram roots, meaning that more than one third of the dry weight of the hexane extract was due to non-volatile compounds.

To test the influence of the two extraction methods on the volatile components of the oil, roots from the same batch were extracted with hexane or distilled with a P-buffer (P-dist/roots). Half of the hexane extract was distilled (P-dist/hexane extract) to observe changes that might result from the high temperature. Distillation of roots liberated more oil than the extraction with hexane did. After distillation of the hexane extract more oil was detected than in the hexane extract before distillation, but still less than with direct distillation

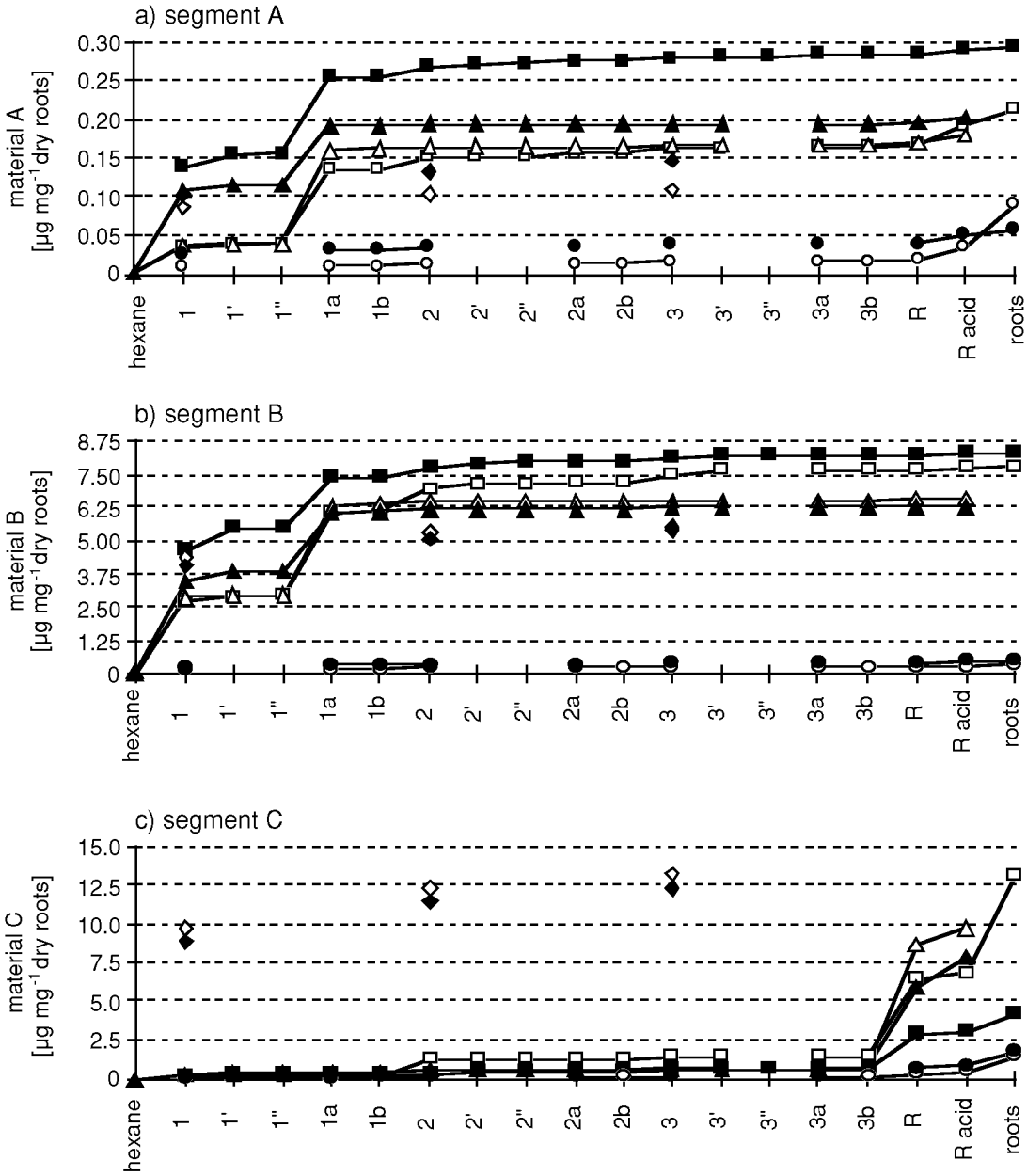

Figure 6.8. Comparison of distillation and hexane extraction. To compare the influence of the extraction method on the amount and the composition of the vetiver oil, vetiver roots were extracted by distillation with phosphate buffer (0.5M, pH8) (□, ■) and by hexane extraction at room temperature (◆, ◇). To test the influence of high temperatures and the efficiency of hexane extraction, hexane extract and the roots after hexane extraction were distilled (P-dist/ hexane extract (△, ▲); P-dist/extracted roots (○, ●)). The distilled water fractions were extracted three times (samples x, x', x") and the cooler was rinsed twice (sample xa, xb). All samples were analyzed by GC. The yield of material A, B and C was followed over the three extractions and the three distillation steps (a, b, c).
R: remaining water or buffer extracted with MTBE; R acid: remaining water or buffer acidified before extraction with MTBE; roots: distilled roots extracted with MTBE

(Figure 6.8). To compare the extraction efficiency of the hexane extraction, roots after hexane extraction were distilled as well (P-dist/extracted roots). However, the distillate of the extracted roots contained only a small amount of oil (Figure 6.8), less than the difference between the root distillate and the distillate of the hexane extract. This difference is most probably due to peak integration errors.

The differences in the oil content of hexane extract and distillates (direct roots or hexane extract) are probably a result of the heat (100 - 140°C) and the pH. Under these conditions some bound molecules might have broken down and were subsequently detectable by GC.

The ratios of the material Ba - Bf of the distillate (of roots and of extract) and the hexane extract were similar but not identical (Table 6.3). With distillation, the material Bf decreased, whereas the material Ba, Bb and Bc increased slightly. For the distilled extracted roots, only small amounts of oil were distilled (data not shown) and no clear conclusions could be drawn.

Table 6.3. Comparison of the oil composition of distillates from roots, of hexane extracts and of distillates from a hexane extract, as determined by GC

	Ratio of material X normalized to material B [b]					
segment [a]	P-dist/roots [c]		hexane extract [c]		P-dist/hexane extract [c]	
A	0.022	0.035	0.020	0.027	0.026	0.031
Aa	0.007	0.007	0.008	0.009	0.009	0.007
B	1.000	1.000	1.000	1.000	1.000	1.000
Ba	0.061	0.058	0.054	0.050	0.064	0.054
Bb	0.267	0.269	0.248	0.248	0.259	0.261
Bc	0.100	0.097	0.075	0.074	0.079	0.074
Bd	0.171	0.170	0.179	0.176	0.183	0.177
Be	0.128	0.133	0.116	0.122	0.118	0.127
Bf	0.273	0.273	0.328	0.330	0.298	0.306
C	0.185	0.064	2.396	2.291	0.108	0.109

[a] : Division of the segments see figure 6.2
[b] : To directly compare the oil composition of the different distillates the material A, B, C, Aa and Ba to Bf were normalized to the material B
[c] : two repetitions

From the GC analyses we conclude that the composition of the volatile oil components obtained by distillation and by hexane extraction is similar. Only when separate peaks are compared in detail, can small differences in composition be found. Therefore, for the pre-screening of vetiver plantlets, the

less labor-intensive hexane extraction provides a suitable extraction method, if the non-volatile components can either be removed or do not disturb the subsequent GC analysis.

ACKNOWLEDGEMENTS

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Chapter 7: Optimization of small scale extraction, purification and concentration methods for analysis of the essential oil from vetiver roots

Ruth E. Leupin, Charles Ehret, Karl H. Erismann and Bernard Witholt

ABSTRACT

To develop a simple and small scale procedure for the analysis of vetiver oil in multiple vetiver root samples, hexane extraction and water distillation were optimized. As hexane also extracted non-volatile compounds, which complicated subsequent gas chromatography analysis, extracts were further fractionated by thin layer chromatography or column chromatography. Neither method was sufficient. To minimize the scale of distillation and permit distillation of samples in parallel, water distillation was combined with solid phase extraction. An Amberlite XAD-2 column was used to adsorb the vetiver oil from the steam, thus replacing the cooling system.

The choice of the best system for analysis of multiple small scale samples depends on the acceptability of non-volatile compounds in the final analysis assay. If non-volatile compounds are acceptable, as is the case for TLC, hexane extraction is a useful method to prepare many small samples. If non-volatile compounds disturb the analysis, as it is the case for gas chromatography, distillation coupled to the Amberlite column is better. For very small amounts of material, both methods require a concentration step.

INTRODUCTION

After regeneration of vetiver plantlets via tissue cultures (Chapter 3, Leupin *et al.* 2000), all plantlets have to be tested for the amount and composition of the essential oil. Usually, vetiver plants are harvested after 15 - 22 months and the oil is extracted from the roots by steam distillation (Roth and Kornmann 1997; Weiss 1997a). With *in vitro* plantlets, even more time is needed before regenerated plantlets can be tested. A micro assay that permits an earlier pre-screening for changes in the oil composition is therefore needed.

Several methods such as simultaneous distillation - solvent extraction (Bicchi *et al.* 1983; Godefroot *et al.* 1981), headspace solid-phase micro extraction (Field *et al.* 1996), microwave extraction (Craveiro *et al.* 1989) and supercritical fluid extraction (Blatt and Ciola 1991; Sugiyama and Saito 1988) have been described to extract essential oil from small samples. It was shown that the

composition of the oil varies depending on the extraction method used (Boutekedjiret *et al.* 1997; Pino *et al.* 1996; Scheffer 1996; Simándi *et al.* 1999). In previous experiments, we have optimized and compared water distillation and solvent extraction at room temperature to extract oil from larger samples of 5 g dry roots (Chapter 6). For large amounts of roots, distillation is better, as only volatile compounds are extracted, but the distillation of many samples raises practical problems. Solvent extraction has the advantage that many small samples can be extracted in parallel. However, since such extracts also contain non-volatile compounds, these may cause problems during the subsequent analysis by gas chromatography. In the previous study (Chapter 6), it was shown that hexane, methyl *tert*-butyl ether, ethyl acetate and ethanol extracted about the same amount of volatile components but different amounts of non-volatile compounds, of which fewest were extracted with hexane. Nevertheless, the dry weight of the hexane extract (23.7 mg g^{-1} dry roots) is still significantly higher than that of the distillate (14.9 mg g^{-1} dry roots), indicating that the hexane extract does contain non-volatile compounds.

In this chapter we describe the optimization of the small scale hexane extraction, test methods to lower the amount of non-volatile compounds by thin layer chromatography, column chromatography or distillation, and try a method to distill several samples in parallel by a combination of solvent extraction, distillation and solid phase extraction.

MATERIAL AND METHODS

Vetiver roots

The plants and the dry vetiver roots were provided by Mr. Heini Lang, Jakarta. For the extraction experiments, the roots were cut in 5 mm pieces. Fresh roots were harvested from the *Vetiveria zizanioides* of our stock. The plants were grown outside (summer) and in a greenhouse at 10 - 15°C (winter).

Vetiver oil

The vetiver oil Bourbon was provided by Givaudan-Roure Forschung, Dübendorf.

Hexane extraction at room temperature

Dry vetiver roots, cut in 5 - 10 mm pieces, were wetted for about 1 hour with water and subsequently extracted overnight at room temperature with hexane. The hexane was removed (hexane extract 1) and new hexane was added. After another 24 hours stirring, the hexane (hexane extract 2) was again removed and the roots were extracted for the third time with new hexane (hexane extract 3).

For experiments with hexane extract as starting material, 5 g vetiver roots, pre-wetted with 10 ml water, were extracted with 50 ml hexane to avoid variation due to inhomogeneous root material.

To optimize the extraction of small amounts of oil, different starting materials (thick or thin roots, cut or ground with sand), different amounts of water to wet the dry roots (0, 150, 300, 600 or 1200 µl water per 300 mg roots), different amounts of hexane (1.5, 3 or 6 ml hexane per 100 mg roots) and different extraction periods (sequentially for 1, 1 and 3 days, for 2 and 3 days or for 5 days) were tested, as described in the result and discussion section.

The acidic compounds were removed by shaking out the hexane extract with the same volume of a 1M potassium hydroxide solution (KOH). The hexane phase contained the hexane extract without the acids (hexane extract - KOH), whereas the acidic compounds remained in the water phase.

Concentration or purification with silica columns

Pasteur pipettes were plugged with cotton and dry silica (Merck, Kieselgel 60, 0.062-0.200 mm) was added to make simple silica columns. Before use, the columns were washed with 1 ml ethanol, 2 ml methyl *tert*-butyl ether (MTBE) and 3 ml hexane. Hexane extracts were loaded on columns (outflowing hexane:

H) and oil was eluted in 0.5 ml steps (elution with different solvents: s, elution with MTBE: M). All samples were analyzed by gas chromatography (GC).

To analyze the concentration effect of these columns, we tested different amounts of silica (30, 50, 100 and 160 mg), different solvents to elute the extract from the column (MTBE, isopropanol, ethanol, ethyl acetate and diethyl ether) and different amounts of hexane extract or vetiver oil Bourbon (0.5 mg ml^{-1} in hexane). When solvents other than MTBE were used to elute the oil from the column, these were used instead of MTBE to wash the column.

Small scale distillation in combination with solid phase extraction

Optimization of the micro-distillation

Pasteur pipettes were used to make Amberlite columns (Figure 7.1a). Loose cotton was used to retain the column material (Amberlite XAD-2), which was added as a suspension in water. Before use, the columns were washed sequentially with 3 ml ethanol, 3 ml MTBE, 2 ml hexane, 2 ml ethanol and water and dried for several hours at 100°C.

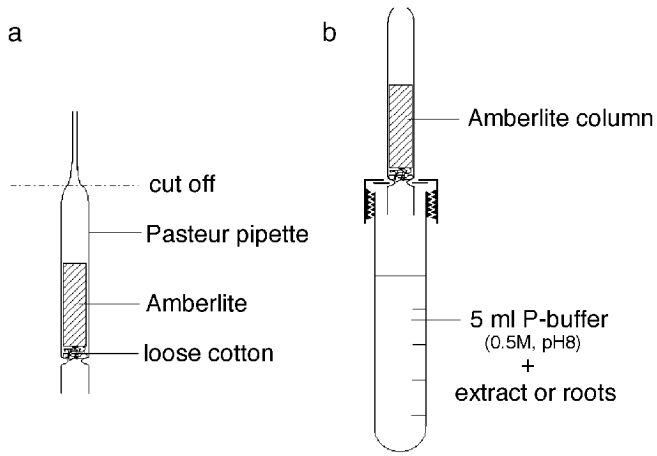

Figure 7.1. Miniaturized distillation apparatus with Amberlite column.
a) Amberlite column made from a Pasteur pipette
b) distillation apparatus with Amberlite column

Samples (distillate or hexane extract) were added to 5 ml phosphate buffer (P-buffer, 0.5M, pH8) in a Pyrex tube and the hexane was evaporated with nitrogen (HexN) or the compounds in the hexane extract were adsorbed on silica (HexSil) and the silica was transferred in the P-buffer. The XAD-2 column was connected to the tube (Figure 7.1b) and put in the oven at 120°C. After

3 - 5 hours about 3 to 4 ml of the P-buffer had been evaporated and the distillation was stopped. The Amberlite column was eluted three times with 2 ml MTBE (M1 - M3), and the residual buffer was extracted with 1 ml MTBE (R). All samples were analyzed by GC.

Comparison of hexane extracts and roots as starting material for micro-distillation

Three different samples were distilled: 1 ml hexane extract 1, concentrated by evaporating the hexane under nitrogen (HexN); 1 ml hexane extract, loaded on 50 mg silica (HexSil) (outflowing hexane: H); and 100 mg of dry roots. After about 3 hours the distillation was stopped. For the second distillation, the residual buffer was filled up with water to 5 ml and new XAD-2 columns were used. After another five hours the distillation was stopped. The Amberlite columns of the first and the second distillations were rinsed three times with 2 ml MTBE (first distillation: M1 - M3, second distillation: M4 - M6). The residual buffer (≈ 1 ml) and the roots were extracted with MTBE (R).

As controls, roots were extracted twice with hexane and the hexane extract was prepared as for the distillation, either by adding the extract to the P-buffer, followed by the evaporation of the hexane with nitrogen (HexN) or by loading the hexane on the silica column (HexSil), but instead of the subsequent distillation, the compounds were then re-extracted or re-eluted, respectively. All samples were analyzed by GC.

Thin layer chromatography (TLC)

For TLC analysis, extracts were separated on silica gel plates (Silica gel 60 F_{254}, MERCK), using hexane and chloroform as consecutive solvents in one dimension. To separate non-volatile compounds from the essential oil, different solvents (hexane, isooctane, isopropanol, diethyl ether, acetonitrile, methanol, methylene chloride, chloroform, toluene) were tested. To detect the different compounds, UV (254 nm), iodine vapor and anisaldehyde-acetic acid-sulfuric acid (2 ml : 20 µl : 40 µl) staining for essential oils (Cosicia 1984; Merck 1970) were used.

Gas chromatography (GC)

A Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector and a DB-WAX column (25 m x 0.32 mm, 0.25 µm film thickness) was employed. Hydrogen was used as carrier gas at a rate of 2 ml min^{-1}. The samples were injected in the splitless mode. Injector and detector temperatures were maintained at 200°C and 300°C, respectively. The column oven temperature was programmed for 80°C (after 4 min) to 220°C at 2.5°C min^{-1} and the final temperature was held for 20 min. Peak areas and retention times were measured by electronic integration (Hewlett Packard 3390A integrator).

Before injecting in the GC, dibutyl phthalate was added to all samples as internal standard. To compare the composition of the extracts, the GC chromatogram (Figure 7.2) was divided in three main segments (A, B, C). Segment A contained mainly hydrocarbons, segment C contained mainly the acidic components and segment B contained residual components (alcohols, ketones,..) (Chapter 5). To detect the effects of the extraction methods on the oil composition in more detail, the segments A and B were subdivided (Aa, Ba - Bf; Figure 7.2). In the following text, X compounds refer to the compounds running within segment X of the GC chromatogram.

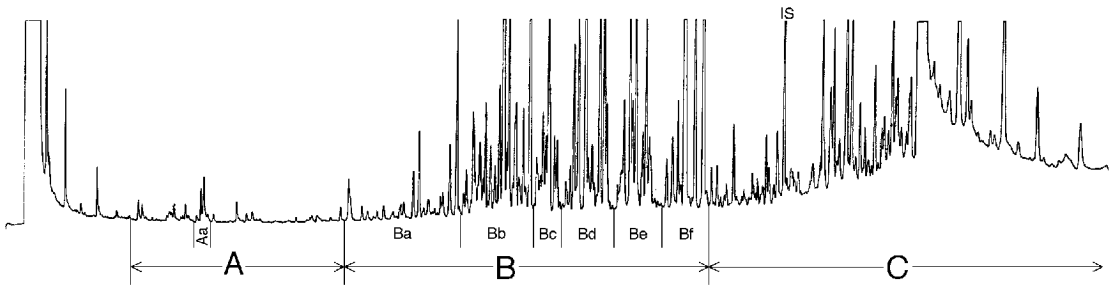

Figure 7.2. Gas chromatogram of vetiver oil on a DB-Wax column. The chromatogram was divided in segments A, B and C for data analysis. Segment A contains mainly hydrocarbons, segment B contains mainly alcohols, aldehydes and ketones and C contains mostly acids. The segment B was subdivided in Ba - Bf. IS: dibutyl phthalate (internal standard)

Estimation of the amount of oil based on GC analysis

To determine the amount of any compound Z from a GC chromatogram, the response factor of that compound Z and the internal standard (IS) has to be determined. The FID response is roughly proportional to the number of carbon atoms present in the compounds, however the response is also affected by hetero atoms and various functional groups (Flanagan 1993). Therefore, for each compound the response factor can be different and has to be determined separately.

$$\text{response factor} = \frac{\text{peak area}}{\text{amount}}$$

Using this response factor, it is possible to estimate an unknown amount of compound Z from a GC chromatogram.

$$\text{amount of compound Z [µg]} = \frac{\text{peak area Z}}{\text{peak area IS}} \cdot \frac{\text{response factor IS}}{\text{response factor Z}} \cdot \text{amount IS}$$

As many vetiver oil components are not isolated and the structure not elucidated, it is not possible to determine the response factor for each single component. Since vetiver oil components are expected to be all sesquiterpenoids, they should have C15 and exceptionally C14. The main differences are the number and the position of the functional groups. To simplify the calculation, we assume that all vetiver oil components have the same response as dibutyl phthatate ($C_{16}H_{22}O_4$). This results in:

$$\text{amount of compound Z [µg]} = \frac{\text{peak area Z}}{\text{peak area IS}} \cdot 1 \cdot \text{amount IS}$$

The amount of oil in each segment X was estimated relative to 1 µl internal standard containing 0.025 µg dibutyl phthalate by summing up all approximate amounts of the compounds within segment X.

$$\text{material in segment X [µg]} = \frac{\sum \text{peak areas in segment X}}{\text{peak area IS}} \cdot 0.025\ \mu g$$

It has to be noted that the calculated amount is only an approximate amount of oil, based on the assumption described above. Depending on the commercial vetiver oil tested (see chapter 5), the calculated amount was up to 1.5 times of the amount used (results not shown).

RESULTS AND DISCUSSION

In this study, we describe experiments to miniaturize the extraction of vetiver oil from a small amount of roots. Given the simple equipment necessary for hexane extraction of roots at room temperature, several samples can be extracted in parallel. However, non-volatile compounds which are co-extracted, disturb the gas chromatographic analysis and methods to remove them should be found.

Water distillation of roots has the advantage that only volatile compounds are extracted, but only a few samples can be distilled simultaneously due to the limitation of the necessary cooling system. To replace the cooling system and therefore make the distillation of several small samples in parallel possible, a solid phase extraction was tested.

Optimization of small scale hexane extraction

In previous experiments, we found that more oil was extracted from the vetiver roots by distillation than by hexane extraction (Chapter 6). Therefore, the extraction of the roots with hexane not only has to be miniaturized, but the extraction procedure must also be further improved.

Effect of solubility

Factors which can limit an extraction are the solubility of compounds in the extracting solvent and the diffusion of solvents into and compounds out of the

plant material. To test if the solubility of the vetiver oil in hexane is a limiting factor, different amounts of hexane were used for the extraction. In previous experiments, 5 g roots were extracted in 50 ml hexane. For 100 mg roots, this ratio is not useful, as 1 ml hexane fails to submerge the roots and the magnetic stirring bar. Therefore, for 100 mg pre-wetted roots, 1.5, 3 and 5 ml hexane were used. The first extraction step was only slightly more effective with 5 ml hexane compared to 3 or 1.5 ml, extracting more acidic compounds (results not shown). Thus, limited solubility of the oil components can not be the main reason for their less efficient extraction.

Since after induction of oil formation, the *in vitro* plantlets contain very small amounts of oil, it is necessary to extract the oil in as little solvent as possible. As the increased amount of hexane did not result in a major improvement, the ratio 100 mg roots in 1.5 ml hexane was used for further extractions.

Effect of extraction time

To test the influence of the extraction time on the extracted amount of oil, 100 mg pre-wetted roots were extracted sequentially for 1, 1 and 3 days, for 2 and 3 days or for 5 days.

Prolongation of the extraction time from 1 to 2 days did not influence the amount of oil extracted. However, the extraction for 5 days resulted in the same material A and B as the sum of the subsequent extractions for 1, 1 and 3 days or 2 and 3 days. Independent of the time intervals, the total yield after 5 days remained within the variation of the experiment (results not shown).

Effect of diffusion distance

To test the influence of the diffusion distances on the extraction yield, the dry roots were separated in thick (≥ 1 mm) and thin roots (< 1 mm), before cutting them in 2 - 5 mm pieces.

Thin roots contained about 1.5 to 2 times less oil than thick roots (Figure 7.3). However, the dry roots were stored for 5 - 6 years and to test whether thin roots contain less oil as a consequence of evaporation during this storage time, fresh roots were harvested, split in only thick and mainly thin roots and extracted with

hexane. Again, more oil was extracted from the thick roots (1.6 - 1.9 times). With both experiments, the oil was not extracted faster from thin roots. Therefore, we concluded that the diameter of the roots did not have an influence on the effectiveness of the extraction.

To increase the surface of the root particles further and simultaneously destroy possible diffusion barriers of the intact root surface, 100 mg samples of thick and thin roots were ground with sand. This treatment did not extract more oil, but it was extracted faster: after one extraction step already about 72 - 82 % of the total extracted oil was recovered, whereas only 63 - 72 % of the oil was extracted from control roots, which were not ground (Figure 7.3). However, as the grinding represents an extra working step and some oil might be lost during this process, it was not used further.

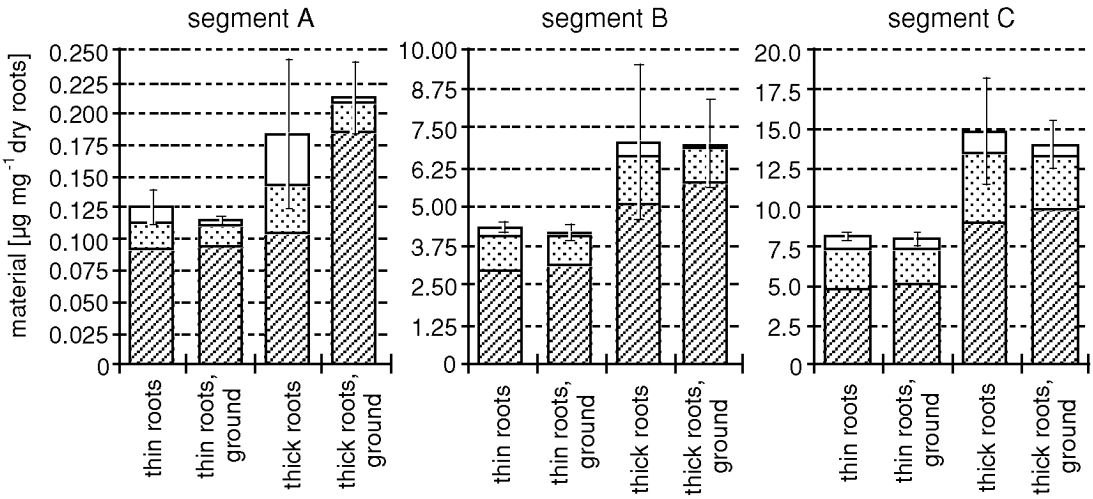

Figure 7.3. Extraction of thin, thick or ground vetiver roots with hexane. Thin and thick vetiver roots were cut in pieces and either directly extracted, or first ground with sand before they were extracted three times with hexane (▨ first extraction, ⬚ second extraction, □ third extraction). The extracts were analyzed by GC. The yield of material A, B and C was determined after each extraction step.

Influence of water on extraction of vetiver oil from dry roots

Up to now all extractions were performed with dry roots, but as after the induction experiments fresh material might also be extracted, it is important to know how water influences the hexane extraction. Therefore, different amounts of water (0, 50, 100, 200, 400 µl per 100 mg roots) were added to dry roots

before extraction with hexane. Without water, many small particles were found floating in the hexane, and the extract needed to be filtrated through a 0.2 µm filter. With 50 µl water, only a few particles were observed and after adding 100 µl water or more, filtration was not necessary. Addition of water also affected the oil composition: by adding more water, less material A was extracted, whereas for polar components (B and C compounds), the addition of water was beneficial (Figure 7.4).

To compare the influence of fresh and dried material on the extract, roots of an *in vivo* grown plant were harvested. One part (1.7 g) was extracted directly with 5 ml hexane and one part was dried for one day at room temperature. The weight loss after one day was between 73 and 76 %. Samples of dried roots (370 to 450 mg) were re-wetted with 600 µl water (61 - 57 %) and extracted with 5 ml hexane. About the same material B and C were extracted from dried and fresh roots. The material A was slightly (24 %) lower for the dried roots (results not shown). We concluded therefore that hexane extraction can be used for both fresh and dry material.

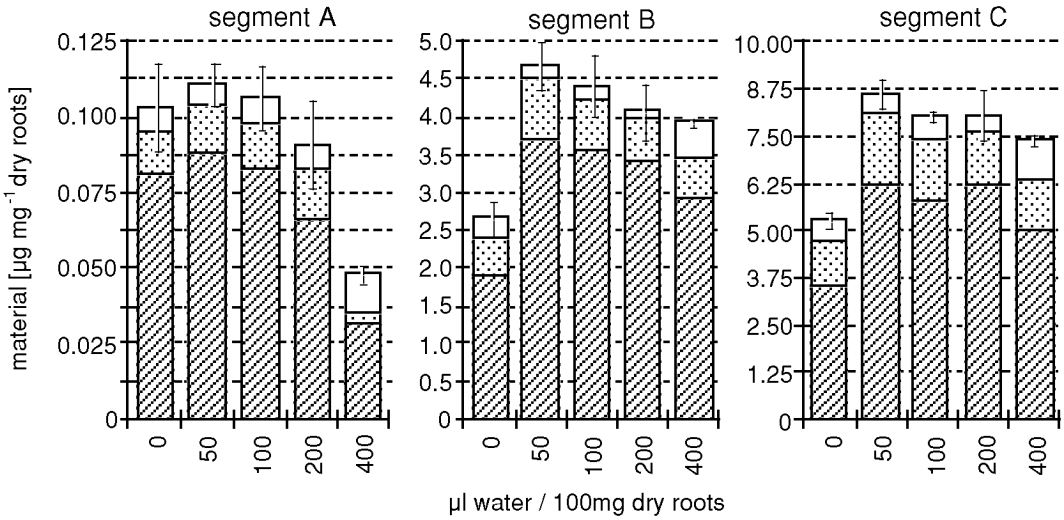

Figure 7.4. Extraction of vetiver roots with hexane after pre-wetting the dry roots with different amounts of water. Vetiver roots were pre-wetted with 0, 50, 100, 200 and 400 µl water per 100 mg roots, before they were extracted three times with hexane (▨ first extraction, ⬚ second extraction, □ third extraction). The extracts were analyzed by GC. The yield of material A, B and C was determined after each extraction step.

Reproducibility of extractions

To test the influence of inhomogeneous material (e.g. thin and thick roots) on the reproducibility of small scale extraction, ten times 100 mg dry roots were pre-wetted with 200 µl water and extracted overnight with 1.5 ml hexane. Each extract was analyzed by GC in duplicate.

The ten hexane extracts were compared and a variation of maximally 8 % for the material B and C, and of 16 % for material A was found (results not shown). Compared to material B and C, only small amounts of material A were extracted from the dry roots used in our experiments. Therefore, GC integration errors and variations in hydrocarbon contents could have a large effect on material A. It is thus difficult to determine if observed changes in the material A are due to the inhomogeneous material, to integration errors or to the extraction treatments.

Concentration of the hexane extract

The *in vitro* tissue may, after induction of the oil production, still contain less oil than the in this study used dry *in vivo* roots. As a result, a concentration step may be necessary. One way to achieve this is to evaporate the hexane with nitrogen; another is to reduce the volume by running it over a silica column.

Evaporation with nitrogen

To determine how evaporation with nitrogen changes oil composition, especially of the more volatile compounds, 500 µl hexane extract 1 and 500 µl of vetiver oil Bourbon (0.5 mg ml^{-1} in hexane) were taken to dryness under nitrogen for 2.5, 5 and 10 minutes. After 2.5 minutes all hexane was evaporated. The dried extracts were re-dissolved in 500 µl hexane and analyzed by GC. The evaporation affected the A compounds. After evaporation for 10 minutes, 30 % of the material A found in the original hexane extract 1 was recovered. Whereas for the vetiver oil Bourbon, which contained 14 times more material A per 500 µl diluted solution than the hexane extract of the dry roots,

only 10 % of this material A was recovered. The loss in the other chromatogram segments was within the variation of the analysis (93 - 110 % of original material B, Table 7.1).

Table 7.1. Influence of the evaporation time on the hexane extract and the vetiver oil composition, as determined by GC

GC segments	hexane extract[1]				vetiver oil[2]			
	material	recovery after evaporation with N2 for			material	recovery after evaporation with N2 for		
	[µg per 500 µl]	2.5 min [%]	5 min [%]	10 min [%]	[µg per 500 µl]	2.5 min [%]	5 min [%]	10 min [%]
segment A [3]	2.35	87	69	29	31.85	110	59	9
segment B [3]	164.83	109	108	110	155.18	110	102	93
segment C [3]	233.45	88	108	94	11.58	99	97	97

[1] : dry vetiver roots extracted in hexane, 100 mg roots ml^{-1} hexane
[2] : vetiver oil Bourbon (Givaudan-Roure), 0.5 mg ml^{-1} in hexane
[3] : see figure 2

Concentration by running over silica column

It is known that hydrocarbons can be isolated from terpenoids or essential oils by chromatography on silica with hexane or pentane as eluents (Croteau and Ronald 1983; Kubeczka 1985). We used this fact to concentrate hexane extracts with small columns containing different amounts of silica gel (30, 50, 100 and 160 mg). 0.5 ml hexane extract 1 - KOH was loaded and subsequently rinsed 5 times with 0.5 ml hexane. Three hexane fractions of 1 ml each were collected and analyzed by GC. The first hexane fraction contained mainly hydrocarbons (segment A). The next two hexane fractions contained only traces of B and C compounds. With 30 mg silica slightly more B and C compounds were found in the hexane fractions than with larger amounts of silica gel. As more solvent is necessary for larger columns, 50 mg silica was chosen for further experiments.

Elution of the hexane extract components from silica columns

To concentrate the hexane extract, it was necessary to use less solvent to elute the compounds from the silica column than the volume of extract loaded

on the column. Different solvents (MTBE, isopropanol, ethanol, ethyl acetate and diethyl ether) were therefore tested. MTBE and ethyl acetate re-extracted about 100 %, ethanol and ether about 120 % and isopropanol only 90 % of the original material B. All solvents tested extracted about 64 - 77 % of the original material C (Figure 7.5).

To determine the minimal amount of solvent necessary for complete elution, the extract was eluted in three steps and analyzed by GC. To recover 98 % or more of the total eluted material B, 0.5 ml isopropanol or ethanol was sufficient, whereas for MTBE or ethyl acetate 1 ml and for diethyl ether 1.5 ml were necessary (Figure 7.5). Since MTBE eluted 100 % of the original material B and it has a low boiling point, which makes it easy to load samples on a TLC plate, MTBE was chosen for routine elution of the oil from silica columns.

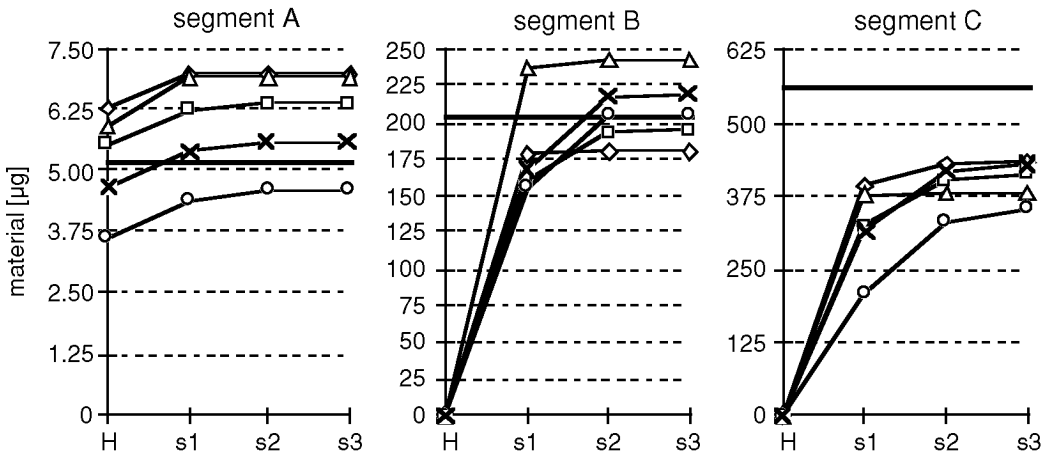

Figure 7.5. Elution of a vetiver root hexane extract from a silica column using different solvents. 2 ml vetiver root hexane extract 2 was loaded on silica columns (50 mg silica). The vetiver oil components were eluted from the column with methyl *tert*-butyl ether (□), isopropanol (◇), ethanol (△), ethyl acetate (○) or diethyl ether (×). The outflowing hexane fraction (H) and the eluted solvent fractions (s1 - s3) were analyzed by GC. The total recovery of material A, B and C was compared with that in the original hexane extract 2 (━).

To further reduce the amount of MTBE needed to elute the oil from the column, we examined the influence of the hexane left after loading the hexane extract. Two columns were eluted directly, whereas the other two columns were first blown dry with a pipetting ball before they were eluted three times with MTBE.

Removal of hexane from the column improved the effectiveness of elution with MTBE: after one or two elution steps, the eluates of the dry columns contained 96 % or 99 % respectively of the total eluted material B, whereas without prior hexane removal only 76 % or 96 % respectively of the total eluted material B was found back (results not shown).

Adsorption capacity of silica columns

The capacity of 50 mg silica columns was determined by loading different hexane extracts and vetiver oil Bourbon in hexane in 0.5 ml steps and analyzing the outflowing hexane per 0.5 ml fraction (H1-Hx). After loading 5 or 10 ml of the extracts, the columns were eluted with MTBE (M1 - M5). All fractions were analyzed by GC.

By loading 10 ml of hexane extract 2 or 3 ml of hexane extract 1, which contain about as much material B, it was shown that the same amount of material B and C was rinsed through (Figure 7.6a, b). This suggests that the loading capacity of the silica is unrelated to the volume loaded onto the column.

The composition of the oil influenced the amount of extract adsorbed on the silica column. After loading 5 ml hexane extract 1 about 10 % of the material B and 22 % of the material C were rinsed through the silica column, whereas after loading 5 ml vetiver oil Bourbon (0.53 mg ml^{-1} in hexane) only about 2 % of the material B and 1 % of the material C were rinsed through the column (Figure 7.6a, c). The differences between the composition of hexane extract 1 and the vetiver oil Bourbon is that the latter contains more of the hydrocarbons (A compounds), whereas the hexane extract 1 contains more acids (C compounds). To test whether the acidic compounds have a negative effect on the ability of the silica to adsorb the oil, they were removed from the hexane extract 1 by KOH extraction. 5 ml of this hexane extract (hexane extract 1 - KOH) were loaded on the column. As was the case for the vetiver oil Bourbon, only about 2 % of the material B and 1.5 % of the material C were rinsed through the column during the loading (Figure 7.6d). We concluded therefore that the acid concentration has a negative influence on the adsorption capacity of the silica column.

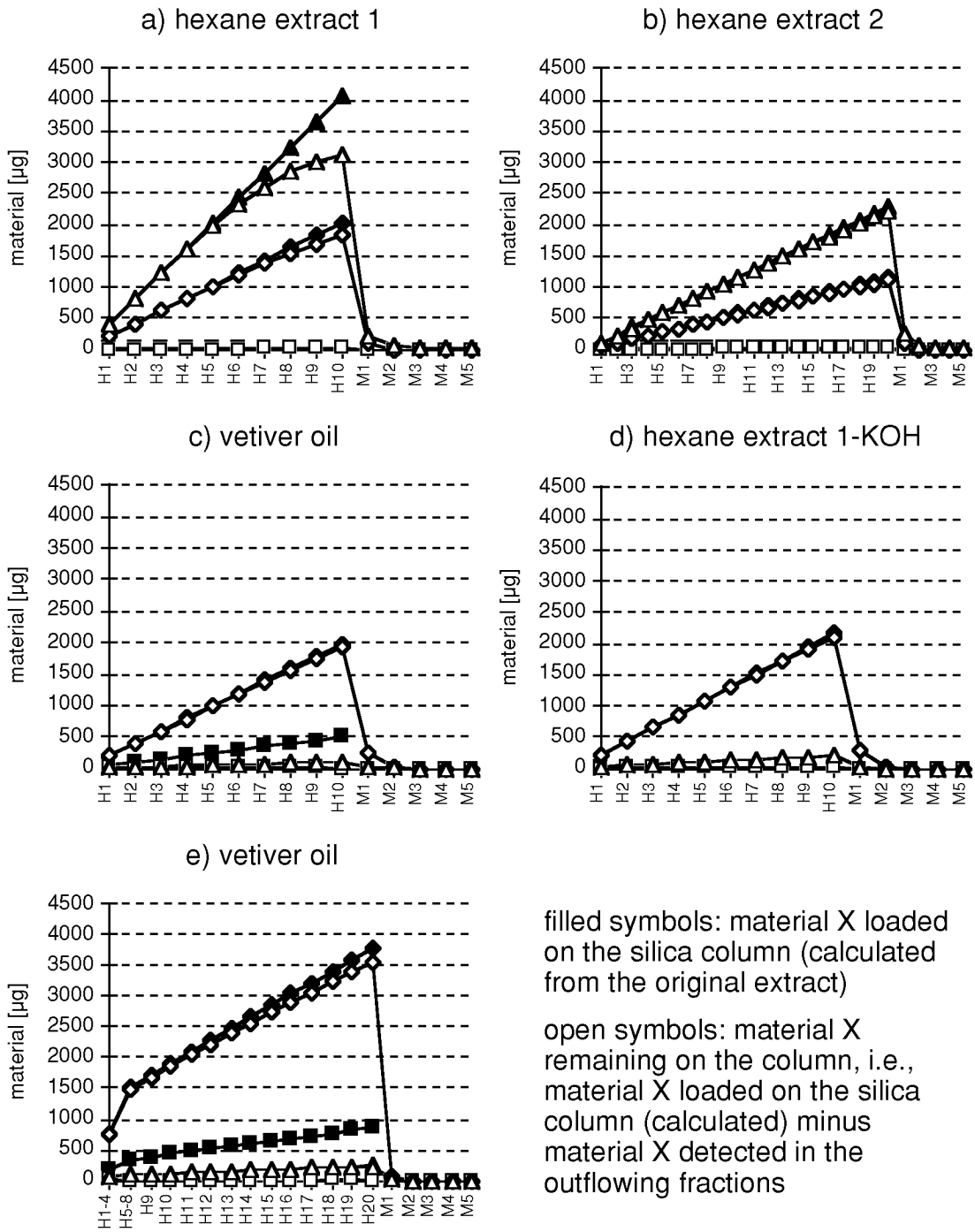

Figure 7.6. Loading capacity of 50 mg silica columns. Different vetiver root hexane extracts of and vetiver oil (Bourbon) in hexane (0.5 mg ml^{-1}) were loaded in 0.5 ml steps on silica columns (50 mg). After loading 5 or 10 ml extracts, the columns were eluted with methyl *tert*-butyl ether (MTBE). The outflowing hexane fractions (H1 - Hx) and the eluted MTBE fractions (M1 - M5) were collected and analyzed by GC. The recovery of material A (■, □), B (◆, ◇) and C (▲, △) was followed over the loading (outflowing hexane fractions) and the subsequent elution.

All of the B and C compounds in 2.5 mg vetiver oil Bourbon were adsorbed on a 50 mg silica column. When 5.3 mg oil was loaded, about 7 % of the material B was rinsed through the column during loading. About 98 % of the adsorbed oil was eluted from the column with 0.5 ml MTBE (Figure 7.6e). The material B of about 5 mg vetiver oil was therefore concentrated from 10 ml hexane to 0.5 ml MTBE. If a loss of 5 - 6 % is accepted, the hexane extract 1 could be concentrated about 8 times. If the hexane extract without acid compounds behaves as does the vetiver oil Bourbon, it could be concentrated even more (up to 18 times) by first removing the acidic compounds.

Comparison of extract concentration procedures

Which of the two above described methods is used to concentrate the extract depends on which compounds of the oil are of interest. By evaporation with nitrogen, A compounds were partially lost, whereas on the silica column they were not adsorbed and therefore not concentrated. For B compounds, it did not matter which method was used. No C compounds were lost during evaporation with nitrogen, whereas a part remained on the silica column after eluting with 0.5 ml MTBE and high concentrations of acids reduced the loading capacity of the silica column.

To concentrate the total oil, evaporation with nitrogen seems very promising for small amounts of extracts, whereas for larger amounts of hexane extracts the silica column method alone (Box 1) or in combination with a subsequent evaporation with nitrogen is useful.

Box 1: Optimized silica column concentration method:

- load 50 mg silica in Pasteur pipette (or column with smaller diameter ≤ 50 mg silica gel)
- wash column with ethanol, MTBE and hexane
- if extract contains a lot of acids: shake out with KOH
- load the hexane extract: A compounds are mainly found back in the outflowing hexane
- remove hexane by blowing air through the column
- elute with ≤ 0.5 ml MTBE

Removal of non-volatile compounds from the hexane extract

One problem of solvent extraction is that the volatiles are isolated together with non-volatile compounds, which during subsequent GC analyses may give rise to problems such as additional peaks or base line shifting. Therefore it is necessary to use some kind of sample preparation, for example TLC or column chromatography, for a preliminary clean-up of the samples (Croteau and Ronald 1983; Scheffer 1996). We have tested the separation ability of different solvents by TLC. None of the tested solvents separated the non-volatile compounds from the volatile compounds (result not shown).

In the concentration experiments, it was found that up to 25 % of the acidic compounds remained on the silica after the first elution with 0.5 ml MTBE (Figure 7.5). If simultaneously the non-volatile compounds, running at the same place and slower than the acidic oil components of the vetiver oil, were to remain on the silica column, a partial cleaning might be possible. To test how efficient the precleaning was, a hexane extract of distilled roots was, after extraction of the acidic compounds with KOH, loaded on silica columns and eluted with different solvents (MTBE, isopropanol, ethyl acetate and diethyl ether). TLC was used to detect the non-volatile compounds. With all solvents used, the major portion of the non-volatile spots eluted in the first fraction (results not shown). The precleaning effect is thus only minor.

Small scale distillation in combination with solid phase extraction

The most efficient method to separate volatile and non-volatile compounds is to use distillation. One disadvantage is that distillation requires a cooling system, which makes it complicate to distill several samples in parallel and may cause losses due to the large surface area of the glass material. A method to avoid these problems is to replace the cooler by a solid phase extraction column, which adsorbs the oil components from the steam.

Adsorption of vetiver oil on Amberlite XAD-2

To avoid plugging of the column and possible overpressure, the column material should not be packed too densely. Machale (1997) reported that with Amberlite XAD-4 it is possible to extract essential oil compounds from condensate water, and Menon (1999) used Amberlite XAD-2 columns to isolate free and glycosidically bound volatiles from a water extract of cardamom. We therefore selected Amberlite, which consists of small spheres (20 - 50 mesh). To test if the apolar Amberlite XAD-2 adsorbs all distilled vetiver oil components from cold water, an Amberlite column was fixed behind the cooler of the distillation apparatus. Only about 3 % of the total material B was found in the distilled water fractions after the Amberlite column (results not shown).

The next step was to test whether the Amberlite XAD-2 also adsorbs the vetiver oil from steam. Distillation with the Amberlite column before the cooler did not work, as the water condensed in the Amberlite column and the overpressure caused the Amberlite to be blown out. The same happened with a Pyrex tube with Pasteur pipette column (Figure 7.1b) in an oil bath at 140°C. An alternative is to place the system in an oven. The Pyrex tube as well as the Amberlite column are heated, the water does not condense in the column and the risk of an Amberlite blow out is reduced. To test whether such Amberlite columns adsorb the volatile compounds from steam, small scale distillations with XAD-2 columns were performed in an oven at 120°C.

Table 7.2. Minimized distillation of vetiver distillate with a XAD-2 column to absorb the oil from the steam

GC segments	original distillate [1]	combined distillation - solid phase extraction percent of original distillate [%]					
		eluate of Amberlite column				extract of	total
	material [µg]	1	2	3	total	residual buffer	
segment A [2,3]	4.78	87.3	13.3	8.7	109.4	7.1	116.4
segment B [2]	432.30	69.7	27.1	0.8	97.6	0.1	97.6
segment C [2]	28.55	67.6	10.9	0	78.4	17.0	95.4

[1] : distillate of vetiver roots dissolved in hexane
[2] : see figure 2
[3] : large variations

The eluate of the Amberlite column contained the major part of the oil. Except for material A, the sum of the eluates and the extracts of the residual buffer contained only a few percent less than the original starting distillate (Table 7.2). The missing oil might remain in the steam or not be extractable from the column material. The material A varied, due to evaporation and small amounts, around 116 ± 15 % (Table 7.2). This experiment showed that the major part of the oil adsorbed on XAD-2.

Effect of Amberlite column height on adsorption

To find an optimal amount of Amberlite, the effect of the Amberlite column height (0.8, 1.8 or 3 cm) on the adsorption of volatile compounds from the steam was examined. With the 0.8 cm high columns up to 60 % of the original material A and about 10 % of the original material B were lost, whereas for the 1.8 and 3 cm columns up to 40 % of the original material A and less than 10 % of the original material B were lost (results not shown).

Due to the loss of material A during evaporation of the hexane, it was not possible to determine how much of material A was not adsorbed by the Amberlite. The elution of the Amberlite columns was not influenced by the height of the column material: with all three column heights about 90 - 96 % of the total eluate was re-extracted with the first 2 ml MTBE and more than 99 % within the second 2 ml (results not shown). Therefore we selected a 2 cm Amberlite column for further experiments.

Sample preparation of hexane extracts for distillation

Due to the explosion danger, hexane has to be removed before the hexane extract is distilled in the oven. Analogous to the concentration experiments described above, this can be done by evaporation of the hexane with nitrogen (HexN) or by adsorption of the oil compounds on silica (HexSil).

Since during preparation of the HexSil concentrate the major portion of A compounds of the hexane extract remained in the outflowing hexane (H), they were neither distilled nor lost due to evaporation with nitrogen. Therefore, the

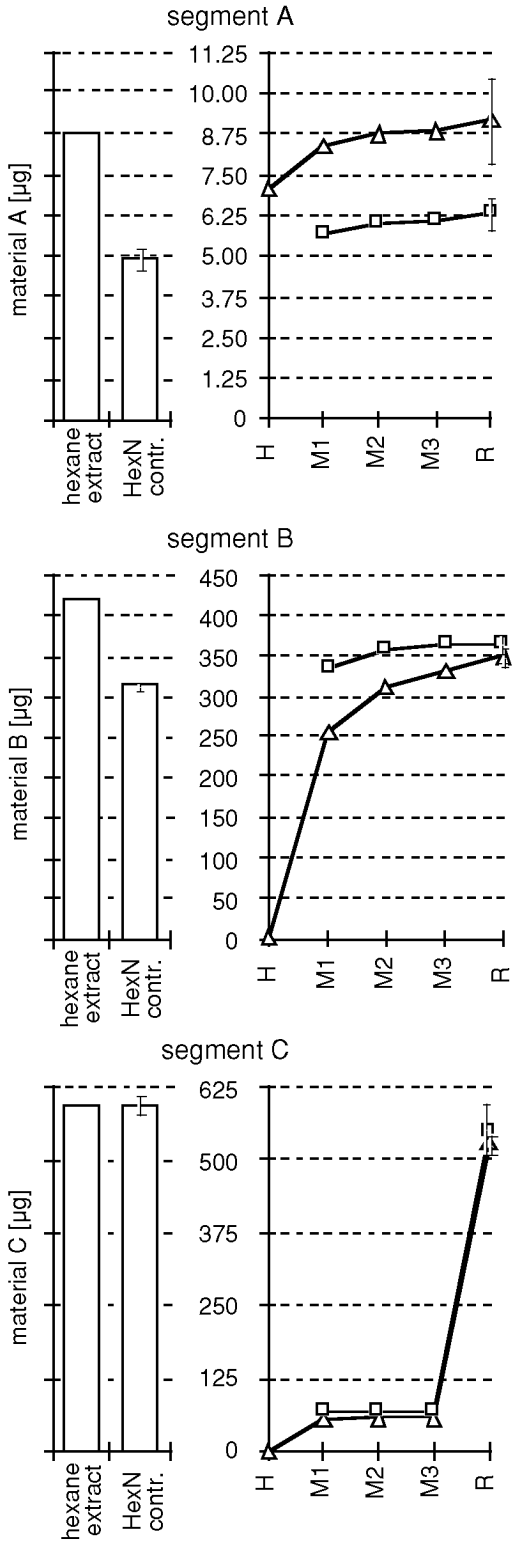

Figure 7.7. Influence of sample preparation of the hexane extract on the distillation behavior. The hexane of the hexane extract was removed either by evaporation with nitrogen (HexN ☐) or by adsorption of the oil components on a silica column (outflowing hexane: H) and suspending the dry silica in P-buffer (HexSil △). HexN and HexSil concentrates were distilled in a miniaturized distillation apparatus with XAD-columns (see Figure 7.1) to retain the vetiver oil components. The XAD-2 columns were eluted three times (M1 - M3) and the residual buffer was extracted (R). The original hexane extract and HexN, reextracted directly from the P-buffer (HexN contr.), were used as controls. All samples were analyzed by GC. The recovery of material A, B and C was determined after each extraction step.

outflowing hexane and the distillate of HexSil concentrate together contained more material A than the distillate of HexN concentrates (Figure 7.7).

After about 4 hours distillation, the eluates of the Amberlite columns of the HexSil distillation contained less material B and C than those of the HexN distillation, but after extraction of the residual buffer (R), both resulted in the same recovery (Figure 7.7). This indicates that it takes longer to distill the oil adhering to the silica than the oil floating free in the phosphate buffer.

Comparison of hexane extracts and roots as starting material for small scale distillation

To compare the effect of the starting material on the distillation performance, hexane extracts (HexSil, HexN) and roots added directly to the P-buffer, were distilled. To obtain a complete distillation, a second distillation step was added.

Analogous to the large scale distillation (Chapter 6), more oil was extracted by distillation from the roots than by solvent extraction: the first and the second distillation steps yielded larger amounts of oil (about 1.3 x material B) than the hexane extracts 1 and 2, respectively.

After the first distillation step nearly all oil components of HexN were distilled, whereas the second distillation step still resulted in 5 - 10 % of the total recovered oil for the HexSil distillation and about 20 % of the total recovered oil for the root distillation. Scheffer (1996) already described that distillation removes the volatiles much faster from solvent extracts than from plants containing them. We found that adsorption of the hexane extract on the silica similarly prolonged the distillation time (Figures 7.7, 7.8).

After the two distillation steps, the HexN and HexSil distillates contained about 90 % material B of the starting hexane extract 1 and extraction of the rest buffer increased this amount maximally by 1 %. Therefore about 9 % of the original material B is missing. This discrepancy can have several causes: integration errors of the GC may cause small variations; some of the oil could be lost during sample preparation before the distillation; some of the oil may still be adsorbed on the Amberlite column and was not removable with MTBE; some of the oil evaporated through the column; or some oil might still be in a not extractable form in the rest buffer or the wet silica. The first option is unlikely,

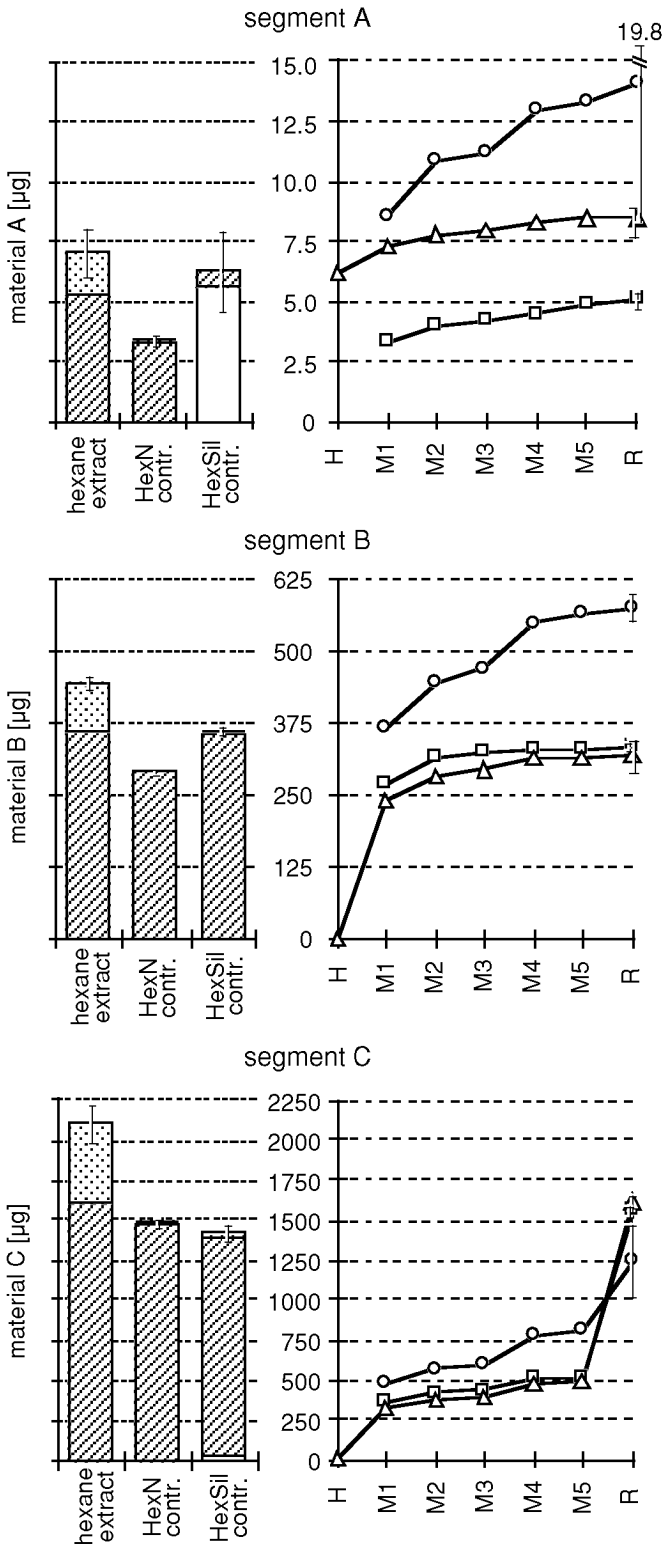

Figure 7.8. Combined distillation-solid phase extraction of hexane extracts and roots. A vetiver root hexane extract, evaporated with nitrogen (HexN □) or adsorbed on silica (HexSil △) and vetiver roots (○) were distilled in a miniaturized distillation apparatus with XAD-columns (see Figure 7.1) in two steps. After the first distillation, the buffer was replenished and the Amberlite column was replaced. After the distillations, the columns were eluted (first distillation: M1 - M3; second distillation: M4, M5) and the residual buffer and the roots were extracted (R). As controls, roots were extracted twice with hexane (first hexane extract ▨; second extract ⬚). The hexane extracts were prepared as for distillation, but instead of a subsequent distillation, the compounds were reextracted (HexN contr.: first extraction ▨, second extraction ⬚) or reeluted (HexSil contr.: outflowing hexane □, first elution ▨, second elution ⬚), respectively. All samples were analyzed by GC. The yield of material A, B and C per 1 ml hexane extract or per 100 mg dry roots was determined after each extraction step.

as all three repetitions resulted in recovery around 90 % of the original hexane extract. All other options might have an influence, - after evaporation smaller amounts of material A were recovered and also after elution of the silica column less oil was found back, - after three times elution of the Amberlite columns with MTBE, a few percent of the oil could still be extracted with ethanol (results not shown) and - from HexN, which was after the evaporation of the hexane, directly reextracted from the P-buffer, only about 85 % of the original material B was recovered (Figures 7.7, 7.8). A part of the oil might still stick on the silica gel, as the eluate from a wet silica column contained only about 40 % of the original hexane extract after 3 times rinsing with 0.5 ml MTBE (results not shown).

From this distillation experiment, we can conclude that a prior extraction with hexane is not necessary. In fact, direct distillation of dried material results in even better yields. The distillation of the roots takes longer than the distillation of the hexane extract, but already after the first distillation step (up to 5 hours), more oil was extracted than after three extractions with hexane.

Adsorption capacity of XAD-2 columns

The distillation of the roots has proven that more oil can be retained by the 2 cm Amberlite column than the amount of oil in 1 ml hexane extract. Therefore different amounts of hexane extract 1 (1, 1.5, 2, 2.5, 3, 4 ml) were loaded on silica and subsequently distilled in P-buffer for about 5 hours, to determine the adsorption capacity of a 2 cm XAD-2 column.

The 2 cm XAD-2 column was able to adsorb all tested amounts of oil from the steam (Figure 7.9). Only for the smallest amounts, 1 and 1.5 ml hexane extract, less oil was recovered in the eluate. Most probably some oil was lost from all samples, for example by sticking to the Amberlite or to the glass, but for smaller amounts of oil such losses had a bigger effect. Between 2 and 4 ml of hexane extract all volatile compounds were adsorbed by the column. Higher amounts of extract were not tested, as with 4 ml hexane extract 1 already 12 % of the total material B and 17 % of the total material C were rinsed through the silica column during loading of the hexane extract. The residual buffers

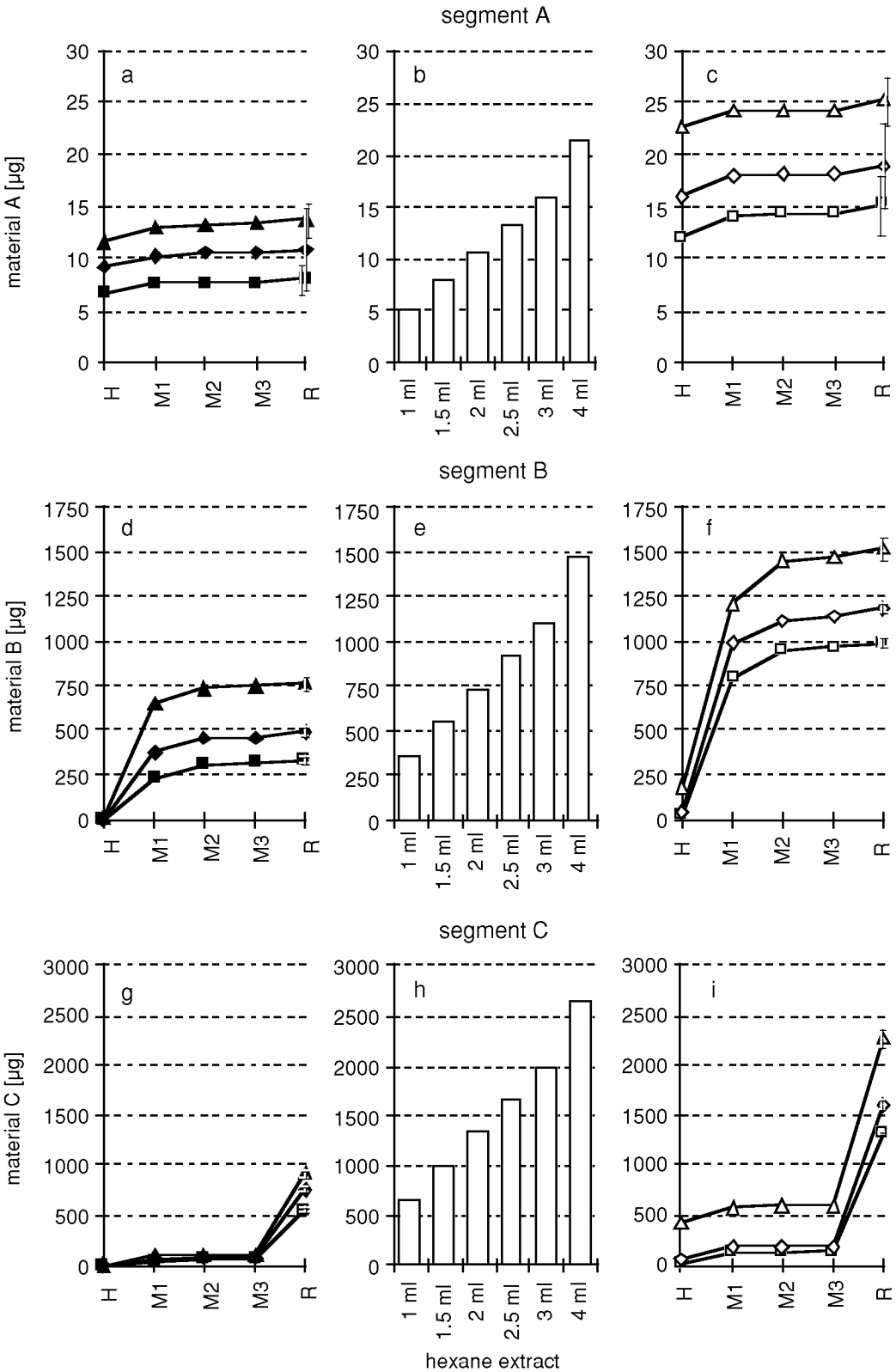
segment A
segment B
segment C
material A [µg]
material B [µg]
material C [µg]
a
b
c
d
e
f
g
h
i
H
M1
M2
M3
R
1 ml
1.5 ml
2 ml
2.5 ml
3 ml
4 ml
hexane extract

Figure 7.9.

contained about 5 % of the total material B and up to 74 % of the total material C (Figure 7.9).

Comparison of small scale hexane extraction and combined distillation - solid phase extraction to screen for oil variants

In this chapter hexane extraction and distillation with phosphate buffer were optimized for a small amount of root material (100 mg). For hexane extraction, roots were pre-wetted and subsequently extracted three time with hexane. For distillation, the roots were distilled in phosphate buffer (0.5M, pH8) in an oven at 120°C, with an Amberlite XAD-2 column on the tube to adsorb the oil from the steam. The column was rinsed three times with MTBE.

For both methods, the major part of the oil was extracted from the roots in the first extraction step and from the Amberlite column in the first elution step. Additional material was extracted in subsequent extraction or elution steps, but the concentration of the total extract decreased, and more solvent needs to be removed to detect the oil components by GC, especially for small amounts of oil. The ratio of the material Ba - Bf was about the same after the first, second or third extraction or elution step, respectively. Therefore, it is possible to use only one extraction or elution step. Due to variations in the recovery of the first eluate compared to the total eluate after three elutions or extractions, the

Figure 7.9. Adsorption capacity of a 2 cm XAD-2 column for small scale distillation combined with solid phase extraction. Different amounts of hexane extract (1 (■), 1.5 (◆), 2 (▲), 2.5 (□), 3 (◇) and 4 ml (△)) were loaded on silica columns (outflowing hexane: H). The silica was distilled in a miniaturized distillation apparatus with a 2 cm XAD-column (see Figure 7.1) to adsorb the vetiver oil from the steam. After the distillation, the XAD-2 columns were eluted with methyl *tert*-butyl ether (M1 - M3) and the residual buffer was extracted (R). All samples were analyzed by GC. The recovery of material A, B and C was determined after each extraction step.
a, c, d, f, g, i) sum of the material X of the outflowing hexane during loading of the silica column, the eluate of the XAD-2 columns after distillation and the residual buffer extracts
b, e, h) material X of extract used as starting material, calculated from GC analyses of 1 ml hexane extract

quantitative analysis will not be exact, but qualitative changes of the vetiver oil will be detectable.

With both methods, the concentration of the very small amounts of induced oil in the eluate (2 ml) or extract (1.5 ml) will still be too low for detection by GC. Therefore, before the analysis of the extract by GC, a concentration step has to be added.

Hexane extraction of the roots is more convenient to perform as the pre-wetted roots have only to be put in hexane and extracted over night, whereas for the distillation, columns have to be prepared and eluted after the distillation. However, the removal of the non-volatile compounds of the hexane extract with the tested methods was either not effective or, as in the case of the combined distillation - solid phase extraction, was not necessary, because roots can be distilled directly. Therefore hexane extraction is a useful method only if non-volatile compounds do not disturb the analysis, as it is for TLC.

Finally, if more sensitive analysis methods, such as GC, should be used, distillation with the Amberlite column is a very useful tool.

ACKNOWLEDGEMENTS

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Chapter 8: Initial study on induction of vetiver oil production and accumulation in tissue culture: material preparation

Ruth E. Leupin, Charles Ehret, Karl H. Erismann and Bernard Witholt

ABSTRACT

To pre-screen regenerated vetiver plantlets for changes in oil composition in tissue cultures, oil production and accumulation must be induced. Since the state of differentiation might influence the induction of essential oil biosynthesis, *in vitro* plantlets, root cultures and calli should be tested. Nevertheless, the oil content of *in vitro* tissue is expected to be low and enough tissue for extraction and analysis is therefore necessary. After about 4 - 6 months growth, about 100 mg (dw) roots can be obtained from *in vitro* plantlets. By varying the plant growth regulator composition in the medium, plants with different root types were obtained, which could be interesting for oil induction experiments. All trials to obtain continuously growing root cultures were unsuccessful. However, continuously growing root cultures might not be necessary for the induction of oil biosynthesis or precursor feeding. The calli from root tips grown on modified MS medium supplemented with NAA are a very interesting tissue for the induction of oil production or accumulation, since some of them smell of vetiver oil. However, here also it takes at least 2 months to obtain large calli for oil extraction.

INTRODUCTION

Vetiver oil is a valuable raw material in perfumery. New vetiver variants containing more of the essential oil or another ratio of the different compounds are therefore of interest. New variants could be obtained either by traditional breeding (Gupta *et al.* 1983; Lal *et al.* 1998; Sethi 1982; Sethi and Gupta 1980) or via tissue cultures (Chapter 3, Leupin *et al.* 2000), potentially altered with additional chemical or physical mutagenesis. To determine whether the regenerated plantlets are oil variants, the oil has to be analyzed and compared with the original oil. By inducing the essential oil biosynthesis and accumulation at an early stage, the screening can be done specific for changes in the vetiver oil. Since undifferentiated cultures only rarely accumulate monoterpenoids or sesquiterpenoids in quantities that are comparable with those present in the parent plants (Charlwood and Charlwood 1991), *in vitro* cultures of vetiver are

expected to contain very small amounts of oil, implying that the oil production or accumulation has to be induced, and enough material has to be available to extract and analyze the oil with the miniaturized procedures described in chapters 5 and 7.

In this work, we studied the usefulness of different tissue cultures such as *in vitro* plantlets, root cultures and callus cultures for the induction of vetiver oil production or accumulation and the feasibility to produce enough tissue to analyze the oil content.

MATERIAL AND METHODS

In vitro plantlets

To study the influence of the growth medium on the root production of *in vitro* plantlets, they were cultured on VRM0, VPM, VRM8 (composition see chapter 2) or VBM (modified MS medium (see Chapter 2) supplemented with 1 mg l^{-1} α-naphthalene acetic acid (NAA)). The plantlets were transferred every 6 - 8 weeks, after reducing the leaf length and removing the roots, to fresh medium. All *in vitro* plantlets were cultured at 23°C with a 12 hours photoperiod.

Root cultures

To obtain a growing root culture which can be subcultured, 5-10 mm long root tips of plantlets grown for several subcultures on VBM, were cut 2 weeks after the last transfer to new VBM and were cultured on different liquid media. These media were 1/2 Murashige & Skoog medium (1/2 MS) (Murashige and Skoog 1962) or VWM (0.25 g l^{-1} KCl, 0.144 g l^{-1} $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.5 g l^{-1} NH_4NO_3, 0.25 g l^{-1} $MgSO_4 \cdot 7\ H_2O$, 2 g l^{-1} NaCl, 0.835 g l^{-1} KH_2PO_4, 0.688 g l^{-1} $Na_2HPO_4 \cdot 2\ H_2O$, 4 mg l^{-1} $FeCl_3 \cdot 6\ H_2O$, 0.5 mg l^{-1} thiamine-HCl, 3.0 % sucrose, pH 7) supplemented with the growth regulators 2,4-dichlorophenoxy acetic acid (0 - 0.1 mg l^{-1} 2,4-D) or NAA (0 - 1 mg l^{-1}) and casein hydrolysate (0, 0.05, 0.5, 5 g l^{-1}). The growth of the roots was measured after 1 month in culture.

To obtain continuously growing cultures, they were subcultured by transferring either the complete roots or root tips (5 mm) to new medium.

Root calli on solid medium

To induce calli from roots, 5 mm long root tips from plantlets grown on VBM or VRM0 were cultured on solid modified MS medium containing various concentrations of NAA or 2,4-D (0.5, 1, 2.5 mg l^{-1}).

RESULTS AND DISCUSSION

The *in vitro* accumulation of monoterpenoids and sesquiterpenoids was reported in callus, suspension cultures and callus with differentiating roots or shoots, as well as in shoots and root organs and in fully differentiated tissue (Charlwood and Charlwood 1991). To induce the essential oil in tissue cultures of vetiver, different starting materials, including *in vitro* plantlets, root cultures, callus cultures or liquid cultures should be considered.

In vitro plantlets

By using *in vitro* plantlets, the same tissues are available as in *in vivo* plants. Precursors or parts of the oil could be produced in any tissue and be transported to the roots. To obtain sufficient roots for the analysis, media containing different growth regulators were tested. On vetiver propagation medium VPM, some roots grew only after a long time without subculture. On VRM8 the roots did not develop side-roots, whereas without any growth regulators (VRM0), the roots have side-roots. On VBM medium calli were first induced, and later thick roots with side-roots were produced. After several subcultures on VBM, the callus production was reduced and the roots were induced faster after subculture. By reducing the NAA content in the medium, the callus induction could already be reduced in the first culture step.

Since VRM0, VRM8 and VBM each resulted in roots with different phenotypes (Figure 8.1), it was of interest to test each of these root types in oil

production experiments. However with all three media, it took more than 6 months until 100 mg dry roots were obtained. To increase the amount of roots formed, the effect of solid versus liquid medium on root growth was examined. For that purpose, plantlets were cultured on VRM0 containing different agar concentrations (0.3, 0.65, 0.9 %) and on liquid VRM0, using wire-screens to support the shoots. After 2 months, no obvious trend was detectable. Only after 4 months, plantlets on the wire-screens produced slightly more roots, but 4 - 6 months were still necessary to obtain 100 mg roots (dw) per plantlet (data not shown). One possible advantage of liquid medium is that subsequently added compounds for the induction of vetiver oil biosynthesis distribute more easily in liquid than in solid medium.

To test the influence of the age of the plantlets on root production, plantlets, which were previously subcultured for 0, 1, 5 or 12 times on VRM8, were grown on VRM0. The resulting dry weights of roots were similar for all plantlets, except for plantlets which were directly transferred from the propagation medium. These produced less root material at the beginning, but over time the difference with plants which were subcultured at least once, dwindled and after 8 months all differences disappeared (data not shown).

With all tested methods, it was not possible to obtain 100 mg dry roots within less than 4 - 6 months and for long cultures the 400 ml beakers are too small. Therefore 1.5 l glass jars were tested, and single plants were cultured in jars on solid VRM0 medium. Some of the plantlets died and the surviving plantlets produced fewer roots in 6 months than in the beakers. After 12 months only 150 - 300 mg roots (dw) were obtained per plant. Hence, the growth conditions need further improvement.

Since a given treatment of a plantlet might have an influence on subsequent oil induction, the plantlets that are used for one induction experiment can not be used for a later repetition. The plantlets must therefore be propagated before induction experiments, especially as the oil induction treatment could harm the plant.

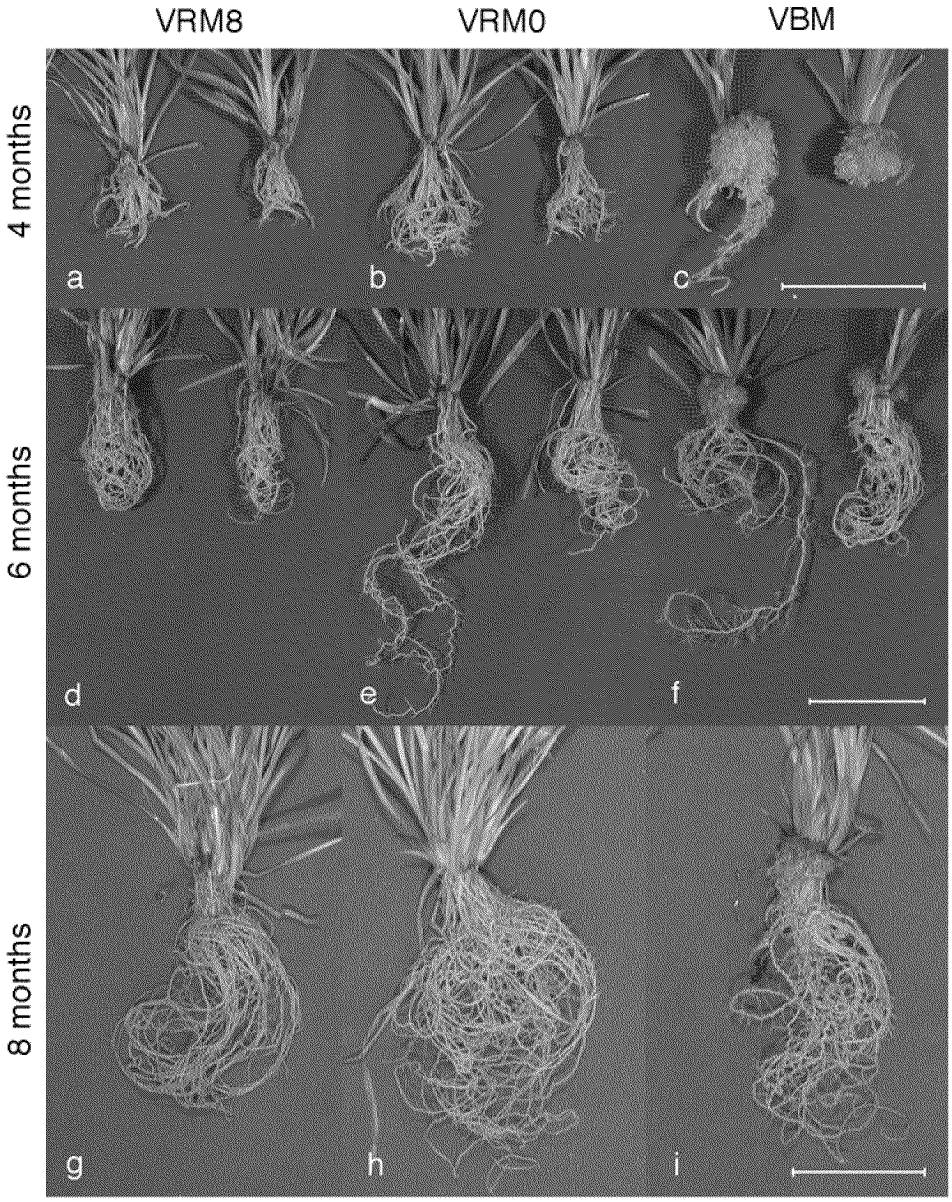

Figure 8.1. Root production of *in vitro* plantlets on different media. Culture of plantlets on media supplemented with different amounts of growth regulators (VRM8: modified MS medium supplemented with 1 mg l^{-1} kinetin and 0.1 mg l^{-1} indole acetic acid (a, d, g); VRM0: modified MS medium with no growth regulator (b, e, h); VBM: modified MS medium supplemented with 1 mg l^{-1} α-naphthalene acetic acid (c, f, i)) resulted in roots with different phenotypes. The plants were harvested after 4 (a, b, c), 6 (d, e, f) and 8 months (g, h, i). bars = 5 cm.

Root cultures

The essential oil is mainly found in the roots, although traces may be found in the rest of the plant (Vietmeyer and Ruskin 1993). Therefore, root cultures would be a useful starting material for oil induction experiments.

To obtain a growing root culture which can be subcultured, root tips were cultured on different liquid media. The best growth was obtained with VWM medium supplemented with 50 mg l^{-1} casein hydrolysate (VWM-C), which not only improved the growth but also the number of side-roots per root. On average, the roots grew to only 20 mm and became thin. These roots did not grow any more after subculturing their root tips (5 mm). The variation between the roots was large (results not shown) and it was not possible to obtain a continuously growing root culture, producing enough roots to analyze the oil content, with any of the tested media. However, for oil induction it might not be necessary to have continuously growing roots; it might be enough to have living roots. Since roots which were transferred to new medium without reducing their sizes still grew a little and continued to produce new side-roots (data not shown), we concluded that they are still alive after culturing for 4 weeks in VWM-C. It might therefore be possible to cut the complete roots of *in vitro* plantlets and culture them during the oil induction in liquid VWM-C. The advantage of such root cultures is that the regenerated plants are not influenced by the induction experiment and the experiment can be repeated as soon as enough roots are available again.

Hairy root cultures

Another approach to obtain continuously growing root cultures is to infect the plant with *Agrobacterium rhizogenes*, resulting in hairy root cultures. *Agrobacterium* is a soil bacterium, that causes gall (*Agrobacterium tumefaciens*) and hairy root formation (*Agrobacterium rhizogenes*) by transferring parts of the bacterial Ti (tumor inducing) or Ri (root inducing) plasmid DNA into the nuclear genome of plant cells (Conner and Dommisse 1992). Hairy root cultures commonly exhibit very high growth rates, can be cultured in the absence of plant growth regulators and generally retain the capability of the parental tissue

to accumulate secondary products (Charlwood 1993; Charlwood and Charlwood 1991). Monocotyledonous plants, particularly cereals, have been considered outside the host range for *A. tumefaciens*, because most monocotyledons do not form tumors as a result of *A. tumefaciens* inoculation (Smith and Hood 1995). However, gene transfer and consequently infection by *A. tumefaciens* have been proven for many monocotyledons, including *Zea mays*, *Triticum aestivum*, *Hordeum vulgare* and *Oryza sativa*. The missing tumor formation in monocotyledons is due to a lack of transcription, or to different endogenous hormone physiology compared with dicotyledons (Smith and Hood 1995). However, if the lack of tumor production after infection with *A. tumefaciens* is due to the different endogenous growth regulator physiology of monocotyledons, the chance to obtain "hairy" root cultures from vetiver after infection by *A. rhizogenes* might be small.

Callus culture on solid or in liquid media

Callus cultures on solid media

Another possibility for screening the regenerated plantlets is to use tissue cultures, like callus cultures on solid or liquid media, for the essential oil induction experiments. To induce calli, leaf slices, crown slices (Chapter 2) and root tips can be used, but since the plant should not be destroyed, root tips are ideally used as explants. Therefore, root tips were cultured on solid media containing various concentrations of NAA or 2,4-D (0.5, 1, 2.5 mg l^{-1}). With both growth regulators, calli were induced. On some calli from plates containing 0.5 or 1 mg l^{-1} NAA, root tips were observed. These calli are also interesting, since the accumulation of monoterpenoids or sesquiterpenoids of undifferentiated cultures could often be improved by some degree of cytodifferentiation, or even morphological differentiation (Banthorpe 1988; Charlwood and Charlwood 1991; Charlwood *et al.* 1989). However, it took at least 1 - 2 months to obtain larger calli (data not shown).

Nevertheless, these cultures are very interesting, because some calli from root tips cultured on modified MS medium supplemented with 0.5 and 1 mg l^{-1} NAA produced a smell similar to that of vetiver oil. A very concentrated methyl

tert-butyl ether extract of these smelly root calli and of their medium showed similar spots on a thin layer chromatogram (silica gel plates, developed with hexane and chloroform as consecutive solvents and stained with anisaldehyde-acetic acid-sulfuric acid; Chapter 5). These compounds ran with the same Rf as the acid components and (very faintly) at the lowest "non-acid" spot of the vetiver oil (Figure 8.2). Unfortunately, the induction of smelling calli was not reproducible. However, the results of these experiments should not be ignored in later stage oil induction experiments.

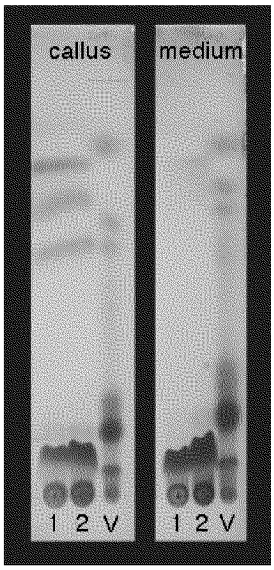

Figure 8.2. Thin layer chromatogram of extracted root calli and their media. Calli from root tips were induced on medium containing 0.5 (sample 1) or 1 mg l^{-1} α - naphthalene acetic acid (sample 2). The calli and the corresponding media were separately extracted with methyl *tert*-butyl ether. The concentrated extracts were analyzed by thin layer chromatography (developed with hexane and chloroform as consecutive solvents and stained with anisaldehyde-acetic acid-sulphuric acid) and compared with vetiver oil (25 µg, sample V).

Liquid cultures

Previously, we obtained well growing liquid cultures that were tested for plantlet regeneration, consisting of compact clumps (Chapter 4; Mucciarelli and Leupin, in press). The starting material for these cultures were compact calli from leaf and crown slices, which are in principle not available when the plant should stay alive. The induction rate of these liquid cultures was low and it took more than 6 months until the cultures were homogenous. Therefore, induction of these liquid cultures is not useful to screen the regenerated plantlets. However, such liquid cultures might be interesting to produce the vetiver oil in reactors. For this purpose, the growth rate, biomass level and yield of vetiver oil

in the *in vitro* material has to be sufficient to compete with oil produced *in vivo* on plantations.

As the occurrence of somaclonal variation increases with the duration of the disorganized phase and the extent of the disorganization (Karp 1994), it has to be taken in account that by using callus or suspension cultures for pre-screening, detected oil changes might arise during the culture and may not be intrinsic to the plant.

CONCLUSIONS

Different methods are now available to obtain *in vitro* vetiver tissue for experiments to induce the oil biosynthesis or accumulation.

In vitro plantlets produce about 100 mg roots (dw) in 4 - 6 months, with different phenotypes of the roots, depending on the plant growth regulator composition in the medium. The plantlets could be used directly for oil induction or the complete roots could be cut off and cultured in liquid medium for oil induction. Another very promising tissue is callus from root tips. To test if a differentiation has to take place for the production and accumulation of vetiver oil, it is necessary to distinguish between unorganized calli and calli with regenerated root tips.

Using the material described in this study, oil induction experiments can be started. These experiments will show whether it is feasible to produce vetiver oil *in vitro*, and whether plants can be screened in an early stage after regeneration.

Chapter 9: Conclusions and outlook

The aim of this project was to obtain vetiver variants with a higher vetiver oil yield or a different oil composition via tissue culture. To reach this goal, we developed procedures to regenerate plantlets via tissue cultures. Additionally we optimized methods to extract the oil from many small samples and detect qualitative and quantitative changes.

1. TOOLS TO OBTAIN VETIVER VARIANTS

1.1. *In vitro* cultivation of vetiver

During this study an efficient plant regeneration system via callus culture was developed for the used non-flowering vetiver variant from Java. In contrast to the other regeneration methods described for vetiver, crown and leaf slices of *in vitro* plantlets were used as starting material. Regenerated plantlets were found within 18 weeks on up to 55 % of the crown slices and on up to 60 % leaf slices. The regeneration via liquid cultures was less successful than via callus, since it took 6 - 14 months before compact structures developed and even longer before the cultures were homogeneous and at that time plantlets did not regenerate anymore. Therefore, regeneration via callus is the more promising method to obtain somaclonal variants.

It is not possible to predict whether our regeneration method via callus would also work for other vetiver variants. Other regeneration methods did not result in regenerated plantlets from our *in vitro* non-flowering vetiver variant from Java. It would be interesting to know whether the lack of regeneration ability is related to the medium or the starting material (*in vivo* or *in vitro* explants). At the same time it would be necessary to test whether our regeneration method is also useful for other vetiver variants.

1.2. Obtaining vetiver variants

First, regenerated plantlets should be screened for somaclonal variants with the desired trait. If the frequency of variation is too low, it can be increased by

inducing mutations by irradiation or mutagenic chemicals. To avoid chimeric plants due to mutation of pre-existing meristems, irradiation of leaf slices, subsequent callus induction and plant regeneration would be a useful procedure. However, the correlation between irradiation dose rate and regeneration rate must still be determined.

To obtain variants via genetic engineering, extensive studies are necessary, since nothing is known about the molecular biology of vetiver and genes that limit the biosynthesis of the vetiver oil components must be identified.

2. IDENTIFICATION OF VARIANTS

To determine whether the regenerated plantlets are oil variants, the oil has to be analyzed and compared with the original oil. Since it takes about 15 - 22 months until the plant contains the complete vetiver oil (Roth and Kornmann 1997; Weiss 1997a), and starting from *in vitro* plantlets even more time is needed, a pre-screening of the plantlets in an earlier stage would be advantageous. DNA based pre-screenings have been developed, based on random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (ALFP), microsatellite repeat polymorphism (also known as simple sequence repeat) and cleaved amplified polymorphic sequence (CAPS) (Jones *et al.* 1997; Rafalski and Tingey 1993; Ridout and Donini 1999). Unfortunately, nothing is known about the relationship between vetiver oil production and such DNA based changes. As a result it is necessary to screen for changes of plant phenotype and especially for the production of more or altered oils.

2.1. Analysis methods

Different analysis methods have been compared for their ability to detect qualitative and quantitative changes of the vetiver oil, as a pre-screen of many small samples from *in vitro* cultures. Neither olfactory detection nor the inhibition of bacterial growth were able to replace the time consuming gas chromatography. The gain of time was too small compared to the loss of information.

With thin layer chromatography, qualitative and quantitative changes and even the non-volatile compounds of the extract were detectable. However, GC analysis provides more detailed information and requires only 0.5 µg oil per analysis. Therefore, TLC is preferred for a preliminary analysis of many samples, whereas GC analysis provides more detailed information for a smaller set of selected samples.

2.2. Extraction methods

To extract the oil from the roots, water distillation and solvent extraction were compared for their effectiveness in extracting all vetiver oil and for the possibility to miniaturize the procedures for many small samples.

The long distillation time necessary for vetiver roots could be reduced by using a phosphate buffer (0.5M) at pH 8 instead of water. Additionally, the replacement of the cooling system by an Amberlite column made it possible to distill many small samples in parallel in an oven at 120°C.

All the tested solvents (hexane, methyl *tert*-butyl ether, ethyl acetate and ethanol) extracted about the same amount of volatile components but different amounts of non-volatile compounds, of which fewest were extracted with hexane. Due to the simple equipment requirements, hexane extraction could be miniaturized to extract 100 mg roots in 1.5 ml hexane.

Although the extraction method has an influence on the oil composition, the gas chromatograms of the hexane extract and the distillate were similar. The choice of the extraction method depends therefore largely on the experiment. Hexane extraction is less labor intensive than combined water distillation with solid phase extraction. However, hexane also extracts non-volatile compounds, which complicated subsequent gas chromatography analysis.

For both extraction methods, one problem is the dilution of the samples. If only trace amounts of the oil are available, a concentration step has to be included, since GC analysis requires at least 0.5 µg oil per analysis. To concentrate the total oil, evaporation with nitrogen is very promising for small amounts of hexane extracts or MTBE eluates, whereas for larger amounts of hexane extracts the silica column method alone or in combination with a subsequent evaporation with nitrogen is useful.

2.3. Amount of tissue necessary for small scale extraction and subsequent analysis

The yield of vetiver oil from *in vivo* vetiver roots varies between 0.1-3.3 % oil, depending on the vetiver variant (Akhila *et al.* 1981; Anonymous 1976; Roth and Kornmann 1997). To analyze the oil by gas chromatography (GC) about 0.5 µg oil is necessary per injection (Chapter 5). However, the minimized extraction does not extract the total oil in one extraction step (Chapter 7) and for better handling we need 100 µl extract. Therefore, about 5 mg dry roots are necessary for roots containing 1 % oil, and for plant material containing less than 0.1 % oil, more than 50 mg dry material is needed. Since undifferentiated cultures only rarely accumulate monoterpenoids or sesquiterpenoids in quantities that are comparable with those present in the parent plants (Charlwood and Charlwood 1991), *in vitro* cultures of vetiver are expected to contain very small amounts of oil, implying that the oil production or accumulation has to be induced, and enough material has to be available to extract and analyze the oil with the miniaturized procedures.

3. INDUCTION OF OIL BIOSYNTHESIS AND ACCUMULATION

The ability to pre-screen regenerated plantlets depends on the possibility to induce the oil production and to accumulate the oil in the plantlets or the *in vitro* cultures. Therefore the next step will be to find a way to induce the biosynthesis and / or to accumulate the vetiver oil in *in vitro* cultures.

To induce the essential oil biosynthesis in the plant, it would be useful to know why the plant produces it. Essential oils are not absolutely required for viability, though especially mono- and sesquiterpenoids are important mediators of plant-plant, plant-insect, and plant-pathogen interactions and help to adapt the plant to different environmental conditions (Charlwood 1993; Kribii *et al.* 1999; Newman and Chappell 1999).

However, nothing is known about the biosynthesis pathway or the regulation of essential oil biosynthesis in vetiver. This implies that the induction of the oil will mainly be a matter of trial and error. Nevertheless, the induction might be

feasible, because some calli from root tips on modified MS medium supplemented with 0.5 and 1 mg l^{-1} NAA did smell of vetiver oil (Chapter 8). The induction of smelling calli, however, was not reproducible, so that a more systematic approach has to be followed to induce sesquiterpenoid biosynthesis or to increase the amount of oil compounds in vetiver tissue cultures.

3.1. Medium composition and environmental conditions

The usual approach to induce secondary product accumulation in plant cell cultures is to manipulate the culture medium by varying carbon and nitrogen sources and their concentrations, the phosphate concentration and the range of plant growth regulators, or to change environmental conditions such as temperature, light and gas composition (Scragg 1997; Stafford *et al.* 1986). Especially different concentrations and ratios of growth regulators are interesting, as they can influence the root type of the *in vitro* plantlets, as described above. For callus cultures, these manipulations may result in changes in cytodifferentiation or morphological differentiation which in turn may result in the production of more essential oil or oil with a composition that differs from that of undifferentiated cultures (Charlwood and Charlwood 1991; Charlwood *et al.* 1989)

3.2. Elicitor treatment

By applying stress to the plant cultures, they may react with secondary metabolite formation. Phytoalexin production is one of the plant-defense mechanisms already recognized against pathogens. Phytoalexins consist of flavonoid/isoflavonoids, terpenoids and polyacetylenes (Weissenborn *et al.* 1995). Their production is triggered either by pathogen attack or by biotic and abiotic elicitors (Chávez-Moctezuma and Lozoya-Gloria 1996). Biotic elicitors include plant pathogens (bacteria, fungi or viruses), applied in the form of living cells or fractions thereof (glucan polymers, glycoproteins, organic acids such as arachidonic acid, or fungal cell-wall material), degradative enzymes and resulting cell wall fragments of the plant (DiCosmo and Misawa 1985; Threlfall and Whitehead 1991), and signal molecules involved in defense reactions such

as methyl jasmonate (Singh *et al.* 1998) or salicylic acid (Ram *et al.* 1997). Abiotic elicitors include stress inducing conditions such as UV light, pH changes, osmotic stress, wounding and heavy metal ions (Holden *et al.* 1988; Threlfall and Whitehead 1988). The effect of any elicitor is dependent on the specificity of the elicitor, elicitor concentration, the duration of treatment and the growth stage of the culture (Holden *et al.* 1988). Treatment of plants or tissue cultures with elicitors leads not only to the accumulation of phytoalexins, but also other, non-antimicrobial compounds can be formed (Holden *et al.* 1988).

The vetiver plant shows resistance to infections by root-knot nematodes (*Meloidogyne spp.*) (West *et al.* 1999), the roots are used to repel insects (Vietmeyer and Ruskin 1993), the oil inhibits bacteria and fungi (Chaumont and Bardey 1989; Dikshit and Husain 1984; Gangrade *et al.* 1991; Gangrade *et al.* 1990; Hammer *et al.* 1999; Maruzzella and Sicurella 1960) (Chapter 5) and some bacteria are observed in the same root cells as the oil (Viano *et al.* 1991a; Viano *et al.* 1991b). The oil or at least some compounds could therefore have a defensive function *in vivo*. The question is whether the biosynthesis of oil is constitutive, inducible or both. The inducible phytoalexin biosynthesis takes place over a short, well-defined period of time (Wolters and Eilert 1983) and the phytoalexins are normally not detectable in healthy plants (Threlfall and Whitehead 1988).

Additional approaches to improve terpenoid biosynthesis are the induction of polyploidy by colchicine treatment, by precursor feeding or by diverting the carbon flux through the isoprenoid pathway towards the lower isoprenoids by inhibiting other pathways using the same precursors/ intermediates (Banthorpe 1988; Charlwood and Charlwood 1991; Charlwood *et al.* 1989).

3.3. Colchicine treatment

Lavania (1988, 1991) obtained a tetraploid vetiver plant with a significant improvement of the oil productivity, by treating germinating vetiver seeds with colchicine. Additionally, the tetraploid plants resulted in vetiver oils with enhanced levels of khusinol and reduced levels of β-vetivone, which caused a shift in optical rotation (Lavania 1988; Lavania 1991).

Since the treatment resulted in mixaploid plantlets (2n = 20 to 40), a selection for diploid and tetraploid plantlets had to be done. For the plants, the selection was done via subsequent sequential separation of tillers from the mixaploid plant (Lavania 1988). In our case, using callus or suspension cultures, it would be practically impossible to select for tetraploid cells, while mixaploid cultures are not useful for testing the parental plants for oil variations as the ploidy influences not only the oil content but also the oil composition.

3.4. Precursor feeding

The oil level of the plant cultures could be increased by precursor feeding. The terpenoid building block, isoprene diphosphate (IPP), can be synthesized in plants via two different biosynthesis pathways: the mevalonate pathway starting from acetyl-CoA via mevalonate (MVA) to IPP, which takes place in the cytoplasm, and the DOXP pathway from pyruvate and glyceraldehyde-3-phosphate via 1-deoxy-D-xylulose-5-phosphate (DOXP) to IPP, which is found in the chloroplasts (Lichtenthaler 1999). Examples of sesquiterpenoid biosynthesis are described, which show that IPP synthesized via both pathways can be used as building blocks for sesquiterpenoid biosynthesis.

The IPP biosynthesis via the mevalonate pathway is involved in the biosynthesis of sesquiterpenoids, since isoforms of (*S*)-3-hydroxy-3-methylglutaryl-CoA reductase (HMGR: enzyme catalyzing the irreversible conversion of HMG-CoA to mevalonate) are induced during the biosynthesis of sesquiterpenoid phytoalexins (Stermer *et al.* 1994). Incorporation experiments using radioisotopes of acetate or mevalonate resulted in efficiently labeled sterols or sesquiterpenoids in the cytoplasm (Rohmer 1999).

In contrast, in secretory cells isolated from glandular trichomes of peppermint, it was shown with precursor feeding, that the cytoplasmic pathway is blocked at HMG-CoA reductase and that the IPP utilized for both monoterpenoid and sesquiterpenoid biosynthesis was biosynthesized exclusively in plastids (Ramos-Valdivia *et al.* 1997).

The combination of IPP from both IPP pathways should also not be excluded, since for chamomile sesquiterpenoids two of the isoprene building blocks were predominantly formed via the DOXP pathway, whereas the third

unit was of mixed origin, being derived from both mevalonate and the DOXP pathway (Adam and Zapp 1998).

Therefore, precursors like acetate and mevalonate for the mevalonate pathway and pyruvate, deoxy-D-xylulose and methyl deoxy-D-xylulose (Lichtenthaler 1999) for the DOXP pathway should be examined. Since acetate and pyruvate are also involved in several other biosynthesis pathways, the intermediate mevalonate, deoxy-D-xylulose and methyl deoxy-D-xylulose would be more specific for terpenoid biosynthesis. Other precursors can also be tested, but it has to be considered that the precursors have to be taken up by the plant material.

3.5. Diverting the carbon flux

By diverting the carbon flux through the terpenoid biosynthesis pathway or more exactly through the sesquiterpenoid biosynthesis pathway and by inhibiting competing pathways, the amount of vetiver oil might be increased. However, acetyl-CoA and pyruvate are involved in the primary metabolism of plants (e.g. glycolysis, TCA-cycle, photosynthesis and fatty acid synthesis) and as these pathways are essential for viability, inhibition might be lethal to the cultures.

Within the terpenoid biosynthesis, sterol-, carotenoid- and other terpenoid synthesis might compete with the sesquiterpenoid biosynthesis for the IPP building blocks or for farnesyl diphosphate. In some cases plants redirect the precursor into the needed pathway by inhibiting competing pathways. Thus after a pathogen attack or an elicitor treatment with arachidonic acid, some plants inhibit the sterol biosynthesis and increase the sesquiterpenoid biosynthesis to improve the phytoalexin production (McCaskill and Croteau 1998; Newman and Chappell 1999; Weissenborn *et al.* 1995). In plant cultures, the essential oil accumulation has been increased in some cases by using carotenoid or sterol synthesis inhibitors. However, in *Pelargonium tomentosum* shoot proliferation cultures, a progressive dedifferentiation resulting in non-accumulating aggregate cultures was observed, dependent on the inhibitor used (Charlwood *et al.* 1989). Thus, it might be possible to improve the vetiver oil biosynthesis by inhibiting the sterol biosynthesis.

4. END PRODUCT TOXICITY

To be able to analyze the essential oil in the culture, the oil biosynthesis must not only be induced but the oil should also accumulate. Since it has been demonstrated that many monoterpenoids and sesquiterpenoids are toxic to plant cells, end-product toxicity may be the regulating factor in oil accumulation in undifferentiated cultures (Banthorpe 1988; Charlwood *et al.* 1989). However, a lack of product accumulation does not always mean a lack of pathway enzyme expression. Some cultures that did not accumulate detectable quantities of monoterpenoids or sesquiterpenoids, nevertheless appeared to possess the full enzymatic machinery (Banthorpe 1988; Charlwood 1993). To accumulate product, the rate of its synthesis has to be larger than the rate of product catabolism. In several suspension cultures, it was shown that the conversion of the monoterpenoids was higher than the rate of their synthesis (Charlwood 1993) and thus the cultures do not toxify themselves.

To obtain terpenoid accumulation, a possibility is to induce sufficient differentiation to allow formation of storage or excretion sites, like secretory cells, resin or oil ducts, hairs, glandular epithelial cells, and trichomes, without reaching plantlet regeneration (Banthorpe 1988).

An alternative strategy that may minimize end-product toxicity and enhance productivity of the terpenoid accumulation would involve the continuous removal of monoterpenoids and sesquiterpenoids from the culture. In the case of an excretion of the essential oil to the medium, the accumulation of the terpenoids in undifferentiated cultures could be increased by using a lipophilic second phase (e.g. Miglyol) or by adding adsorbing resin (e.g. XAD) (Banthorpe 1988; Scragg 1997). Loss by volatilization of the excreted products could be avoided, labile products could be stabilized and by removing the toxic compounds the viability of the cultures could be maintained (Banthorpe 1988).

5. TOWARDS A PRACTICAL PROTOCOL FOR THE PRODUCTION OF VETIVER OIL VARIANTS

Since the culture condition and the culture type can influence the composition and content of the essential oil (Charlwood *et al.* 1989; Scragg 1997), the induced oil of the *in vitro* cultures may have a different composition than that produced by plants grown *in vivo*. However, if there are differences in the vetiver oil produced by the original plant and the regenerated plantlets when these are treated the same way, it is possible that there will also be differences after 15 - 22 months. There is the risk that oil variants are not detected because some compounds might not be induced with the selected treatment, or that observed changes are a matter of the treatment and not of genetic variation. It is important therefore for a useful pre-screening method, that the induced oil has a composition similar to that of the oil extracted from plants cultivated for 2 years on soil. Furthermore, the oil production and accumulation *in vitro* should take place in less time than needed *in vivo*. Finally, to prove that the obtained compounds are oil compounds, it is necessary to analyze them by GC-MS.

After developing methods to induce vetiver oil synthesis, regenerated plantlets are pre-screened. The selected putative oil variants must subsequently be cultured for 2 years on soil, to confirm that they are variants. Variants can then be analyzed genetically or biochemically for the mutated trait, ultimately perhaps permitting a search for variants by analysis of DNA modifications in plantlets developed *in vitro*.

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