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Differential incorporation of 1-deoxy-D-xylulose into (3*S*)-linalool and geraniol in grape berry exocarp and mesocarp

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Abstract

In vivo feeding experiments with [5,5-$^2H_2$]mevalonic acid lactone (MVL) and [5,5-$^2H_2$]-1-deoxy-D-xylulose (DOX) indicate that the novel mevalonate-independent 1-deoxy- D-xylulose 5-phosphate/2C-methyl- D-erythritol 4-phosphate (DOXP/MEP) pathway is the dominant metabolic route for monoterpene biosynthesis in grape berry exocarp and mesocarp and in grape leaves. The highly uneven distribution of the monoterpene alcohols (3*S*)-linalool and geraniol between leaves, berry exocarp and berry mesocarp can be attributed to a compartmentation of monoterpene metabolism. In grape berries incorporation of [5,5-$^2H_2$]-DOX into geraniol is mainly restricted to the exocarp, whereas (3*S*)-linalool biosynthesis can be detected in exocarp as well as in mesocarp tissue. The results demonstrate that grape berries exhibit an autonomic monoterpene biosynthesis *via* the novel DOXP/MEP route throughout the ripening process.

Keywords: *Vitis vinifera* L.; Vitaceae; Monoterpenes; Biosynthesis; 1-Deoxy-D-xylulose; Mevalonic acid

1. Introduction

Monoterpenes are biologically active molecules that belong to the structurally diverse group of isoprenoids. In higher plants, monoterpenes participate in a wide variety of functions and are synthesized and stored in specialized structures like glandular trichomes in several essential oil producing plants (Wise and Croteau, 1999). Numerous nonglandular plants, including many crop and ornamental species, produce at least trace levels of monoterpenes, some of which are components of herbivore or pathogen defense systems (Boland et al., 1998). In flowers volatile monoterpenes act as pollinating insect attractants (Raguso and Pichersky, 1999) and the odor impression of several edible fruits is determined by monoterpenes (Rouseff and Leahy, 1995). Particularly linalool has been shown to contribute to the characteristic varietal aroma of grapes of *Vitis vinifera* cultivars Muscat and White Riesling (Ebeler, 2001). In addition to the free odour-producing forms of monoterpenes, several glycosidically bound forms of monoterpenes have been described. Upon chemical or enzymatic hydrolysis these bound monoterpenes can contribute significantly to the characteristic floral aroma in Muscat and related aromatic grape varieties. These findings have been a major stimulus to research on free and bound monoterpenoids in grapes, especially on their structure elucidation, their distribution within the grape, and their changes in levels during grape maturation and vinification (Strauss et al., 1986). However, relatively little is known about the biosynthesis of monoterpenoids in *V. vinifera*. Free and bound monoterpenes were detected in blades and petioles of vine leaves as well as in the exocarp and mesocarp of berries (Gunata et al., 1985, 1986). The principal biosynthetic capability and contribution of each of these organs or tissues to the total biosynthesis of monoterpenes in *V. vinifera* is still unclear and it has been assumed that the bulk of monoterpenes is synthesized in the leaf blade and transported to the berries in glycosidically bound form (Gunata et al., 1986). Feeding experiments with the cultivar Muscat of Alexandria with ^{14}C-labelled mevalonic acid indicated that monoterpene glycosides were translocated from the leaves to the berries (Shoseyov, 1988). Nevertheless, it could be shown by

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approach grafting experiments that biosynthesis of monoterpene glycosides in the grape berry is independent of other parts of the vine and is determined by the genotype of the grape bunch (Gholami et al., 1995). In support of this finding is the demonstration of glucosidation activity in detached grape berries grown in vitro (Bravdo et al., 1990). This points to an autonomy of grape berries concerning terpenoid metabolism and, indeed, plastids isolated from cell suspension cultures of cv. Muscat de Frontignan berries are able to import isopentenyl pyrophosphate (IPP) which is incorporated into geranyl diphosphate by a geranyl-diphosphate synthase located within the plastids (Feron et al., 1990; Soler et al., 1993; Clastre et al., 1993). However, the cell suspension cultures used in these studies did not accumulate detectable amounts of monoterpenoids (as it is frequently observed in plant cell cultures of essential oil accumulating plants due to an increased activity of catabolic enzymes (Falk et al., 1990)) and the principal biosynthesis and origin of IPP in grapes remained unclear. Thus, the question of how monoterpene metabolism is compartmentalized between and within different tissues of complex structures such as fruit is largely unanswered. An immunohistochemical study of the compartmentation of sugar, organic acid and amino acid metabolism during the development of the flesh and seeds of grape berries has shown that the distribution of the corresponding enzymatic activities was extremely heterogeneous even in apparently structurally homogeneous tissues (Famiani et al., 2000).

Against this background it is important to note that recently a mevalonate-independent pathway has been discovered in bacteria, algae and plants that leads to the formation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the two basic precursors of monoterpenoids (for reviews see Lichtenthaler et al., 1997; Lichtenthaler, 1999, 2000; Rohmer, 1999; Eisenreich et al., 2001). According to the key intermediates 1-deoxyxylulose-5-phosphate (DOXP) and 2*C*-methyl-D-erythritol-4-phosphate (MEP) this novel route is called the DOXP/MEP pathway and operates in the plastids of higher plants where hemiterpenes, monoterpenes, diterpenes and carotenoids are formed. Isoprenoids synthesized in the cytoplasma and mitochondria like sterols and sesquiterpenes are formed predominately via the classical mevalonate route (MVA). However, the compartmental separation of the two pathways is not absolute and the extent of this crosstalk depends on the species and the physiological conditions (Arigoni et al., 1997; Piel et al., 1998; Adam et al., 1999). It is still unclear to which extent the MVA and DOXP/MEP route are utilized for monoterpenoid biosynthesis in *V. vinifera* and to which extent the demonstrated uptake of IPP in isolated plastids in vitro contributes to the biosynthesis in vivo. Therefore, the biosynthesis of monoterpenes in *V. vinifera* was investigated by in vivo feeding experiments using different berry tissues and deuterium labelled DOX and MVL with subsequent enantioselective multidimensional GC/MS analysis of the formed metabolites. The results demonstrate that in grape berry exocarp and mesocarp as well as in grape leaves the novel DOXP/MEP route is almost exclusively utilized for monoterpene biosynthesis. This localization of monoterpene biosynthesis in grape berry is an essential prerequisite for the selection of suitable grape tissues for more detailed studies on isolation and characterization of enzymes involved in monoterpene metabolism.

2. Results and discussion

For investigating the monoterpene biosynthesis in *V. vinifera* the cultivar Muscat Ottonel was chosen. This cultivar belongs to the so called Muscat group of the different grape varieties and exhibits a unique floral character because of high concentrations of volatile odiferous monoterpenes like linalool and geraniol (Ebeler, 2001). Total monoterpene concentration in this Muscat group can be as high as 6 mg/l in grape berry juice which largely represents the vacuolar sap of the mesocarp tissue (Strauss et al., 1986). The distribution of the main monoterpene alcohols linalool, geraniol, nerol, and citronellol in different berry tissues and in leaves of Muscat Ottonel cultivar is illustrated by Fig. 1. A highly uneven distribution of these monoterpenes is observed. The main monoterpene alcohol in leaves is geraniol (15 μg/g). Both enantiomers of linalool are present with an excess of the (3*S*)-enantiomer (*S*/*R* = 65/35). In grape berry exocarp the dominating monoterpene is linalool (0.2 μg/g) of high enantiomeric purity (*S*/*R* = 98/2). In grape berry mesocarp the relative proportion of linalool is even higher and all other monoterpene alcohols are only present at a very low level. However, the concentration of linalool (0.2 μg/g) is in the same range like in grape exocarp. The enantiomeric purity of linalool in berry exocarp and mesocarp is practically identical. The highly uneven distribution of monoterpenes in the different organs and tissues has also been observed in other Muscat varieties like Muscat de Frontignan and Muscat of Alexandria (Gunata et al., 1985; Wilson et al., 1986) and are qualitatively comparable to the results in this study.

The reason for this uneven distribution is still unclear and it has been suggested that the bulk of monoterpenes is synthesized in the leaf blade and transported to the berries in glycosidically bound form (Gunata et al., 1986). The distribution pattern would thus be a result of uneven translocation rates. However, as shown by Ambid and coworkers, plastids isolated from cell suspension cultures of cv. Muscat de Frontignan berries are able to import exogenous isopentenyl pyropho-

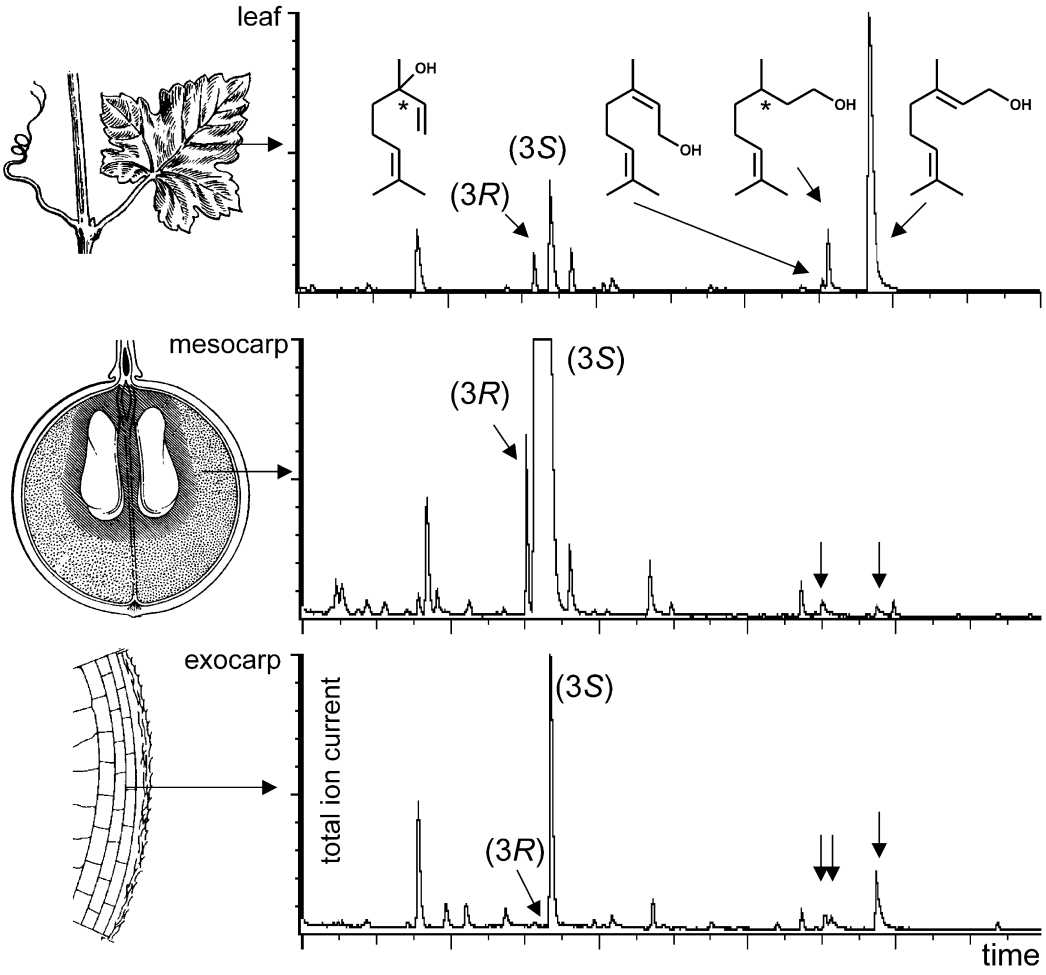


Fig. 1. Distribution of the main monoterpene alcohols linalool, nerol, citronellol and geraniol in different organs and tissues in *Vitis vinifera* cv. Muscat Ottonel.

sphate (IPP) which is incorporated into geranyl-diphosphate by a geranyl-diphosphate synthase located within the plastids (Feron et al., 1990; Soler et al., 1993; Clastre et al., 1993). The origin and biosynthesis of IPP was not investigated in these studies and the used cell suspension culture did not accumulate detectable amounts of monotoerpenes. That plastids of the grape berry pericarp nevertheless may play a central role in the synthesis of these constituents in vivo was examined by transmission electron microscopy using berries of cv. Traminer (Hardie et al., 1996). In this study it has been assumed that plastids in grape mesocarp cells and in dermal tissue cells are associated with monoterpene synthesis. The similarity of the appearance of the pleomorphic plastids throughout the tissues of the pericarp throughout its development appeared to indicate a degree of synchronised autonomic monoterpene biosynthesis within individual cells, and thus render unnecessary translocation of monoterpenes from the dermal tissue or leaves. The uneven distribution of monoterpenes in the different berry tissues and leaves would thus be a consequence of different enzymatic activities. In support of this assumption is the uneven enantiomeric purity of linalool in leaves and berries as it has been described above for the cultivar Muscat Ottonel pointing to different linalool synthases.

A suitable experimental design for addressing the question of compartmentation of monoterpene biosynthesis in grapevine would be in vivo feeding experiments with different grape berry tissues and grape leaves using suitable precursors, that allow the differentiation between the classical cytosolic mevalonic acid pathway and the novel plastidic mevalonate-independent DOXP/MEP pathway. Such experiments have not been performed yet with grapevine but would be a prerequisite to the selection of suitable tissues for any enzymatic studies on monoterpene biosynthesis in this plant. Therefore, [5,5-$^2H_2$]mevalonic acid lactone ([5,5-$^2H_2$]-MVL) and [5,5-$^2H_2$]-1-deoxy-D-xylulose ([5,5-$^2H_2$]-DOX) were chosen that are frequently used in incorporation studies to determine the biosynthetic origin of the basic building units IPP and DMAPP (Schwender et al., 1997; Arigoni et al., 1997; Piel and Boland, 1998; Sagner et al., 1998; Zeidler and Lichtenthaler, 2001).

Due to the low concentrations of monoterpenes in all parts of grapevine the recently developed stir bar sorptive extraction method (SBSE) was chosen for rapid isolation of the target compounds (Baltussen et al., 1999). SBSE was coupled to a thermal dersorption-enantioselective multidimensional GC–MS system and allows the sensitive detection of labelled metabolites and the concomitant enantioselective analysis of chiral compounds like linalool (Kreck et al., 2001).

For in vivo feeding studies aqueous equimolar solutions (19 μmol/ml) of the labelled precursors [5,5-$^2H_2$]-MVL and [5,5-$^2H_2$]-DOX were administered to grape leaves 2 weeks before anthesis using terminal shoots (approx. 10 g weight) and the cut stem method. Within 48 h the plant took up 7.5 μmol of each labelled precursor per gram tissue. Fig. 2 shows the main column chromatogram of linalool obtained from the leaf extract when [5,5-$^2H_2$]-DOX is admininstered. Highly labelled (3*S*)- and (3*R*)-[1,1,5,5-$^2H_4$]linalool is clearly detecable and separates well from the genuine linalool enantiomers due to the inverse isotope effect in gas chromatography of deuterium labelled compounds (Matucha et al., 1991). Also minor amounts of the enantiomers of [1,1-$^2H_2$]linalool and [5,5-$^2H_2$]linalool are detectable. The fragmentation pattern of the deuterium labelled linalool isotopomers is in agreement with the incorporation of one ([1,1-$^2H_2$]linalool and [5,5-$^2H_2$]linalool) or two ([1,1,5,5-$^2H_4$]linalool) units of labelled IPP/DMAPP that is generated from administered [5,5-$^2H_2$]-1-deoxy-D-xylulose (see Fig. 3). It is noteworthy that no loss of ^2H is detected in the formation of IPP/DMAPP from 5-^2H-labeled 1-deoxyxylulose. This result is in agreement with similar studies (Boland et al., 1998; Piel et al., 1998).

Fig. 4 shows the main column chromatogram of geraniol obtained from the same leaf extract. Again, deuterium labelled isotopomers of geraniol are clearly detectable and well separated from genuine geraniol. The fragmentation pattern proves incorporation of deuterium labelled IPP/DMAPP units. The incorporation rate of [5,5-$^2H_2$]-DOX into the main monoterpene alcohol geraniol in grape leaf was 1.2% (117 nmol

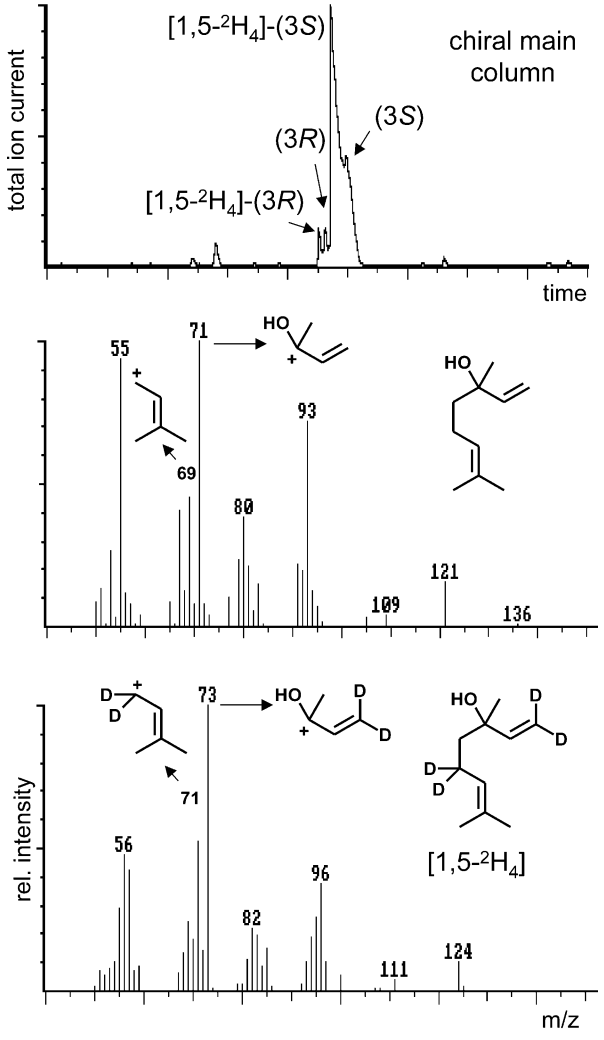


Fig. 2. Chiral main column chromatogram and MS spectra of unlabelled and labelled linalool obtained from a leaf extract when [5,5-$^2H_2$]-DOX is administered.

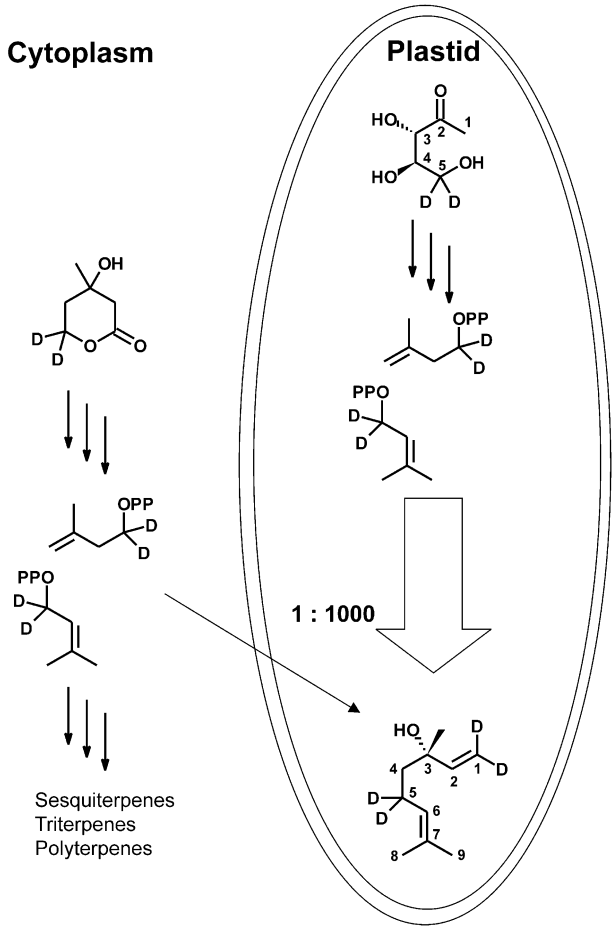


Fig. 3. Expected labelling pattern of (3*S*)-linalool upon incorporation of two units [5,5-$^2H_2$]-DOX and compartmentation model of monoterpene biosynthesis in grape.

[5,5-$^2H_2$]-DOX per gram tissue; see also Table 1) and exceeds the rates achieved with [1,2-^{14}C]-DOX and rooted shoots of *Mentha piperita*, *Mentha pulegium*, *Pelargonium graveolens*, and *Thymus vulgaris* by factors of at least 5–10-fold (Sagner et al., 1998). The relatively low incorporation rates in these essential oil producing plants was attributed to a limited transport of labelled DOX into the plant glandular trichomes or to a limited enzyme involved in the utilization of exogenous DOX (e.g. a kinase). When [5,5-$^2H_2$]mevalonic acid lactone is administered to grape leaves only trace amounts of labelled geraniol are detetable. The incorporation rate was below 0.001% (<0.1 nmol/g tissue; see Table 1). An incorporation into linalool was not detectable. This indicates that the novel DOXP/MEP pathway is the dominant metabolic route for monoterpene biosynthesis in grape leaves and exceeds the MVA pathway by a factor of at least 1000.

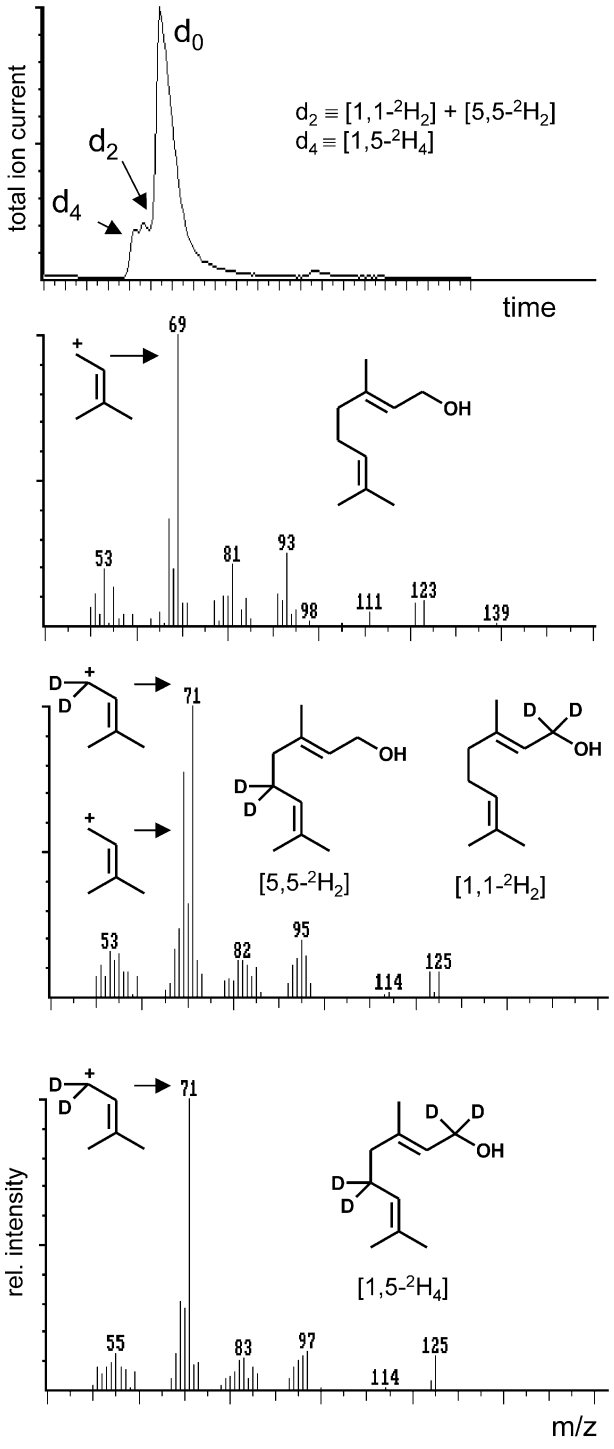
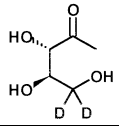
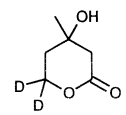


Fig. 4. Main column chromatogram and MS spectra of unlabelled and labelled geraniol obtained from a leaf extract when [5,5-$^2H_2$]-DOX is administered.

Fig. 5 shows the main column chromatogram of linalool and geraniol obtained from the vacuolar mesocarp sap when [5,5-$^2H_2$]-DOX is directly injected into the mesocarp of ripening grape berries. A significant incorporation of exogenous [5,5-$^2H_2$]-DOX into linalool is detectable. (3*R*)-[1,1,5,5-$^2H_4$]linalool and genuine (3*R*)-linalool are well separated. Due to the high concentration of genuine (3*S*)-linalool and (3*S*)-[1,1,5,5-$^2H_4$]linalool the column is slightly overloaded and a separation could not be achieved. However, the mass spectra measured in the first part and in the last part of the linalool peak allow to distinguish clearly between labelled and genuine linalool. The incorporation rate into linalool was 0.2% (3.6 nmol/g tissue) and is considerably lower when compared to the leave tissue. This may be attributed to a more efficient distribution of the exogenous precursor via the phloem system of the twig and leaf compared to the locally restricted injection and diffusion of the precursor in grape mesocarp. Additionally, the larger vacuoles of the juicy mesocarp tissue result in a lower cell density and thus in a lower biosynthetic capability per gram tissue (Gholami et al., 1995). It is remarkable that no labelled geraniol could be detected. This is in agreement with the observed uneven distribution of monoterpenes in the different grape tissues and

Table 1
Incorporation of [5,5-$^2H_2$]-DOX and [5,5-$^2H_2$]-MVA into the main monoterpene alcohols in different organs and tissues of *Vitis vinifera* cv. Muscat Ottonel

Tissue/organ	Incorporation of precursor (nmol/g tissue)	
		
Leaves (before anthesis)	117[a] (1.2)[b]	<0.1
Berry mesocarp (ripening stage)	3.6[c] (0.2)	n.d.
Berry mesocarp (ripe stage)	0.4[c] (0.04)	n.d.
Berry exocarp (ripe stage)	2.0[c]	n.d.

n.d., Not detectable.

[a] Incorporation into geraniol.

[b] Values in parentheses indicate the incorporation rate in % of the administered precursor.

[c] Incorporation into linalool.

shows that this phenomenon is directly correlated with the enzymatic activities within the tissues rather than with a differential translocation rate of the monoterpene alcohols. Thus, the dominance of (3*S*)-linalool in grape mesocarp is certainly due to a high (3*S*)-linalool synthase activity in this tissue. The incorporation rate into linalool in ripe berries was significantly lower (0.04%) when compared to ripening berries. No incorporation was detecable in ripening or ripe berries when [5,5-$^2H_2$]-MVL was injected into the mesocarp. This shows that grape berries exhibit, indeed, an autonomic monoterpene biosynthesis *via* the novel DOXP/MEP pathway throughout the ripening process and is in agreement with the observed accumulation of monoterpenes in berries during ripening (Gunata et al., 1985; Noble, 1994).

Fig. 5 shows the main column chromatogram of linalool and geraniol obtained from isolated grape skins (exocarp) of ripe berries that were incubated with an aqueous [5,5-$^2H_2$]-DOX solution. Again, a significant incorporation of exogenous [5,5-$^2H_2$]-DOX into linalool is detectable. As opposed to the mesocarp a significant incorporation is also evident for geraniol and suggests that the cells of the exocarp are sites of biosynthesis of this compound and that the cells of the mesocarp exhibit onyl a restricted geraniol synthase activity. The incorporation rate into linalool could not be determined in this incubation experiment because uptake of [5,5-$^2H_2$]-DOX from the feeding solution was not determined. However, 2.0 μmol of [5,5-$^2H_2$]-DOX per gram exocarp tissue were incorporated into linalool and this compares to an incorporation of 0.4 μmol of [5,5-$^2H_2$]-DOX per gram mesocarp tissue. No incorporation was detectable when skins were incubated with [5,5-$^2H_2$]-MVL.

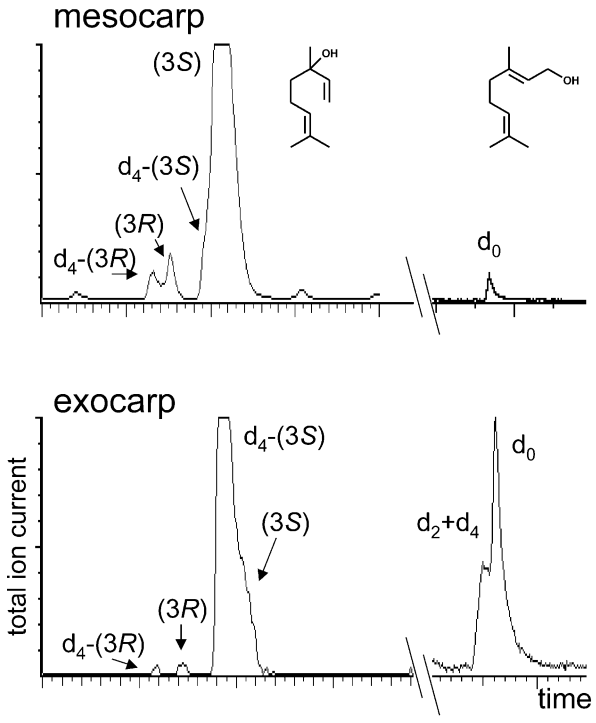


Fig. 5. Chiral main column chromatogram of unlabelled and labelled linalool and geraniol obtained from a mesocarp and exocarp extract when [5,5-$^2H_2$]-DOX is administered.

The results of the feeding experiments show that monoterpene metabolism is compartmentalized between different grape berry tissues: Geraniol biosynthesis is mainly restricted to grape berry exocarp, whereas linalool biosynthesis can be detected in exocarp as well as in mesocarp. Thus, the distribution of the corresponding enzymatic activities within the berry is extremely heterogeneous. If one assumes plastidic monoterpene biosynthesis in mesocarp and exocarp cells via the DOXP/MEP pathway the observed import of exogenous IPP into isolated plastids from cell suspension cultures (Soler et al., 1993) may be only a minor pathway for monoterpene biosynthesis under normal conditions (that means in the absence of specific inhibitors or stress conditions). However, observations with induced plants strongly suggest a cooperative use of both the MVA- and DOXP/MEP pathways in biosynthesis of terpenoid volatiles (Piel et al., 1998; Jux et al., 2001). This dynamic allocation of resources in induced plants may also be operative in grapevine and further detailed studies are needed.

3. Concluding remarks

The differential incorporation of DOX into geraniol and linalool within the berry may also be of importance for further studies on enzyme isolation and characterization. Isolation of the native enzymes from plant tissues is complicated by the presence of low molecular weight compounds such as phenolic materials which (particulary in the presence of phenol oxidases) can inhibit or denature the enzymes of interest (Wise and Croteau, 1999). It is therefore of importance to note that the concentration of phenolic compounds in grape berry skins is very high if compared to the concentration in grape mesocarp (Ribérau-Gayon et al., 2000). Hence, the suitable tissue for the isolation of a linalool synthase would be grape berry mesocarp.

If one compares the amount of monoterpene alcohols, that are generated from exogenous [5,5-$^2H_2$]-DOX within 48 h, to the amounts of genuine monoterpene alcohols, in some cases the amount of labelled compounds exceeds the amount of genuine compounds (see Figs. 2 and 5). This suggests that 1-deoxy-D-xylulose-5-phosphate synthase is a limiting enzyme for plastidic isoprenoid biosynthesis in grapevine as has been demonstrated with transgenic *Arabidopsis* plants that over- or underexpress this enzyme (Estévez et al., 2001).

When the regulatory mechanisms inducing fruit to produce monoterpene flavourants become known it

may be possible to alter varietal character of grapes by down- or upregulating activities of limiting enzymes such as 1-deoxy-D-xylulose-5-phosphate synthase or 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Mahmoud and Croteau, 2001).

4. Experimental

4.1. Plant material

Leaves and bunches of the *V. vinifera* L. cultivar Muscat Ottonel were obtained from the Research Centre Geisenheim, Department for Grapewine Breeding and Grafting (Geisenheim, Germany) during the vintage 2001.

4.2. Chemicals

(3*R*)-Linalool, (±)-linalool and (±)-citronellol were obtained from Fluka (Taufkirchen, Germany). Nerol, geraniol, and geranyl acetate were obtained from Roth (Karlsruhe, Germany). [5,5-$^2H_2$]Mevalonic acid lactone was prepared according to Simpson et al. (1997). [5,5-$^2H_2$]-1-Deoxy-D-xylulose was prepared according to Jux and Boland (1999). [8,9-$^2H_6$]Geraniol was prepared according to Pyun et al. (1993) and [8,9-$^2H_6$]-(3*R*)-linalool was prepared using the same procedure by substituting (3*R*)-linalyl acetate, which was prepared according to Vidari et al. (1999), as starting material for geranyl acetate. Spectral data of the labelled compounds were in all cases in good agreement with the data given in the references cited above.

4.3. Administration of labelled precursors

Approximately 2 weeks before anthesis equimolar aqueous solutions (19 μmol/ml) of [5,5-$^2H_2$]-1-deoxy-D-xylulose and [5,5-$^2H_2$]mevalonic acid lactone, respectively, were fed through a terminal cut stem into twigs and leaves (about 10 g of plant material). During a 48 h period 75 μmol of each precursor were taken up by the plant. For administration of labelled precursors to grape berry mesocarp two ripening stages were chosen: pre ripe stage (sugar content 3.1%) and ripe stage (sugar content 18.0%). A 20 μl volume of an aqueous solution (95 μmol/ml) of each labelled precursor was injected into the mesocarp of detached grape berries using a microliter syringe. The berries were incubated for 48 h in the dark at room temperature. In an field experiment the same volume of precursor solution was injected into berries of a bunch attached to the vine. The berries were detached after 48 h. For administration of labelled precursors to grape berry exocarp the skin was peeled from the berries and the inner surface was gently abraded in a mortar containing 10 ml buffer (100 mM potassium phosphate pH 7.5, 20% (v/v) glycerol, 50 mM ascorbate, 2.5 mM EDTA), 0.5 g Amberlite XAD-4 resin, and a spatula tip of purified sea sand per berry to ensure complete removal of mesocarp cells. A small piece (about 1 cm^2) of the skin was incubated with 200 μl of an aqueous solution (15 μmol/ml) of each labelled precursor. All feeding experiments were conducted in triplicate with appropriate controls.

4.4. Isolation of free and bound monoterpenes

Monoterpenes were extracted using the novel stir bar sorptive extraction method (SBSE). Theory and practice of SBSE have been described recently (Batussen et al., 1999). Stir bars were obtained from Gerstel (Mühlheim, Germany) under the trade name Twister®. Leaves or isolated berry skins were ground in liquid nitrogen and extracted with 4 ml of water per gram tissue. After centrifugation at 3000 *g* for 5 min the pellet was discarded and the supernatant was incubated with 2 mg of Glucanex® per gram tissue (Novo Nordisk, Dittingen, Switzerland) for 24 h at room temperature to release the glycosidically bound monoterpenes. The extract was saturated with sodium chloride and extracted for 30 min by SBSE. Grape berry mesocarp (flesh) was homogenized in a mortar and the juice was decanted. Glycosidically bound monoterpenes were released and extracted as described above.

4.5. Quantification of monoterpenes

Total amounts of linalool and geraniol in leaves and berries were determined using isotope dilution mass spectrometry (Heumann, 1986). [8,9-$^2H_6$]-(3*R*)-Linalool and [8,9-$^2H_6$]geraniol were used as spike compounds and added to the extracts that were prepared as described above. The monoterpenes were extracted by headspace solid phase microextraction (HS-SPME) as previously described (De La Calle García et al., 1998) using a fiber coated with polyacrylate (100 μm film thickness).

4.6. Instrumental methods

^1H NMR—the spectra of the synthetic products were recorded on a Bruker AMX 500 at room temperature in $CDCl_3$/TMS or DMSO-d_6.

The GC–MS analysis of the synthetic products and the SPME extracts were performed on a Fisions Instruments GC 8065, coupled to a Fisions Instruments MD 800 mass spectrometer, equipped with a HTFS capillary column (30 m × 0.25 mm; coated with SE-52; film thickness 0.25 μm). GC conditions: carrier gas helium 50 kPa; split 20 ml/min; injector temperature 230 °C; oven temperature 40 °C (5 min isothermal), then 5 °C/min to 250 °C (30 min isothermal); ion source tempera-

ture 200 °C; mass range 40–250 amu; electron energy 70 eV. The molecular ions (M^+) and fragment ions are given as m/z with relative peak intensities in% of the most abundant peaks.

The SBSE extracts of free and enzymatically released monoterpenes were analyzed by thermal desorption-enantio-MDGC–MS (enantioselective multidimensional gas chromatography–mass spectrometry). The system has been described in detail elsewhere (Kreck et al., 2001).

The sugar content of the grape berries was determined by refractometry using an Abbé refractometer (Carl Zeiss, Germany).

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