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Biotransformation of constituents of essential oils by germinating wheat seed

Nativ Dudai [a,*], Olga Larkov [a], Eli Putievsky [a], Henri R. Lerner [b], Uzi Ravid [a], Efraim Lewinsohn [a], Alfred M. Mayer [b]

[a] *Division of Aromatic Plants, ARO, Newe Ya'ar, PO Box 1021, Ramat Yishai 30095, Israel*
[b] *Department of Botany, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

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Abstract

Wheat seeds, when exposed to essential oils, are able to metabolise certain monoterpenes. The actual amounts of the compounds and their derivatives in the endosperm and embryo of wheat seeds, after exposure to the monoterpenes were determined. Neral and geranial, which are the constituents of citral, are reduced and oxidised to the corresponding alcohols and acids. Similarly citronellal, pulegone and carvacrol are converted partly to the corresponding reduction and oxidation products. The aromatic compound vanillin is partly reduced to vanillyl alcohol or oxidised to vanillic acid. In all cases it seems that part of the compounds applied are degraded, as indicated by the inability to account for all the compounds, which were supplied to the germinated seeds. In most cases the derivatives of the essential oil applied were less toxic than the parent compound. The possible role of non-specific enzymes by which the compounds are oxidised or reduced is discussed.

Keywords: Allelopathy; Germination; Wheat; *Triticum*; Metabolism; Pulegone; Citral; Carvacrol

1. Introduction

Inhibition of growth of plants by other plants in their vicinity has been known for a long time. The chemical interaction between plants, which can cause enhancement or inhibition of growth, has been termed "allelopathy" (Molisch, 1937) and has been extensively studied (Rice, 1984; Einhellig, 1995). Evidence for allelopathic interactions in nature caused by aromatic plants containing volatile allelochemicals has been described frequently. Essential oils were found as inhibitors of germination and growth (Muller et al., 1964; Asplund, 1968; Fischer, 1986; Muller, 1986; Reynolds, 1987). We have previously reported on the inhibitory effect of monoterpenes from a number of plants on the germination of wheat (Dudai et al., 1993, 1999) and have also shown that wheat seeds exposed to a defined monoterpene, such as citral, are able to metabolise it (Dudai et al., 2000).

The metabolism of essential oils has been investigated in various plant tissues which contain or produce these compounds (Aviv and Galun, 1978; Aviv et al., 1982; Gershenzon et al., 1989; Funk et al., 1992). The biosynthesis of these compounds and some of the enzymes involved in their synthesis has also been determined (Karp and Croteau, 1988; Croteau and Gershenzon, 1994; Adam et al., 1998). A geraniol dehydrogenase from lemongrass has been characterised by Singh Sanwan et al. (1993). The formation of volatile compounds by seeds has been studied by Zhang et al. (1994). Germination inhibition by essential oils, when applied to the dry seeds has been reported (Zhang et al., 1995). However, the question of what is the fate of exogenous monoterpenes in germinating seeds does not seem to have been studied previously. In addition, the problem of whether metabolism of such essential oils is part of a detoxification mechanism is important in understanding their mechanism of action as germination inhibitors. In particular the question of whether the compounds are modified or completely degraded in the seeds is important.

In this study we describe the fate of a number of components of essential oils, when they are applied to

* Corresponding author. Tel.: +972-4-9539510; fax: +972-4-9836936.
E-mail address: dudai-n@netvision.net.il (N. Dudai).

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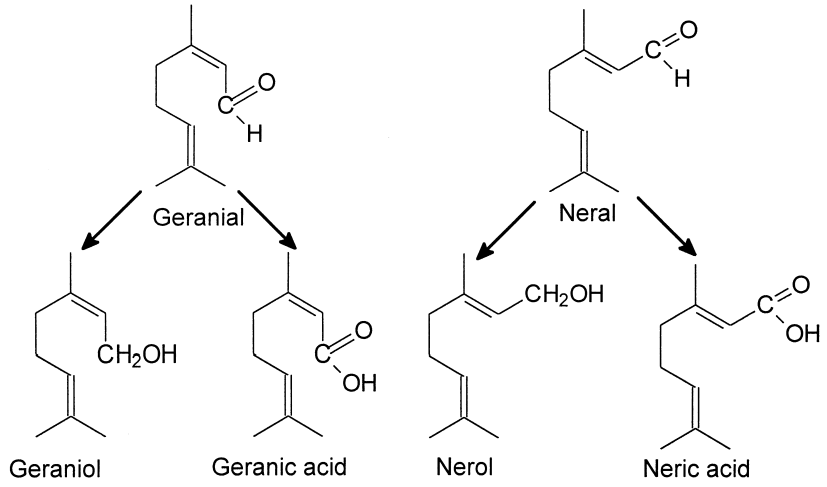
wheat seeds as inhibitors of germination. In particular we address the questions of what are the effective amounts and the concentrations of the inhibitors taken up in the different parts of the wheat seed, i.e. in the embryo and the endosperm and what are the metabolic products, if any, found in the seeds after exposure to essential oils.

2. Results

Germinated seeds were exposed to citral (neral and geranial, see Scheme 1) in the gaseous phase and the amount of citral and its derivatives determined in the endosperm and embryo using gas-chromatographic (GC) analysis combined with mass spectrometry (MS). When citral was applied at 80 nl/ml, which totally inhibits germination, 0.5 n mol of citral were found per embryo and 5 n mol per endosperm (see Fig. 2 and Table 1). When citral was supplied to the seeds, GC–MS showed the presence of new compounds in the treated seeds. The appearance of geraniol, nerol, geranic acid and neric acid was observed.

These compounds were detected both in the endosperm and the embryo. The derivatives did not appear when heat killed seeds were used indicating that the formation of these substances is due to the biological activity of the seeds and not spontaneous. As expected, the seeds which were treated by autoclave did not germinate at all. No metabolites of citral were found in this treatment (Fig. 1). Transformation of citral to oxidation and reduction derivatives took place in an aseptic system, when seeds were disinfected by pretreatment with hypochlorite, with or without antibiotics (see Experimental). Mainly reduction products were detected under these conditions (Fig. 1). When wheat seeds were used without disinfection the resultant derivatives nerol, geraniol, neric acid and geranic acid arose due to the combined activity of the seeds and the contaminant microorganisms.



Scheme 1. Metabolism of citral.

Table 1
Weight of embryo and endosperm of wheat seeds and concentration of citral and total monoterpenes recovered from the seeds, after exposure to 80 or 160 nl citral, after 24 or 48 h

Citral concentration (nl/ml)	Part of seed	Time after sowing (h)	Weight (mg)	Citral concentration (μM)	Total monoterpenes concentration (μM)
80	Embryo	24	3.3±0.4	150	800
		48	6.0±0.6	40	210
	Endosperm	24	61.4±1.1	70	690
		48	63.6±0.9	40	400
	Total	24	64.7±1.5	70	680
		48	69.6±1.5	40	380
160	Embryo	24	2.5±0.2	460	2660
		48	2.7±0.3	440	2600
	Endosperm	24	59.2±0.8	110	1130
		48	61.7±0.3	130	1250
	Total	24	61.7±1.0	120	1190
		48	64.4±0.6	150	1300

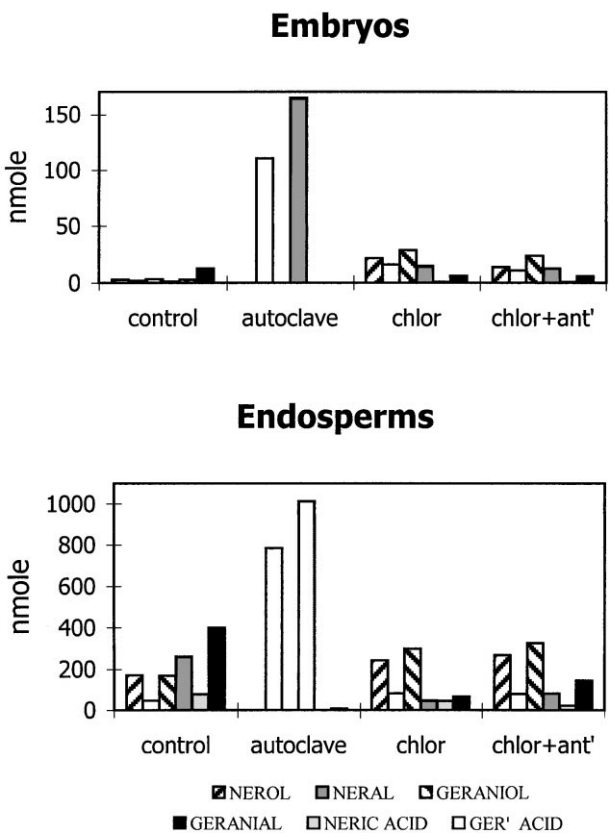


Fig. 1. Effect of sterilisation or disinfection of wheat seeds treated with citral on the amount of the different compounds recovered 48 h after sowing (citral 160 nl/ml in the gaseous phase applied to 20 seeds): 1. Untreated (control). 2. Heat inactivated (autoclave). 3. Disinfected with hypochlorite (chlor). 4. Disinfected with hypochlorite and antibiotics added during germination (chlor + ant').

Geraniol and geranic acid, when applied at 80 nl/ml were less toxic than citral itself and at least 50% of seeds germinated, while citral itself at this concentration caused 100% inhibition (Dudai et al., 1999a,b). We examined the kinetics of the appearance of the derivatives of citral. The transformation occurs both in the embryo and endosperm already at the beginning of imbibition. The citral and its metabolites accumulated during the first day of imbibition (Fig. 2). Their amount either remained constant when the dose of citral applied was high (160 nl/ml) or decreased when lower amounts were applied (80 nl/ml).

An attempt was made to make a balance sheet of the amount of citral applied and the amount of citral and its derivatives recovered in the seeds, and in the water present in the vials. Two surprising results were obtained. The amount of citral, lost from the filter paper in which it was applied, was significantly smaller in the absence of seeds than in their presence. This would indicate that the seeds act as a sink for the monoterpenes, thereby increasing loss from the source. In addition in the complete system comprising the filter paper with citral, the seeds and the solution in which they were germinated, there was a net loss of recoverable citral and its derivatives of the order of 30–40% of the total applied, depending on the initial amount added. The conclusion from this must be that the part of the citral was degraded to compounds which were not detected by the GC–MS of seed extracts. It is possible that citral was degraded to carbon dioxide or lost in the vapour phase.

From these experiments and information about the weight of the embryo and endosperm, it is possible to calculate the actual concentration of citral and its derivatives in the different parts of the seeds. As can be seen from Table 1, the concentration was dependent on the amount applied in the gaseous phase as could be expected. At the lower concentration of citral supplied, the concentrations actually decreased between 24 and 48 h, consistent with the idea that part of the essential oils applied were actually completely degraded. At the higher concentration this was not observed suggesting that perhaps the system responsible for degradation was either inhibited or saturated.

It was important to determine whether other monoterpenes and related compounds were also metabolised. As can be seen from Fig. 3, the aldehydic monoterpene citronellal was also metabolised, with the formation of the reduced and oxidised derivatives citronellol and citronellic acid. Similarly the aromatic aldehyde, vanillin, was converted to vanillyl alcohol and vanillic acid (Fig. 4). This suggests the possible existence of non-specific mechanisms, which can result in the oxidation or reduction of aldehydes.

In the case of the ketonic monoterpene pulegone, the appearance of small amounts of the less toxic compounds menthofuran and menthone as well as isopulegol, again by oxidation and reduction was observed (Fig. 5). However, the amount of pulegone was much higher, as compared to citral, as can be seen from Table 2. As also observed in the case of citral, when constructing a balance sheet of the amount of pulegone and its derivatives a considerable amount of monoterpene was lost, again indicating that part of the monoterpene was degraded to compounds not detected in our system. The phenolic monoterpene carvacrol was metabolised to thymoquinone and hydrothymoquinone; the kinetics are shown in Fig. 6 The concentrations found in the seeds are shown in Table 3. The toxicity of these of two compounds was not examined. Their occurrence in seeds of *Nigella* has been reported (Aboutabl et al., 1986), and since they are quinones they are probably inhibitors of germination (Houghton et al., 1995). Carvacrol was less toxic to the seeds than citral and pulegone. Fifty percent inhibition of germination of wheat was obtained with 16 nl/ml pulegone, 25 nl/ml citral and 28 nl/ml carvacrol.

An examination of the metabolism of carvacrol showed that the concentration in the seed and the

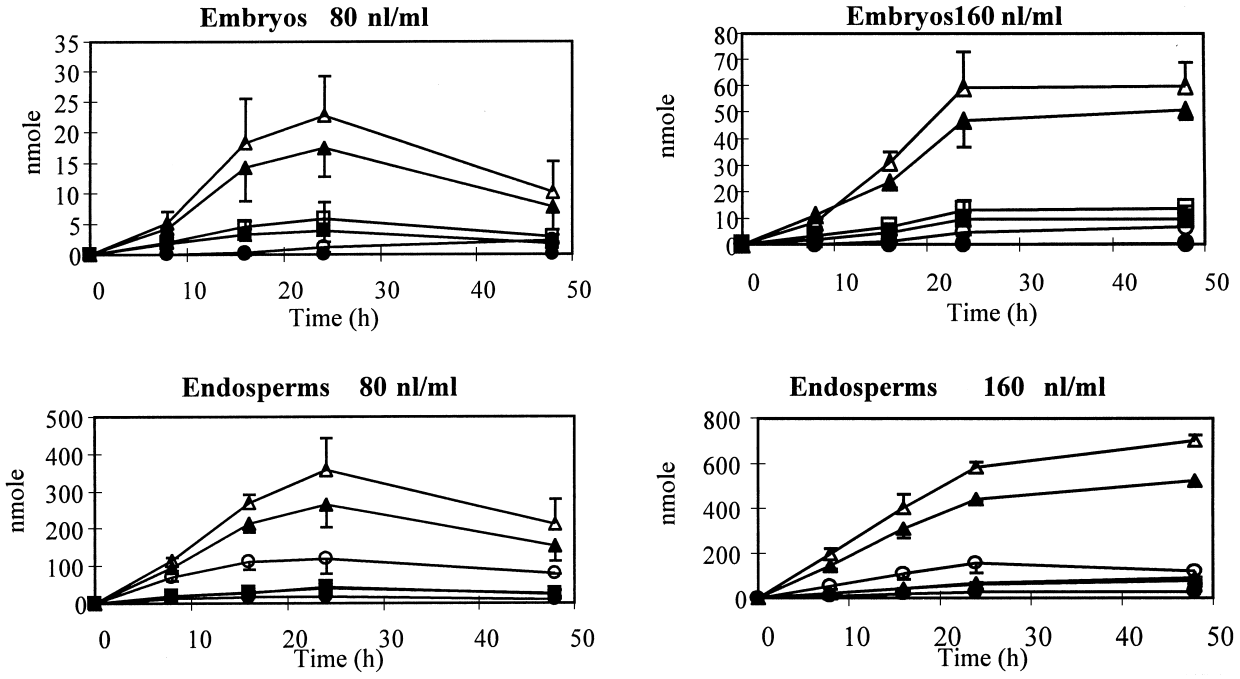


Fig. 2. Content and metabolism of monoterpenes in 20 disinfected wheat seeds during 48 h, after application of 80 nl or 160 nl/ml of citral in the gaseous phase (neral □; geranial ■; neric acid ●; geranic acid ○; nerol ▲; geraniol △).

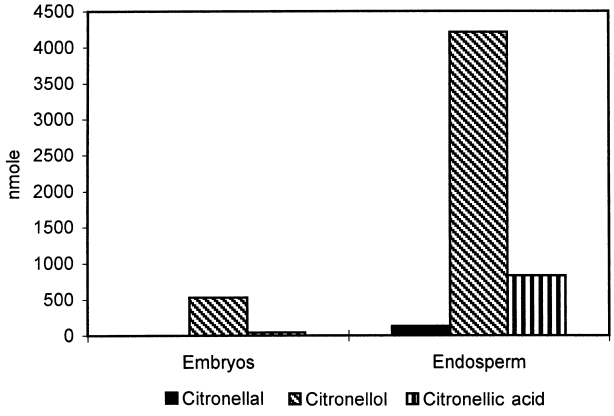


Fig. 3. Content and metabolism of citronellal in 20 disinfected wheat seeds, after application of 160 nl/ml in the gaseous-phase. The content of metabolites was measured 48 h after sowing.

absolute amounts found were of the same order of magnitude as in the case of citral and were lower than those of pulegone. A net loss of carvacrol and its derivatives was noted, which we must ascribe to degradation of the compounds. The loss of carvacrol was of the order of 25%.

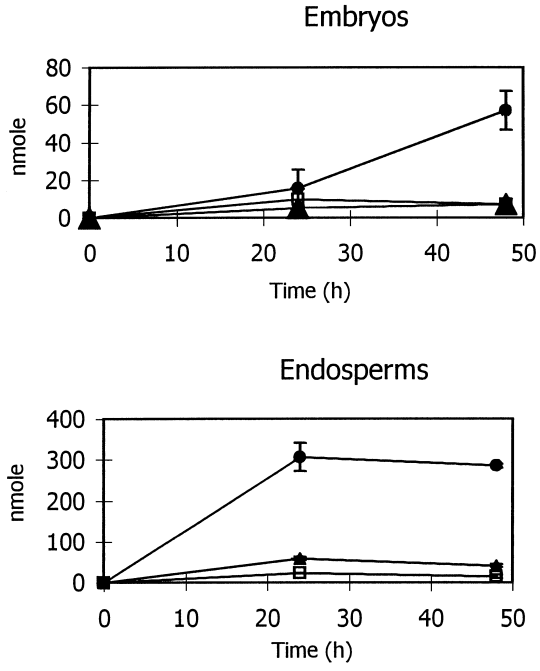


Fig. 4. Metabolism of vanillin in 20 wheat seeds after application of 2.2 mM during 48 h. Vanillin was applied in the liquid phase (vanillin □; vanillic acid ▲; vanillyl alcohol ●).

3. Discussion

The results presented here show that wheat seeds, which do not contain monoterpenes are able to metabolise these and closely related compounds. The compounds and their metabolic derivatives are shown in Schemes 1–5. The metabolic products are primarily the result of oxidation and reduction, although it is clear that part of the compounds are completely degraded,

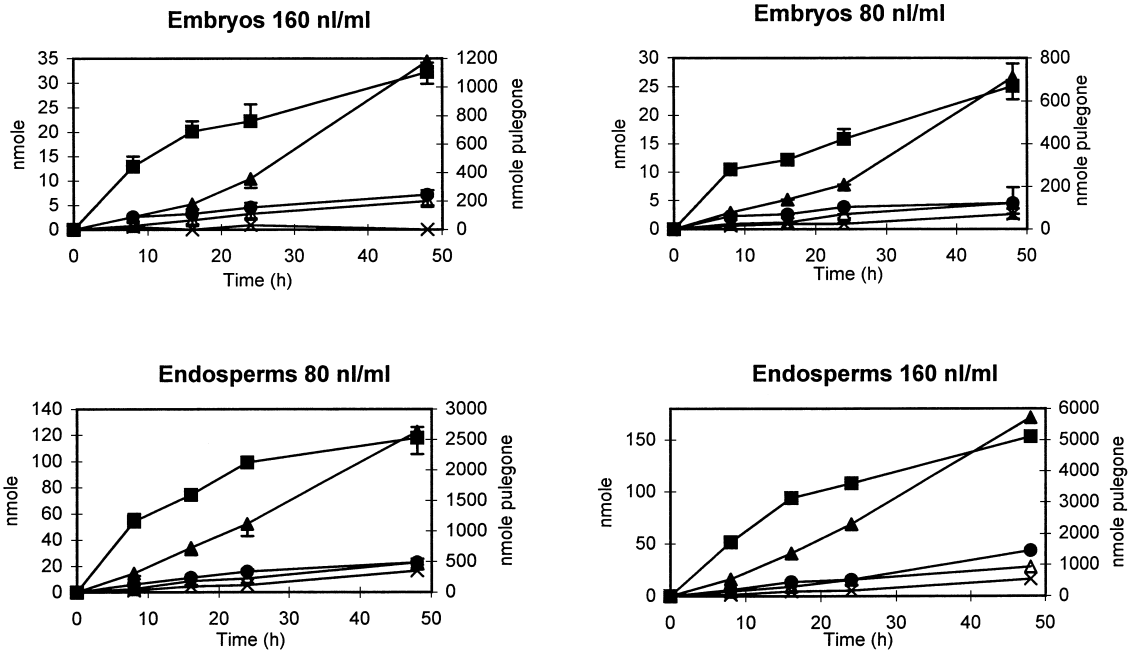


Fig. 5. Content and metabolism of monoterpenes in 20 disinfected wheat seeds during 48 h, after application of 80 nl or 160 nl/ml of pulegone in the gaseous phase (pulegone ■; isopulegone ●; isomenthone ▲; menthone △).

Table 2
Concentration of pulegone and its derivatives in different parts of wheat seeds after treatment with 80 or 160 nl/ml of pulegone for up to 48 h

Pulegone concentration (nl/ml)	Part of seed	Time after sowing (h)	Weight (mg)	Pulegone concentration (μM)	Total monoterpenes concentration (μM)
80	Embryo	24	2.9±0.4	7280	7540
		48	2.9±0.2	11,540	12,200
	Endosperm	24	64.0±1.1	960	980
		48	68.0±0.9	1140	1180
	Total	24	66.9±1.5	1390	1980
		48	70.9±1.1	1570	2420
160	Embryo	24	2.7±0.1	14,710	14,350
		48	2.6±0.1	22,600	21,670
	Endosperm	24	69.2±0.9	2690	2610
		48	67.7±1.3	3940	3750
	Total	24	71.9±2.2	3140	3110
		48	70.3±1.4	4210	5060

Table 3
Weight of embryo and endosperm of wheat seeds and concentration of carvacrol and total monoterpenes after exposure to carvacrol during 48 h

Carvacrol concentration (nl/ml)	Part of seed	Time after sowing (h)	Weight (mg)	Carvacrol concentration (μM)	Total monoterpenes concentration (μM)
80	Embryo	24	2.8±0.2	510	1090
		48	4.2±0.9	230	530
	Endosperm	24	62.0±3.0	170	220
		48	67.0±1.0	90	210
	Total	24	64.8±3.2	190	260
		48	74.8±1.9	90	120
160	Embryo	24	2.6±0.3	1130	2210
		48	3.0±0.6	870	1780
	Endosperm	24	60.0±1.1	300	410
		48	67.0±0.8	280	390
	Total	24	62.6±1.4	330	490
		48	70.0±1.4	310	450

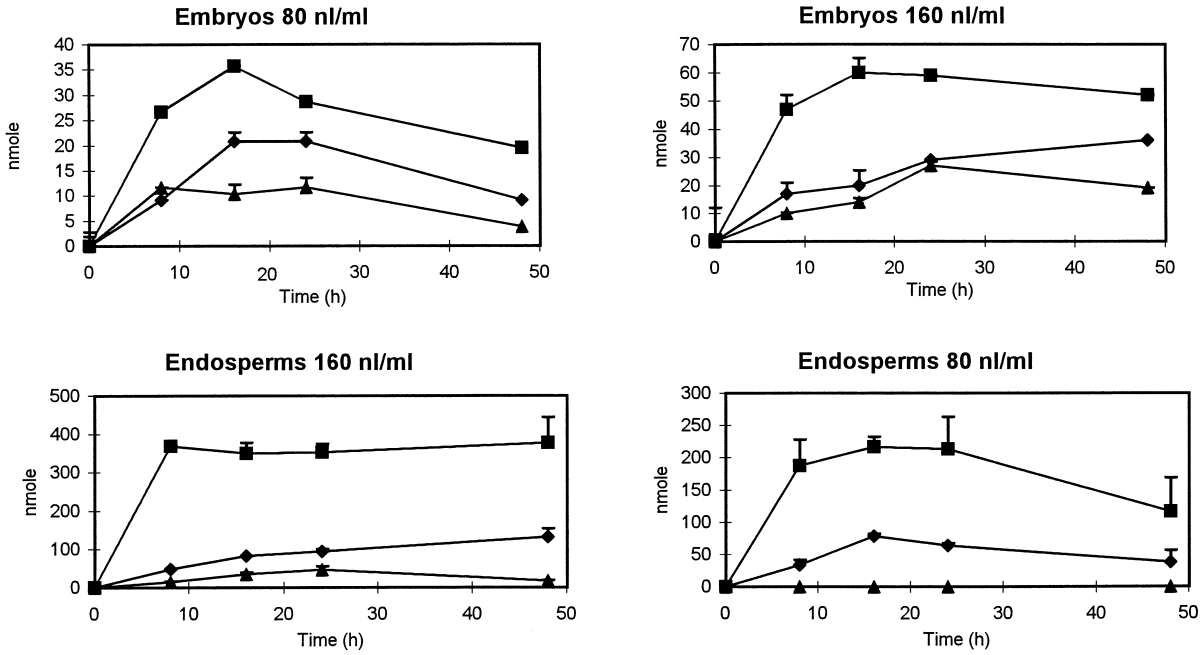
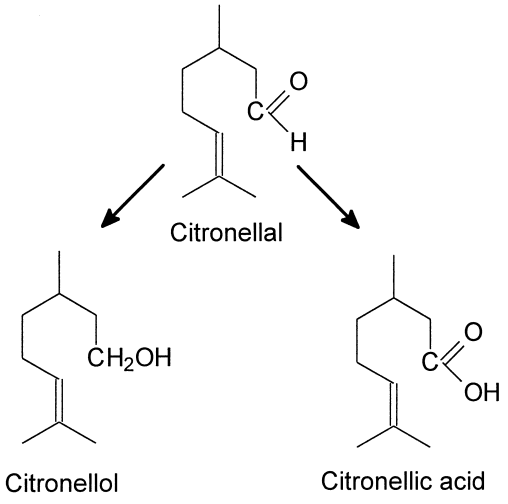


Fig. 6. Content and metabolism of monoterpenes in 20 disinfected wheat seeds during 48 h, after application of 80 or 160 nl/ml of carvacrol in the gaseous phase (carvacrol ■; thymoquinone ● thymohydroquinone ▲).

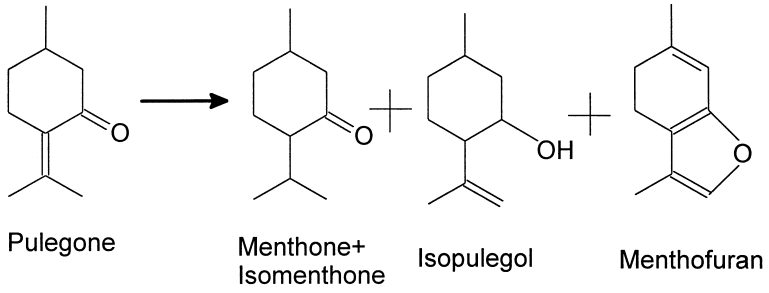


Scheme 2. Metabolism of citronellal.

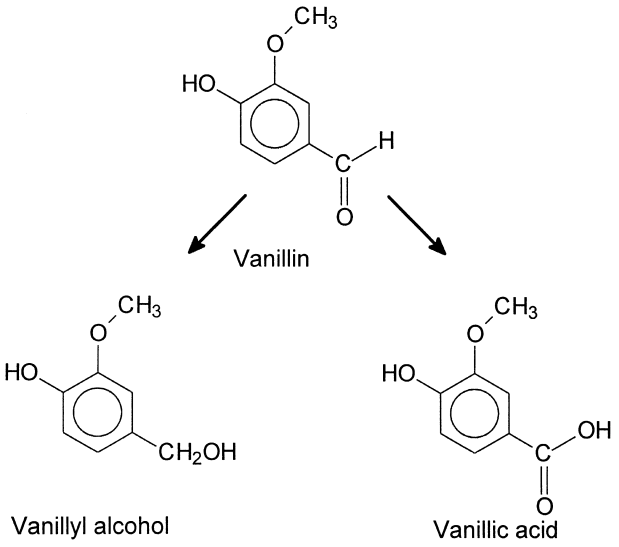
since we were unable to detect any of the derivatives using gas chromatography and mass spectrometry. From Table 1 it can be seen that at 80 nl/ml citral, total momoterpenes decrease from 683 to 382 μM between 24 and 48 h while at 160 nl/ml it increases from 1185 to 1304 μM during the same time period. Moreover, during this same time period weight of the embryo increases from 3.3 to 6.0 mg when seeds are exposed to 80 nl/ml citral, while it only increases from 2.5 to 2.7 mg when seeds are exposed to 160 nl/ml citral. It is probably that the rate at which citral arrives at the seed is a function of the amount of citral applied, while total monoterpenes content is the difference between arrival and catabolism of citral.

There seems to be a correlation between inhibition of embryo development and inhibition of rate of citral catabolism suggesting that the catabolism of citral requires a step which also plays a central role in development. This interpretation would relate the accumulation of monoterpenes to the mechanism of inhibition of development by citral and by essential oils in general.

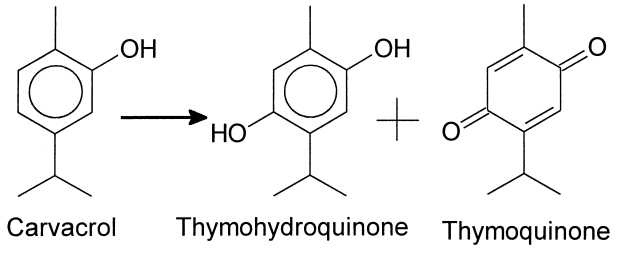
The fact that a number of quite different substances are either reduced or oxidised might indicate that non-specific enzyme systems are involved. The degradation of essential oils requires the activity of several enzyme systems. Thus reductive processes might be catalysed by non-specific dehydrogenases (Plapp et al., 1993) and oxidation might be due to the presence of cytochrome *P*-450 type enzymes, which today are known to be present in plant tissues and are involved in the biosynthesis of terpenes (Mihaliak et al., 1993; Halkier, 1996). The fact that different compounds accumulate to different extents in the seeds, and at different levels in the endosperm and the embryo should be noted. For the inhibition of germination the determining factor is undoubtedly the concentration within the embryo. However, since appreciable amounts can be detected in the endosperm, it is likely that after the initial uptake there might be movement of the active compounds from the endosperm to the embryo. It is tempting to speculate that the metabolic changes which the compounds undergo is a mechanism of detoxification. However, at the present there is no



Scheme 3. Metabolism of pulegone.



Scheme 4. Metabolism of vanillin.



Scheme 5. Metabolism of carvacrol.

concrete evidence that this is the case. In fact the observation that quite different compounds are metabolised in basically the same way, i.e. are either reduced or oxidised, suggest that the metabolism is quite unspecific and cannot be regarded as a real detoxification process. Our results show for the first time that monoterpenes act on seeds at very low levels, and that their amounts in the different parts of wheat seeds differ. The metabolism of monoterpenes by wheat seeds, and by implication, other seeds also, has been shown for the first time. It may be assumed that metabolism of allelochemicals by seeds and other plant tissues is a more general phenomenon and must be taken into account in studies of the interaction of plants with allelochemicals.

It must be stressed that the mechanism of action of monoterpenes as germination inhibitors has not been fully elucidated. Further investigations of the metabolism and mechanism of action of monoterpenes are clearly needed.

4. Experimental

4.1. Essential oil

The monoterpenes citral (55% geranial and 45% neral), pulegone, citronellal and carvacrol and the aromatic compound vanillin were obtained from Sigma.

4.2. Seed germination

Wheat seeds, *Triticum aestivum* L. cv. Dariel were germinated in glass vials, 25 ml, on three layers of filter paper (Whatman No. 1) wetted with 1.5 ml distilled water. Vials containing 20 seeds were incubated at 27°C in the dark. To test the inhibitory effect of the essential oils, a known amount of oil was loaded immediately when the seeds were sown (using a calibrated glass microcapillary) onto a piece of filter paper, which was attached to the inner side of the cover of the vial. The vials were closed hermetically. Amounts of up to 4 μl of oil were applied in this way. One microlitr of oil per vial is equivalent to 40 nl/ml. Experiments were repeated in five replicates. After suitable periods of time the seeds were separated into endosperm and embryo and extracted with methyl-*tert*-butyl ether (MTBE).

Seeds were disinfected by treatment with 70% ethanol for 10 s followed by 1% hypochlorite containing 0.1% Tween 20 for 10 min. and then rinsed three times with sterile double distilled water (Ben-Hod et al., 1991). For heat treatment the seeds were autoclaved for 30 min at 121°C. The effectiveness of these treatments was tested by sowing seed samples on Petri dishes containing A2R

Difco agar, which were incubated for 96 h and then observing the formation of colonies of microorganisms. No microorganisms were detected in the treated seeds.

4.3. Determination of content of monoterpenes and vanillin an their derivatives in the seeds or parts of the seed

The seeds or their parts were extracted with MTBE, containing 10 μg/ml iso-butylbenzene as an internal standard, for 24 h with gentle shaking at room temperature.

The extracts were analysed following the procedures of Lewinsohn et al. (1993) and Dudai et al. (1999b). The samples were analysed using an HP-GCD apparatus equipped with a HP5 (30 m×0.25 mm) fused silica capillary column. Helium was used as the a carrier gas. Injection temperature was 250°C, detector temperature 280°C. Column conditions: 70°C for 2 min, following 4°C/min to 200°C. The components were identified by co-injection with authentic samples and by comparison of the EI–MS (electron impact–mass spectroscopy) obtained with computerised libraries.

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