Transformation of Terpenoids in Grape Must by *Botrytis cinerea*

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Experiments were done to investigate the volatile components in botrytized grape must and transformation of terpenoids in terpene-supplemented grape must by *Botrytis cinerea*. Twenty-eight compounds were identified in the volatile concentrate of botrytized must with a combined gas chromatograph-mass spectrometer. No terpenoids were detected in the concentrate. Linalool or terpinen-4-ol decreased a lot when *Botrytis cinerea* was cultured in the must with these terpenes for 15 days. In linalool-supplemented botrytized must 9 identified and 3 unidentified terpenes were found, while only geranial was detected in terpinen-4-ol-supplemented botrytized must. *Botrytis cinerea* did not produce terpenoid in grape must without terpenes, but transformed linalool added to grape must into some other monoterpenes.

Mono and sesquiterpenoids are primarily plant products and are well known as characteristic components of a variety of essential oils. The presence of these compounds in grapes and wines was reported by many investigators^{1~6)} and about 20 monoterpenoids have hitherto been found in Muscat and wine grapes.^{7~10)}

Biosynthetic pathways of terpenoids in plants have already been proposed by Croteau¹¹⁾ and the formation of these compounds by fungi has been elucidated by Collins *et al.*^{12,13)} Furthermore, Tressl *et al.*¹⁴⁾ have reviewed in detail the isomerization and stereospecific reaction of terpenoids by fungi. Recently, advanced studies were performed on the formation of terpenoids by yeast¹⁵⁾ and bacteria.¹⁶⁾

Noble wine is made from botrytized grapes. Involvement of the fungus, *Botrytis cinerea*, and wine yeasts is responsible for noble wine making^{17,18)} and especially for characteristic fragrant flavor development.^{19,20)} One step of this flavor formation seemed to be that terpenoids in grapes are changed into more fragrant flavors by the action of *Botrytis cinerea* attacking the grape skin. However, only a few studies have been reported on the flavor com-

ponents formed by this fungus.²¹⁾

This paper deals with the identification of volatile components produced by *Botrytis cinerea* using combined gas chromatography-mass spectrometry and with the transformation of terpenes added to artificially botrytized grape must into other terpenic compounds by this fungus.

MATERIALS AND METHODS

Microorganism. *Botrytis cinerea* BIG (IFO 5964) was obtained from the culture collection of microorganisms of the Institute of Microbiology and Biochemistry, Geisenheim, Federal Republic of Germany.

Grape must. The must used in this experiment was supplemented with sucrose to 25% sugar content. The prepared must contained 3.18 g total acids per liter (pH 3.70) and was free of terpenic compounds.

Cultivation. To 300 ml prepared must 120 mg sodium salt of adenosine-3',5'-cyclic monophosphate (c-AMP) was added as a growth stimulator of *Botrytis cinerea*²²⁾ and in some cases 6 mg each of linalool, linalool oxide (mixture of *cis* and *trans* forms), terpinen-4-ol or α -terpineol was further added. After inoculation of a loopful of *Botrytis cinerea* spores, the must in 500 ml Sakaguchi flasks was incubated on a reciprocal shaker with 70-mm amplitude at 130 strokes per minute at 15°C for 15 days.

Chemicals. Sodium salt of c-AMP was donated by Seishin Pharmaceutical Co. Ltd., Tokyo. Linalool from Tokyo Kasei Kogyo Co. Ltd., Tokyo, and linalool oxide, α -terpineol and terpinen-4-ol from Merck, München, were of analytical grade for gas chromatography, respectively.

Preparation of volatile concentrate. Volatile components from 200 ml out of 300 ml of botrytized musts were directly extracted twice with 100 ml of purified dichloromethane by shaking for 30 minutes at room temperature. The separated dichloromethane layer was washed 3 times with distilled water and dried over anhydrous Na_2SO_4 . The solvent layer was concentrated to about 0.2 ml at room temperature *in vacuo*.

Calibration curves and determination of terpenes. Calibration curves to determine terpenes were obtained as follows. Standard reagents of linalool, linalool oxide, terpinen-4-ol and α -terpineol were diluted to 5, 10, 20, 30 and 40 μg per liter each with 20 ml of the must not containing detectable amounts of these compounds. To the above must 20 μg of diethyl succinate (DES) as the internal standard and 20 ml of purified dichloromethane were added. After the mixture was mechanically stirred for 15 minutes, the solvent layer was separated and concentrated at room temperature *in vacuo*. A portion of the concentrate was injected on a glass column (2 mm i.d. $\times$ 3 m) packed with 60 to 80 mesh chromosorb W (AW-DMCS) coated with 10% PEG 20M TPA. The oven temperature was programmed from 70°C to 250°C at a rate of 5°C/min. Both injection and detection temperatures were set at 300°C and the flow rate of carrier gas (N_2) was 30 ml/min. Then, the standard curves were got by plotting the ratio of peak height on the gas chromatogram of terpenes to that of the internal standard (DES) *versus* concentration. In the determination of terpenic compounds in botrytized musts, terpenes were extracted with purified dichloromethane after mixing a 2 ml sample, 18 ml deionized water and 20 μg DES.

Capillary column gas chromatographic-mass spectrometric analysis (Capillary GC-MS). Volatile components in the concentrates from botrytized musts were identified with a combined gas chromatograph-mass spectrometer using a capillary column. The all-glass jet separator used at the GC-MS interface was heated at 250°C. The conditions for gas chromatography were as follows: a 0.28 mm (i.d.) $\times$ 30 m glass capillary column coated with FFAP (G-SCOT), helium pressure of 0.2 kg/cm² (flow rate: 0.7 ml/min), split ratio of 100:1 and injection temperature of 230°C. The oven temperature was programmed from 50°C to 170°C at a rate of 3°C/min. The mass spectrometer was operated under the conditions of ionization electron energy of 70 eV and an ion source temperature of 240°C.

RESULTS AND DISCUSSION

The calibration curves of 4 terpenes were linear up to 40 μg of each terpene under the assay conditions mentioned above and were not influenced by sucrose up to 25% or by ethanol up to 20%.

Table I shows the changes of the 4 terpenes artificially added to botrytized must. Although linalool oxide was unchanged, the amounts of the other 3 terpenes decreased, more or less, during incubation with *Botrytis cinerea*. However, no relation was seen between the decrease of terpenes and the fungal growth. It was assumed, therefore, that the 3 terpenes might be converted by the fungus to other compounds. To confirm this presumption, volatile concentrates prepared from the botrytized musts with or without terpene were subjected to GC-MS.

TABLE I. CHANGES OF THE TERPENIC COMPOUNDS IN TERPENE-SUPPLEMENTED BOTRYTIZED MUST

Terpenes added	Amounts (mg/liter)		Growth of <i>Botrytis cinerea</i> ^b
	Added	Residual	
None (control)	0		100
Linalool	20	7.14	40
Furan-linalool oxide ^a	20	9.11 (<i>trans</i> -form) 11.20 (<i>cis</i> -form)	90
Terpinen-4-ol	20	5.12	80
α -Terpineol	20	15.74	50

^a Mixture of *trans*- and *cis*-forms.

^b Percentage of mycelial weight of the fungus to that of control.

FIG. 1. Gas Chromatograms of Volatile Concentrates from Botrytized Grape Musts.

A: In the absence of terpene. B: In the presence of linalool. Peak numbers correspond to those in Table II.

TABLE II. IDENTIFIED VOLATILE COMPONENTS IN NON-SUPPLEMENTED AND LINALOOL-SUPPLEMENTED BOTRYTIZED MUSTS

Peak Nos.	Compounds*	Methods of identification**
1	Carbon dioxide	MS
2	Dichloromethane (Solvent)	MS
3	1-Propanol	MS, GC
4	2-Butanol	MS, GC
5	2-Methyl-1-propanol	MS, GC
6	1-Butanol	MS, GC
7	3-Pentene-2-ol	MS
8	β -Pinene	MS
9	3-Methyl-1-butanol	MS, GC
10	1-Pentanol	MS, GC
11	2-Methylpyrazine	MS
12	1-Hydroxy-2-propanone	MS
12 ^a	Ethylpyrazine	MS
13	Acetic acid	MS, GC
14	2-Furaldehyde	MS, GC
15	<i>trans</i> -Furan-linalool oxide	MS, GC
16	<i>cis</i> -Furan-linalool oxide	MS, GC

17	Benzaldehyde	MS, GC
18	Propionic acid	MS, GC
18 ^a	2,3-Butanediol (<i>levo</i>)	MS, GC
19	2-Methylpropionic acid	MS, GC
19 ^a	Pyrazine	MS
20	Linalool (Added)	MS, GC
21	5-Methyl-2-furaldehyde	MS
22	Butanoic acid	MS, GC
23	γ -Butyrolactone	MS, GC
23 ^a	<i>p</i> -Menthane-1-ene-3-one	MS
24	α -Furfuryl alcohol	MS, GC
25	3-Methylpentanoic acid	MS, GC
26	α -Terpineol	MS, GC
27	<i>trans</i> -Pyran-linalool oxide	MS
28	<i>cis</i> -Pyran-linalool oxide	MS
29	5-Hydroxypentanoic acid lactone	MS
30	Geraniol	MS, GC
31	Benzyl alcohol	MS, GC
32	2-Phenylethanol	MS, GC
33	Unidentified terpene	MS
34	2,6-di- <i>tert</i> -Butyl-4-methylphenol	MS
35	2-Acetopyrrole	MS
36	2-Methoxy-6-methylpyrazine	MS
37	Methyl furoate	MS
38	N. I.	
39	N. I.	
40	<i>p</i> -Menthane-1,8-diene-9-ol	MS
41	2,5-Hexadione	MS
42	<i>tert</i> -Butyl-2-methoxyphenol	MS
43	N. I.	
44	Unidentified terpene	MS
45	2-Methyl-2- <i>tert</i> -butylphenol	MS
46	N. I.	
47	N. I.	
48	Unidentified terpene	MS

* N. I., not identified.

** MS, mass spectrometry; GC, retention time on gas chromatography with authentic samples.

Peak numbers correspond to those in Figs. 1-A and 1-B.

Figures 1-A and 1-B respectively show the gas chromatograms of volatile concentrates from the botrytized musts in the absence and presence of linalool. Table II lists the identified compounds in both musts. Most of the 28 compounds detected in ordinary botrytized must were further confirmed by gas chromatography with authentic samples. They were flavor components of the grape must and the products of *Botrytis cinerea*. Various furan and nitrogenous compounds were discovered in the flavor components. However, no ter-

TABLE III. MASS SPECTRAL DATA AND AMOUNTS OF TERPENIC COMPOUNDS FOUND IN TERPENE-SUPPLEMENTED BOTRYTIZED MUSTS

Added	Found		M ⁺ or MW*	m/z (Relative intensity, %)	Amounts** (mg/liter)
	Peak Nos.	Compounds			
Linalool	8	β -Pinene	136	93 (100), 41 (75), 69 (49), 39 (35), 27 (32), 79 (20)	0.05
	15	<i>trans</i> -Furan-linalool oxide	170*	59 (100), 43 (43), 94 (42), 111 (34), 55 (30), 68 (30)	0.65
	16	<i>cis</i> -Furan-linalool oxide	170*	59 (100), 94 (42), 43 (41), 111(33), 55 (31), 68 (30)	0.69
	23 ^a	<i>p</i> -Menthane-1-ene-3-one	152	82 (100), 110 (56), 95 (23), 41 (23), 137 (17), 109 (17)	—
	26	α -Terpineol	154	59 (100), 93 (75), 136 (72), 121 (72), 43 (42), 81 (35)	0.39
	27	<i>trans</i> -Pyran-linalool oxide	170*	68 (100), 59 (86), 94 (75), 67 (47), 43 (39), 55 (21)	0.75
	28	<i>cis</i> -Pyran-linalool oxide	170*	68 (100), 59 (85), 94 (75), 67 (47), 43 (38), 55 (23)	1.01
	30	Geraniol	154	69 (100), 93 (35), 41 (28), 68 (24), 84 (15), 123 (15)	0.15
	33	Unidentified terpene	134	43 (100), 93 (98), 79 (43), 41 (43), 91 (36), 119 (23)	0.81
	40	<i>p</i> -Menthane-1,8-diene-9-ol	152	79 (100), 93 (93), 92 (75), 91 (74), 41 (69), 119 (63)	0.53
	44	Unidentified terpene	154	71 (100), 43 (63), 55 (43), 69 (29), 41 (29), 56 (21)	0.39
	48	Unidentified terpene	152	43 (100), 71 (97), 67 (94), 55 (58), 68 (47), 82 (41)	7.47
Terpinen-4-ol		Geraniol	152	69 (100), 41 (65), 84 (30), 39 (22), 94 (16), 27 (11)	0.24

* Molecular weight.

** Determined by gas chromatography. —, not determined.

FIG. 2. Mass Spectra of *trans*-Furan- and *trans*-Pyran-linalool Oxides Isolated from Botrytized Must and Their Suggested Fragmentations.

A: *trans*-Furan-linalool oxide. B: *trans*-Pyran-linalool oxide.

penoids were found in the must although many unknown non-terpenic peaks appeared on the gas chromatogram. From the above results, it was clarified that *Botrytis cinerea* did not produce terpenoid in the must under the culture conditions previously described. On the other hand, in the presence of linalool 12 more terpenoids in addition to the above compounds were detected in the volatile concentrate of the botrytized must. They were β -pinene (peak No. 8), *trans*-furan-linalool oxide (peak No. 15), *cis*-furan-linalool oxide (peak No. 16), *p*-menthane-1-ene-3-one (peak No. 23^a), α -terpineol (peak No. 26), *trans*-pyran-linalool oxide (peak No. 27), *cis*-pyran-linalool oxide (peak No. 28), geraniol (peak No. 30), *p*-menthane-1,8-diene-9-ol (peak No. 40) and 3 unidentified monoterpenes (peak Nos. 33, 44 and 48). Some of them were further confirmed by gas chromatography with authentic samples. Table III displays the mass spectral data and amounts of these terpenes. Williams *et al.*¹⁰⁾ have already reported the presence of 7

of the above monoterpenes except *p*-menthane-1-ene-3-one and *p*-menthane-1,8-diene-9-ol in the must of Muscat of Alexandria grapes without description of their amounts. It is noteworthy that the decreased amount of linalool is very close to the total amounts of the 12 terpenes produced (see Tables I and III). Figure 2 illustrates the mass spectra of furan and pyran types of *trans*-linalool oxide isolated from the must and their suggested fragmentations. The spectra of stereoisomers (*cis* and *trans* forms) of furan-linalool oxide or pyran-linalool oxide were respectively very similar to each other. The above results suggested that *Botrytis cinerea* transformed linalool to some other monoterpenes.

In the presence of terpinen-4-ol only geranial was found as a terpenic compound and no other terpene was detected in the botrytized must (Table III). Most of the terpinen-4-ol is considered to be transformed to other non-terpenic compounds by *Botrytis cinerea* based on the amounts of terpinen-4-ol and geranial in the must (Tables I and III).

In view of the above results it is presumed that acyclic monoterpenoids such as linalool in grapes are easily converted to other terpenic compounds by *Botrytis cinerea* in the process of noble wine making from botrytized grapes and this transformation is one of the factors in the development of the characteristic flavor of noble wine.

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