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Variability in the Production of Volatile Metabolites by *Trichoderma viride*

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The first indication of microorganisms able to produce aromas more or less distinct and pleasant is credited to Omelianski (1) to whom is also credited the observation that the aroma produced by a microbial culture evolves in time in relation to the changes of the substrate composition on which the culture has been grown.

Numerous studies (2-8) have subsequently shown that microorganisms are able both to synthetize aromatic molecules and to modify their structure and aromatic properties. The deuteromycete *Trichoderma viride* Pers. ex S.F. Gray, a fungus commonly present in the soil, noted for its cellulase activity (9) is included among the microorganisms able to produce aromatic substances.

Under ideal culture conditions *T. viride* is able to originate an intense coconut aroma (10), found for the first time by Collins and Halim (11) and linked to the presence in the substrate of 6-pentyl-α-pyrone, a lactone obtained also synthetically (11) and already found in extracts of peach (12).

Although Moss *et al.* (13) have found in extracts of *T. viride* only 6-(1-pentenyl)-α-pyrone, without aroma, subsequent studies (10) (14), confirming the relation between the coconut aroma and the presence in the substrate of 6-pentyl-α-pyrone, have shown that the sources of carbon and nitrogen and the culture method influence only the quantity, but not the chemical nature of the aroma.

The aim of the present work has been to evaluate the effect of different substrates and cultural conditions on three different isolates of *T. viride* and to measure qualitatively and quantitatively any possible variations in the volatile metabolic products.

METHODS

Three isolates of *T. viride* identified as TV 25, TV 44/2a and TV 2611 furnished by the Istituto di Patologia Vegetale of the University of Torino and maintained on potato dextrose agar (OXOID) at 3°C, have been tested. A conidial suspension in water prepared from four day cultures on potato dextrose agar was used as inoculum. The culture media used were:

a) potato extract; glucose, 10%; $CaCO_3$, 0,2 g/l; $MgSO_4 7H_2O$, 0,2 g/l (11);

b) as in a) substituting the potato extract with rice extract (50 g soluble rice starch in 600 ml water held at 120°C for 2 hours);

c) $NaNO_3$, 2g/l; KH_2PO_4, 1 g/l; $MgSO_4 7H_2O$, 0,5 g/l; KCl, 0,5 g/l; $FeSO_4 7H_2O$, 0,1 g/l; sucrose, 30 g/l (13);

d) as in c) substituting the sucrose with glucose;

e) as in c) adding $(NH_4)_2SO_4$ 1 g/l;

f) $NaNO_3$, 2 g/l; $(NH_4)_2SO_4$, 1 g/l; $CaCO_3$, 0,2 g/l; $MgSO_4 7H_2O$, 0,2 g/l; glucose, 100 g/l;

g) yeast extract, 1 g/l; caseine hydrolysate, 2 g/l; KH_2PO_4, 1,5 g/l; $MgSO_4$, 1 g/l; glucose, 15 g/l.

The cultures were grown in 500 ml flasks containing 50 ml of substrate incubated at room temperature without agitation for three or more days. The submerged cultures were obtained by magnetic stirring.

Samples of 100 ml of filtered culture medium were extracted four times with 30 ml of methylene chloride. The extracts were dried over anhydrous sodium sulfate and the solvent was eliminated under mild evaporation conditions in water bath at 39°C.

For quantitative analysis an internal standard of δ-decalactone was added before concentration.

All the samples were analyzed by GLC and GC-MS. A series of blank experiments on different culture media without inoculation of *T. viride* was also made.

Gaschromatopraphic analyses were performed with a Hewlett-Packard 5890 instrument equipped with two fused silica capillary columns (DB-1 and DB-1701 - J&W: 30 m; 0,25 mm i.d.) mounted in the same injection port and two flame ionization detectors. Injector temperature: 280°C. Detector temperature: 300°C. Carrier gas: helium at 1 ml min^{-1}.

Oven temperature program: 50°C for 3 min followed by an increase of 5°C*min^{-1} to 285°C then hold 10 min.

The double column signals were recorded simultaneously and elaborated on a Hewlett-Packard 5890A workstation connected with the gaschromatograph. Kovats retention indices of the peaks, referred to n-alkanes, were calculated (15) and compared with the indices of pure standards.

Gaschromatopraphy-mass spectrometry was used to identify chemical structures: a Finnigan-Mat 4021 series quadrupole equipped with a DB-1 column was employed. Gaschromatographic conditions were set up as previously reported.

All the spectra were recorded in the electron impact mode at an ionization voltage of 70 eV and a scan speed m/e = 33 ÷ 350 in 1 second.

RESULTS AND DISCUSSION

The results of the GLC and GC-MS analyses of the fungal cultures extracts are reported in Tables 1 and 2.

Relative FID peak area percentages arise from electronic integration of the GC peaks. Of the three isolates of *T. viride* tested TV 25 never produced volatile metabolites, neither in submerged cultures nor in surface cultures.

TABLE 1 — *Major volatile metabolism compounds of* T. viride *with surface cultures.* (Relative abundance per cent).

	Substrate (a)			Substrate (g)	
	TV 44/2a[a]	TV 44/2a[b]	TV 2611[b]	TV 44/2a[b]	TV 2611[b]
Acetic acid	—	—	9,19	—	—
Ethyl acetate	—	6,41	—	—	—
Propanol	2,02	0,19	0,40	—	—
Isobutanol	3,17	1,10	7,71	—	6,50
Diacetyl	1,32	0,03	—	—	—
2-methylbutan-2-col	19,16	1,50	7,12	0,02	—
3-methylbutan-1-ol	17,26	1,07	7,98	—	21,85
2-methylbutan-1-ol	2,51	0,56	4,19	—	9,87
Acetoin	23,94	5,03	0,09	—	36,15
Butan-2,3-diol	—	34,75	19,26	—	—
2-phenylethanol	4,86	0,57	5,75	—	15,87
6-propyl-α-pyrone	—	0,03	0,07	—	—
6-pentyl-α-pyrone	21,03	45,62	—	94,42	—
δ-decalactone	—	0,08	—	0,03	—
6-(1-pentenyl)-α-pyrone	0,44	1,62	37,34	4,30	7,85
6-(2-pentenyl)-α-pyrone	—	—	0,77	—	0,27
6-(1-heptenyl)-α-pyrone	—	1,21	—	1,16	—
Limonene	4,29	0,15	0,12	0,07	1,64

[a] 3 day culture
[b] 12 day culture
— not detected

In the surface cultures of TV 2611, characterized by the absence of conidia and coconut aroma, the most abundant derivative of α-pyrone was 6-(1-pentenyl)-α-pyrone, while in the surface cultures of TV 44/2a, characterized by the presence of conidia and an outstanding coconut aroma, the principal pyrone derivative was found to be 6-pentyl-α-pyrone.

The composition of the culture substrate influenced the quantity of the pyrone derivatives but, while 6-pentyl-α-pyrone characterized extracts of TV 44/2a, 6-(1-pentenyl)-α-pyrone characterized those of TV 2611.

This, beyond justifying the constrasting results of Collins and Halim (11) and Moss *et al.* (13), seems to indicate the existence of a different enzymatic pathway in the two isolates, indipendently of the substrate composition.

Other pyrone derivatives have been detected, among them 6-propyl-α-pyrone, identified by comparison with mass spectra reported in the literature (16) and two other pyrone derivatives whose structures have been hypothetically attributed to 6-(1-heptenyl)-α-pyrone [192 (M^+, 56), 124 (22), 123 (69), 122 (69), 110 (28), 107 (35), 95 (62), 94 (100), 79 (30), 77 (33), 55 (16), 39 (44)] and to 6-(2-pentenyl)-α-pyrone [164 (M^+, 8), 146 (1), 136 (1), 110 (2), 96 (6), 95 (100), 68 (4), 53 (3), 41 (79), 40 (3), 30 (42), 38 (3)].

Present in variable quantities are the alcohols, derived, with the exception of 2-methylbutan-2-ol, from the corresponding aminoacids via the Ehrlich pathway.

TABLE 2 — *Major volatile metabolism compounds of* T. viride *isolated TV 44/2a identified from 12 day surface cultures.* (Relative abundance per cent).

	Substrate			
	(c)	(d)	(e)	(f)
Acetic acid	—	—	—	—
Ethyl acetate	—	—	—	—
Propanol	—	—	—	—
Isobutanol	—	—	—	—
Diacetyl	—	—	—	—
2-methylbutan-2-ol	62,11	7,81	3,84	2,23
3-methylbutan-1-ol	—	—	—	—
2-methylbutan-1-ol	—	—	—	—
Acetoin	—	—	—	—
Butan-2,3-diol	—	—	—	—
2-phenylethanol	—	—	—	—
6-propyl-α-pyrone	—	—	—	—
6-pentyl-α-pyrone	24,87	82,60	94,02	95,00
δ-decalactone	—	—	—	—
6-(1-pentenyl)-α-pyrone	10,05	8,97	1,86	2,22
6-(2-pentenyl)-α-pyrone	—	—	—	—
6-(1-heptenyl)-α-pyrone	—	—	—	—
Limonene	2,97	0,62	0,28	0,55

— not detected

The isolate TV 44/2a has synthesized, regardless of the cultural substrate, δ-decalactone, one of the metabolites of *Ceratocistis moniliforme* (7).

The production of limonene, already known from extracts of *Cronartium fusiforme* and *Gyromitra exculenta* (18), was not related to the isolate or to the substrate.

In order to evaluate the evolution in time of the volatile components, cultures of TV 44/2a were analysed after 3 and 12 days of growth. The concentrations in the substrate increased (Table 1) for the pyrone derivatives and diminished for limonene, for the alcohols, and for diacetyl and acetoin which were transformed into butan-2,3-diol.

The substrate a) favored, for both isolates, the biosynthesis of volatile metabolites while substrate g) accentuated, for isolate TV 44/2a, the production of 6-pentyl-α-pyrone to the detriment of the other metabolites and, for isolate TV 2611, limited the synthesis of 6-(1-pentenyl)-α-pyrone, which remained however, the principal pyrone derivative and, due to the greater availability of free aminoacids, stimulated the production of alcohols.

Substrate b), not reported in Table 1, inoculated with TV 44/2a has shown the formation of traces of 6-pentyl-α-pyrone while with isolates of TV 2611 traces of 6-(1-pentenyl)-α-pyrone.

The substrate c), d), e) and f) have limited the production of volatile metabolites for isolate TV 44/2a to only four compounds whose concentrations were in strict report with the composition of the culture medium (Table 2).

The behavior of the isolate TV 2611 has not been tested on the substrates c), d), e) and f) because it is not a producer of 6-pentyl-α-pyrone, the most interesting volatile metabolite.

The glucose and the $(NH_4)_2SO_4$ stimulated the biosynthesis of 6-pentyl-α-pyrone, as already observed (10) (14), who also underlined that the presence of ammonium nitrogen increases the production of 6-pentyl-α-pyrone, independently from the source of carbon present in the substrate.

The quantitative analysis of 6-pentyl-α-pyrone (Table 3), done on the extracts obtained from the cultures of the isolate TV 44/2a on substrate g), showed a greater productive capability with respect to that on substrates a) and f). Furthermore, the submerged culture, in accordance with Yong and Lim (14), was less active than the surface culture.

TABLE 3 — *Production of 6-pentyl-α-pyrone by* T. viride *isolated TV 44/2a (mg/l).*

	Substrate		
	(a)	(f)	(g)
Surface culture[b]	14	70	78
Submerged culture[b]	—	17	27

— not detected
[b] 12 day culture

The quantity of 6-pentyl-α-pyrone produced by the isolate TV 44/2a, inferior to that reported by Collins and Halim (11), can be explained by the different isolate utilized. The choice of an efficient microorganism-culture medium system is therefore the basis of the industrial utilization of *T. viride* for the production of 6-pentyl-α-pyrone.

SUMMARY

Several volatile metabolites including lactones, alcohols, terpene derivatives, and in particular derivatives of α-pyrone have been recovered from isolates of *Trichoderma viride* in different cultural conditions. The fungal isolate, the substrate and the culture method have influenced qualitatively and quantitatively the production of these volatile metabolites. The compound that gives rise to the aroma of coconut characterizing some cultures of *T. viride* has been confirmed to be 6-pentyl-α-pyrone.

RIASSUNTO

Da isolati di *Trichoderma viride* in differenti condizioni colturali sono stati rilevati diversi metaboliti volatili quali lattoni, alcoli superiori, derivati terpenici ed in particolare derivati dell'α-pirone. L'isolato fungino, il substrato e le modalità di coltura hanno influito quali-quantitativamente sulla produzione dei metaboliti volatili medesimi. Il composto che origina l'aroma di noce di cocco caratterizzante alcune colture di *T. viride* è stato confermato essere il 6-pentil-α-pirone.

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