MOULDY-EARTHY FAULTS IN WINES: GEOSMIN IDENTIFIED AS THE MAIN COMPOUND RESPONSIBLE

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The harvest of botrytized grapes displaying fungal or mouldy aromas is not a new phenomenon (*1;2*). However, over the last few years, several aromatic defects with a fungal, mouldy or earthy characteristic associated with a more or less evident occurrence of moulds on the grapes have been found in wines from different viticultural areas (Bordeaux, Val de Loire, Burgundy).

First studies have identified the presence of a compound with a strong wet earth and beetroot aroma in wines. It is (-)-geosmin, a well known water pollutant (*3*). This compound is found in musts before fermentation and its presence is always associated with grapes that were at least partially affected by grey mould (*4;5*).

However, geosmin is not the only compound responsible for the mouldy-earthy aromas of wines. Other earthy defects have also appeared in famous Burgundy appellations.

The significance of the quality degradation caused by these problems in wines produced from numerous grape varieties (Cabernet Sauvignon, Semillon, Gamay, Chenin, Pinot noir) led to an extensive study. Its objectives were to characterize the nature of the faults associated to these fungal and earthy aromas, and also to determine their biological origin and the conditions leading to their formation in the vineyard.

I. Characterization of the fungal and/or earthy faults in grapes, musts and wines

Two types of fungal and/or earthy faults can be responsible for organoleptic deviations. Some are found in grapes and musts but are not perceived in wines. On the other hand, others are present in grapes and musts and remain after fermentation, affecting wine quality. Thus, the thorough understanding of the chemical nature of the compounds responsible for such faults is essential to comprehend their origin in the vineyards.

I.1.Identification of fungal/earthy faults in grapes and musts

Grapes, grape juices and wines produced from Cabernet Sauvignon, Gamay, Pinot and Semillon at least partially affected by grey mould and presenting fungal and earthy characteristics were analyzed by gas chromatography-olfactometry (GC/O). This allowed the determination of the most characteristic aroma regions in the chromatograms.

A relatively large diversity of aromatic regions corresponding to fungal and earthy aromas could be detected (Table 1) according to the grape variety, the stage of development and the type of mould present on the berries (*6*). Among the compounds frequently identified, 1-octen-3-ol, 1-octen-3-one, 2-octen-1-ol and 2-heptanol were systematically associated with the fungal aromas found in grapes affected by grey rot.

Other compounds with camphorated and earthy notes were also identified either in white (Semillon, Sauvignon), or in red grape varieties (Cabernet Sauvignon, Pinot noir, Gamay), which had been harvested after grey mould development due to *Botrytis cinerea*: i.e. fenchol, fenchone, and 2-methylisoborneol. Among earthy aroma compounds, the latter seems to be the main cause for the mouldy-earthy fault in red grapes affected by *B. cinerea*.

Odour	Compounds	Varieties[b]	Sensory threshold (µg/l)[c]			Levels measured in musts (µg/l)	Contribution to the faulty character of wines
			water	Model solution	Wine		
Mould	1-octen-3-one	CS - M - S	0.003	0.03	0.07	0 – 0.01	yes, sometimes
	1-octen-3-ol	CS - M G - S	2	20	40	0 - 20	yes
	2-octen-1-ol	S	20	-	-	0 – 0.01	limited
	2-heptanol	S	70	-	-	0 – 0.06	limited
	not identified	S	-	-	-	-	limited
	not identified	S	-	-	-	-	limited
Earthy [a]	2-methylisoborneol	CS - M - PN	0.012	0.04	0.055	0 – 0.07	yes, limited over time
	fenchol	S	50	-	-	0 – 0.01	yes, sometimes
	fenchone	S	500	-	-	0 – 0.025	limited
	geosmin	CS - M - G PN - C - S	0.01	0.04	0.05	0 - 1	yes
	not identified	S	-	-	-	-	limited
Moss	not identified	S	-	-	-	-	limited

[a] The aromatic regions associated with the earthy faults of Pinot noir do not appear in this table
[b] CS: Cabernet Sauvignon, M: Merlot, S: Semillon, G: Gamay, PN: Pinot noir, C: Chenin
[c] References: (*7*)

Table 1: Main volatile compounds with mouldy and /or mouldy-earthy aromas frequently found in musts and wines made from affected harvests.

The majority of these molecules is degraded during alcoholic fermentation in less odorous compounds, especially 1-octen-3-one and 2-methylisoborneol (MIB), and thus, do not affect wine aroma. However, the levels of fenchol and fenchone change only slightly during alcoholic fermentation but remain in wines at concentrations below the sensory detection threshold (*6*). Thus, they are not responsible for the earthy faults in wines.

I.2. Study of the physio-chemical properties of (-)-geosmin

(-)-geosmin has very low sensory thresholds of approximately 10 ng/l in water, 40 ng/l in a wine mimicking model solution, 40-50 ng/l in white wine and 50-80 ng/l in red wine (thresholds determined by the Oenological Faculty of Bordeaux).

This compound is only slightly degraded during alcoholic fermentation: only 20% were degraded in 2 weeks. Geosmin is also relatively stable during storage since it takes approximately 2 months at 20°C to reduce the initial levels by half, and 8 months at 10°C. Thus, the temperature is a crucial factor for the chemical degradation of geosmin in wines.

Considering the stability of geosmin, numerous curative treatments were carried out in order to remove geosmin from wines (Table 2). Whole milk and grape seed oil allowed to remove up to 60% and 80% of the geosmin, respectively.

Thus, treatments with fat containing compounds offer a good efficiency with regards to geosmin. However, as of today, they are not allowed in oenology. Moreover, thermal treatments at 70°C for 24 hours in closed bottles lead to the degradation of 80% of geosmin. Volatilization by nitrogen or argon sparging has also been considered at different temperatures but the results were not satisfactory.

	Treatment			
	Whole Milk (1,5l/hl wine)	**Grapeseed oil** (0,5l/hl wine)	**Heat** (24h at 70°C)	**Gas sparging** (N_2 at 3l/l wine; 1h at 25°C)
Geosmin				
before treatment	290	320	700	310
after treatment	120	70	100	280
Efficiency	**58.6%**	**78.1%**	**85.7%**	**9.7%**

Table 2: Efficiency of curative treatments on geosmin reduction in wines

In spite of their relative efficiency, these curative treatments are not selective with regards to geosmin and lead to a significant aroma losses in treated wines. Therefore, they can only be considered as an emergency measure and not as a permanent solution. Consequently, the management of this problem requires preventive measures.

II. Origin of geosmin, a compound responsible for an earthy aroma in wines

II.1. Characterization of the microorganisms associated with the formation of geosmin in the vineyard

The biological origin of geosmin has been elucidated by Gerber and Lechevalier (*8*). This compound was found initially in bacterial cultures of Actinomycetes (*Streptomyces* sp.). Its production by blue algae and several *Penicillium* species was demonstrated later (*9-14*). Geosmin was never detected in healthy grapes harvested from plots affected by this problem, which implies that the presence of this compound is systematically linked to mouldy grapes. Since October 1999, numerous microorganisms were isolated from healthy and mouldy grapes harvested in geosmin affected vineyards from the Bordeaux, Loire valley, Burgundy and Beaujolais areas. This included white and red varieties (Cabernet Sauvignon, Semillon, Gamay, Chenin, Pinot noir). The berry microflora was analyzed and the capacity of the isolated microorganisms to produce geosmin was assessed (*15*). The main suspects with regards to the formation of geosmin in vineyards were characterized thanks to molecular biology tools.

II.1.a. Analysis of the grape microflora

The analysis of the microflora of grapes presenting an earthy aroma due to geosmin showed that the *Penicillium* genus was well represented (Table 3). Numerous species were occasionally found with less than 5 isolates per plot according to the area and the year; i.e. *P. thomii*, *P. purpurogenum*, *P. glabrum*, *P. brevicompactum* and *P. carneum*. Regardless of the plot studied, none of these species was systematically isolated over the entire study period of 3 years. However, as characterized by molecular biology (*16*), a single species (*P. expansum)* was always found in grapes containing geosmin in every plot studied and at every harvest during the study period. On average, 5 to 10 isolates were obtained per plot from the 4 sites in the Bordeaux area studied between 1999 and 2004, and frequent isolations were registered in samples from other French vineyards.

Some *Streptomyces* sp. isolates belonging mainly to three species were occasionally found in the plots. However, in a given plot, the presence of specific *Streptomyces* species was never continuous over the years (Table 3).

B. cinerea, the causative organism of grey rot, was omnipresent and found in relative abundance every year and on every site studied with more than 10 isolates per plot (Table 3).

MICROORGANISMS	BORDEAUX				BEAUJOLAIS			BURGUNDY		LOIRE VALLEY			
	M 1 [1]	M 2	S 1	S 2	BJ 1	BJ 2		BG 1	BG 2	VL 1	VL 2	VL 3	VL 4
Year of study	1999 - 2004	1999 - 2004	1999 - 2002	1999 - 2001	2002	2002	2004	2002 - 2005	2005	2002	2002 - 2005	2002	2004
P. expansum	+++ [2]	+++	+++	++	+++	+++	+	++	+	+++	++	+++	+
P. thomii	-	+	-	-	-	+	+	-	-	+	+	-	-
P. purpurogenum	-	+	-	-	-	-	++	-	-	+	-	-	+
P. glabrum	-	-	+	-	-	-	+	++	+	-	-	++	-
P. brevicompactum	+	-	-	-	+	-	-	+	-	+	+	-	-
P. carneum sect *roqueforti*	+	-	-	-	-	-	-	-	-	-	-	-	-
Total *Penicillium* spp.	++	+++	++	++	+++	+++	+++	+++	++	+++	+++	+++	+++
S. coelicolor ou *S. lividans*	-	+	-	+	+	-	+	-	-	-	+	+	+
Streptomyces griseus	-	-	-	-	-	-	-	-	-	+	+	+	+
Streptomyces flavogriseus	-	+	-	+	+	+	-	-	-	+	+	+	+
Other *Streptomyces* spp.	-	-	-	-	-	-	-	-	-	+	-	+	+
Total *Streptomyces* spp.	-	+	-	+	+	+	+	-	-	++	++	++	++
Botrytis cinerea	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++

[1] M1 and M2: Medoc sites with Cabernet Sauvignon; S1 and S2: Sauterne sites with Semillon; BJ1 and BJ2: Beaujolais sites with Gamay; BG1: Burgundy sites with Pinot noir; VL1 to VL4: Loire Valley sites with Gamay in 2002 and 2005 and Chenin in 2004
[2] -: microorganisms not detected; +: few isolates (<5); ++: some isolates (5-10); +++: high number of isolates (>10)

Table 3: Principal microorganisms found on mouldy grapes containing geosmin and sampled from 4 French viticultural areas

Numerous moulds and yeasts were also found on healthy grapes. Among the moulds, *Aureobasidium pullulans* was most frequently found, and several species of *Alternaria, B. cinerea*, *Cladosporium* sp. and *Epicoccum nigrum* were equally well represented. Among the yeasts observed, *Rhodotorula* sp. was systematically found. Other moulds were occasionally detected: *Penicillium* spp.*, Aspergillus niger, Coniothyrium* sp.*, Phoma* sp.*, Cephalosporium* sp.*, Pestalotia* sp. and *Chaetomium* sp.

II.1.b. Ability of the microorganisms to produce geosmin

The geosmin production potential of the microorganisms previously identified was then assessed.

None of the *B. cinerea* strains tested produced geosmin, but certain isolates synthesized an other compound with earthy and camphorated aromas. Among the *Penicillium* spp. isolated, only the representatives of the 2 phenotypically characterized species produced geosmin; i.e. *P. expansum*, which was found on every site, and *P. carneum*, which was occasionally identified. The other *Penicillium* spp. isolated did not produce geosmin in spite of their ability to synthesize various aromas (mould, cave). The *Streptomyces* spp., which were isolated sporadically from mouldy grapes originating from the different plots studied, produced the earthy aroma characteristic of geosmin. However, none of the other moulds tested did produce geosmin.

Therefore, the analysis of the microflora of healthy or mouldy berries has demonstrated the omnipresence and abundance of *B. cinerea* in clusters containing geosmin. Moreover, *P. expansum* was systematically found in association with *B. cinerea* every year and in every plot affected by geosmin. This fungus and the soil bacterium *Streptomyces* sp. were able to produce geosmin. A study of the effect of time and cultivation medium on their geosmin production metabolism was thus initiated.

II.1.c. Preliminary study of the production metabolism of *P. expansum* and *Streptomyces sp*. potentially responsible for the formation of geosmin in vineyards

Five media types were inoculated with *P. expansum* or *Streptomyces* sp.: Malt Agar (MA) and "grape juice" (GJ) media (at pH values from 3 to 7), whole clusters, berries and stems.

The filamentous bacteria produced geosmin in MA and GJ at pH values above 6, but could not grow on the acidic media, such as berries, grape juice or stems (pH 3-4). The hypothesis that they could be involved in the formation of geosmin in the vineyard, which occurs during grape maturity, could thus be abandoned. Consequently, the studies focussed on *P. expansum*.

Paradoxically, *P. expansum* grew and produced geosmin in MA medium regardless of the pH, whereas in grape juice and on berries it was able to grow but could not synthesize geosmin. Therefore, the geosmin production metabolism of *P. expansum* was investigated.

II.2. Study of the geosmin production metabolism of P. expansum

Initially, the metabolic study investigated the influence of the environment and juice composition on the production of geosmin by *P. expansum*. For this purpose, the mould was first inoculated on Petri dishes containing Malt Agar (MA) or grape juice (GJ) and certain abiotic factors were modified (i.e., light intensity, temperature and oxygen concentration) in order to reproduce vineyard conditions as closely as possible. Then, diverse treatments were applied to grape juices to assess their possible stimulating or inhibitory influence on the production of geosmin by *P. expansum* (pH, polyphenols, fatty acids). Afterwards, the effect of the nitrogen composition of the culture medium was specifically considered. Finally, the geosmin biosynthesis pathway was studied with a biochemical approach (results not shown in this article).

Among the environmental factors studied, only oxygen concentration stimulated the formation of geosmin by *P. expansum* in MA medium, but none of the environmental factors had a direct effect on the onset of the geosmin production of the mould.

Among the grape components, linoleic acid, a fatty acid found in the grape bloom, allowed *P. expansum* to produce geosmin. Moreover, elevated amino acid levels, comparable to those found in healthy grape juice, allowed *Penicillium* growth but inhibited the synthesis of geosmin in a model medium. On the other hand, ammonium in the berries favoured geosmin formation and the stimulation was proportional to the quantity added.

II.3. Effect of Botrytis cinerea on the production of geosmin by P. expansum

Vineyard observations demonstrated that rotten clusters containing geosmin always had *B. cinerea* infection foci in association with *P. expansum.* In addition, it was shown that nitrogen deficiencies in the grape juice induced the production of geosmin by *P. expansum* and this deficiency can be caused by *B. cinerea* growth on grapes.

Thus, we decided to investigate the influence of *B. cinerea* on geosmin synthesis by *P. expansum* using two complementary approaches. First, we studied the effect of co-inoculation of grapes, stems or grape juice with *B. cinerea* and *P. expansum*. Then, we assessed the role of the *B. cinerea* metabolism on grapes more closely.

II.3.a. **Importance of the association of *B. cinerea* and *P. expansum* on the formation of geosmin in grape juice and grape mash**

Numerous co-inoculation experiments were performed with whole clusters, berries, stems, grape mash and grape juice using *B. cinerea* and *P. expansum*. Different grape varieties, isolates, environmental conditions and intervals between inoculations were evaluated.

We could demonstrate the ability of *P. expansum* to synthesize geosmin in grape juice and mash previously cultivated with *B. cinerea*. All *P. expansum* strains isolated from different sites were able to produce the earthy compound after *B. cinerea* growth. On the other hand, only 22% of the *B. cinerea* strains taken from a Beaujolais plot in 2004 and tested in co-inoculations modified the grape juice composition in such a way as to allow production of geosmin by *P. expansum* (*17*).

Subsequently, different *B. cinerea* metabolites (gluconic acid, glycerol, mannitol, galactose) were added to grape juice based culture media. A stimulatory effect of mannitol on the formation of geosmin was clearly demonstrated for several *P. expansum* strains and this stimulation was proportional to the quantity of mannitol added. In addition, the inhibitory effect of grape juice amino acids could be confirmed.

Thus, the fundamental effect of *B. cinerea* resides in the degradation of amino acids, which inhibit the formation of geosmin. In addition, mannitol, a polyol produced by *B. cinerea*, also allowed the production of this compound by *P. expansum* and could have a stimulatory effect on geosmin formation.

II.3.b. Interpretation of the variability among the ability of *B. cinerea* species to prepare the grape for the synthesis of geosmin by *P. expansum*

After demonstrating the effect of the association between the two fungi *B. cinerea* and *P. expansum* in the formation of geosmin in the vineyard, we have shown that the metabolism of certain *B. cinerea* strains could promote the expression of geosmin by *P. expansum*. Therefore, we tried to evaluate what difference(s) could exist between those *B. cinerea* strains "favourable" to the formation of geosmin by *P. expansum* in grape juice (called "bot +") and the "unfavourable" ones (called "bot –").

First, the concentrations of amino acids, ammonium ions and mannitol in several grape juices pre-cultivated with "bot +" or "bot –" strains were compared, but no differences were found.

Afterwards, we demonstrated that the "bot–" strains synthesized a compound that inhibited the production of geosmin by *P. expansum*, and several purification steps determined some characteristics of this compound: it was heat resistant, acidic, with a size ranging from 30 to 50 kDa and of polysaccharidic nature. The characterization of this compound is still underway.

These results suggest that all *B. cinerea* strains have the capacity to "prepare" grape juice, especially by modifying the amino acid content, in order to allow the production of geosmin by *P. expansum.* However, the vast majority of *B. cinerea* strains simultaneously synthesize an inhibitor of geosmin formation.

Conclusion

In summary, this study has contributed to the characterization of mouldy and earthy organoleptic defects of grapes, musts and wines thanks to new tools for the chromatographic analysis and the enhanced sensitivity of mass detectors. While the alcoholic fermentation allows the degradation of some of these compounds, other defects, those compounds linked to the presence of (-)-geosmin remain and permanently affect the organoleptic quality of wines. This work has demonstrated the microbial origin of some of these defects and thus revealed the emergence of associations between *Botrytis cinerea* and diverse fungal species in our vineyards with temperate climates. These fungal species had been considered to be marginal until now, specifically *P. expansum*.

A thorough study of the origins of (-)-geosmin allowed to establish the systematic presence of both *B. cinerea* and *P. expansum* on clusters containing geosmin. It also demonstrated the possible formation of geosmin by consecutive growth of *B. cinerea* and *P. expansum* strains in grape juice and mash. Some explanations for this phenomenon could be proposed, notably the effect of nitrogen deficiencies in grape juices following *B. cinerea* growth. This study has equally demonstrated the variability among *B. cinerea* strains with regards to their ability to synthesize a polysaccharidic macromolecule capable of inhibiting the formation of geosmin by *P. expansum*.

A deeper knowledge not only of the chemical nature of these organoleptic defects and their origin, but also of the ecology of the fungal species in the vineyard appears essential to address these problems as rapidly and efficiently as possible in the future.

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