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ORIGINAL PAPER

Impact of the *Botrytis cinerea* strain and metabolism on (−)-geosmin production by *Penicillium expansum* in grape juice

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Abstract Geosmin, an off-flavour of some rotten grapes, has been implicated in wine defects. *Botrytis cinerea* and *Penicillium expansum* were the most common among the numerous microorganisms isolated from rotten grapes. *P. expansum* produces geosmin on model media but not healthy grape juice. However, geosmin synthesis by *P. expansum* was demonstrated in grape juice and on crushed grapes that had been pre-cultured with certain *B. cinerea* strains. 34 out of 156 *B. cinerea* strains ([bot +] phenotype) isolated from the centre of grape bunches were able to induce high geosmin production, up to 494 ng/l, by *P. expansum* in grape juice. A study of the impact of grape juice composition on geosmin synthesis by *P. expansum* revealed the importance of nitrogen composition, particularly amino-acid deficiency. Metabolism of amino acids by *B. cinerea* was shown to be favourable to geosmin synthesis by *P. expansum*. However, the amino-acid and ammonium concentrations in grape juices pre-cultured with *B. cinerea* [bot -] and [bot +] strains were very similar implying that other factors are involved as well. Indeed, an ethanol-precipitable fraction, probably a polysaccharide, synthesized by *B. cinerea* [bot -], but not [bot +] strains, inhibited geosmin production by *P. expansum*.

Keywords *Botrytis cinerea* · (−)-Geosmin · Grape · Metabolism · Nitrogen · *Penicillium expansum*

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Introduction

Grape rot, mainly due to the *Botrytis cinerea* fungus, is one of the major causes of degradation of grape components, leading to deterioration in wine quality. In particular, over the past ten years, winegrowers have observed organoleptic defects with mushroom, mouldy, camphor, or earthy odours in must made with rotten grapes and, sometimes, in wine (La Guerche et al. 2006). Among the compounds implicated in these defects, (−)-geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol), a powerful aromatic terpen with an earthy smell, was identified (Darriet et al. 2000).

The distribution of the *microbiota* associated with rotten grapes containing geosmin was studied on different sites (La Guerche et al. 2004, 2005b). Some microorganisms commonly present on grapes were

isolated (Hewitt 1994, Serra et al. 2005): fungi, e.g. *B. cinerea*, *Cladosporium* sp., *Alternaria* sp., *Rhizopus* sp., *Penicillium* sp., as well as yeasts (*Rhodotorula* sp. and *Kloeckera apiculata)* and bacteria, including *Streptomyces* sp. *B. cinerea*, the fungus responsible for grey rot, was the most recurrent species, isolated every year on grapes containing geosmin. Several species of *Penicillium* were found on the same grapes with *B. cinerea*, constituting specifying bunch rot, including *P. expansum*, *P. thomii*, *P. purpurogenum*, *P. glabrum*, *P. brevicompactum*, *P. carneum*, and *P. miczynskii* (La Guerche et al. 2004, 2005b, 2006). Among the genera identified on rotten grapes containing geosmin, some are able to produce this compound on laboratory media, e.g. *Streptomyces* sp. [*S. griseus* (Harris et al. 1986), *S. coelicolor* (Scholler et al. 2002), and *S. flavogriseus* (La Guerche et al. 2005b)] and some *Penicillium* spp. [*P. expansum* (Mattheis and Roberts 1992), *P. carneum* (La Guerche et al. 2005b), and *P. miczynskii* (La Guerche et al. 2006)]. Our previous study on *Streptomyces* sp. metabolism has demonstrated the inhibiting action of low pH on its development and, consequently, the extremely limited potentiality of this genus to produce geosmin on grapes. Among the *Penicillium* spp., *P. expansum* was the most frequently species found on earthy rotten grapes containing geosmin. This fungus was isolated on grapes with earthy odour of geosmin on all the sites over 6 years (La Guerche 2004, La Guerche et al. 2005a) but, paradoxically, the representatives of this species, all producing geosmin on laboratory media, were not able to synthesize this compound in grape juice or on grapes, under usual conditions of temperature and humidity. In a previous paper, we demonstrated a complementary action of *B. cinerea* and *P. expansum* on geosmin production in grape juice and on crushed grapes (La Guerche et al. 2005b). However, only a few of the *B. cinerea* strains in this complex were capable of inducing geosmin production in grape juice by *P. expansum*, illustrating the complexity of geosmin production during the development of rot on grapes.

Geosmin synthesis in various microorganisms is affected by environmental and nutritional factors. Low concentrations of both nitrate and ammonium were considered to favour geosmin production by *Streptomyces halstedii* (Blevins et al. 1995), whereas the amount of geosmin produced by the cyanobacterium *Anabaena viguieri* increased with the concentrations of ammonium added (Wu et al. 1991). Some amino acids have also been shown to influence geosmin synthesis by *S. griseofuscus*: maximum geosmin production by this bacterium increased considerably in samples containing 10 and 100 mg/l L-methionine (Aoyama 1990). Moreover, leucine and valine have been found to be involved in terpenoid biosynthesis by other *Streptomyces* species (Bentley and Meganathan 1981, Pollak and Berger 1996). The impact of other parameters (e.g. carbon sources, metals) on geosmin production by *S. halstedii* have also been tested (Blevins et al. 1995): mannitol promoted maximal geosmin production and micronutrients as zinc, iron, and copper had the most significant effects on biomass and geosmin production. Moreover, mannitol, a *B. cinerea* metabolite produced on grapes (at levels up to 1 g/l), directly induced geosmin synthesis by *P. expansum* in healthy grape juice (La Guerche 2004).

In this project, *B. cinerea* strains capable of inducing geosmin production by *P. expansum* were studied among a large population of strains isolated from rotten grape bunches. Samples were taken from several vineyards affected by the problem of earthy off-flavours in grapes and wines. The second stage consisted of examining the impact of grape juice composition on geosmin production by *P. expansum*. As one of the main reported effects of *B. cinerea* development on grape is the modification of nitrogen composition, particularly degradation of amino acids (Rapp and Reuther 1971, Sponholz 1991), the impact of the ammonium and amino-acid composition of grape juice on geosmin production by *P. expansum* was investigated. All *B. cinerea* strains isolated from grapes degraded at least 90% of the amino acids in the grape juice, but those unable to promote geosmin biosynthesis were shown to produce an ethanol-precipitable fraction.

Materials and methods

Chemicals and biological compounds

Anhydrous sodium sulphate, 3-octanol, and pentane were from Sigma-Aldrich (Saint Louis, MO, USA). Pentane was distilled in order to improve its purity. Anhydrous ethyl alcohol was from Carlo Erba

(Milan, Italy). Amino acids were from Sigma-Aldrich (Saint Louis, MO, USA). Agar, Malt Agar medium, Czapek medium, and Yeast Extract were from Sigma-Aldrich (Saint Louis, MO, USA).

Origin of the strains tested

Various *Penicillium expansum* isolates from the INRA-UMR Santé Végétale collection (Bordeaux, France) were used: UMRSV00S102 and UMRSV00S203 from sites S1 and S2 (Sauternes; Sémillon grapes) in 2000; UMRSV00M101 and UMRSV01M102 from site M1 (Médoc; Cabernet Sauvignon grapes) in 2000 and 2001, respectively; UMRSV01S101, from site S1 in 2001; UMRSV01M201 and UMRSV01M206 from site M2 (Médoc; Cabernet Sauvignon grapes) in 2001; UMRSV02VL115 from site VL1 (Loire Valley, Gamay grapes) in 2002. The main strains of *Botrytis cinerea* were UMRSV01M103 (INRA-UMR Santé Végétale collection), collected from site M1 in 2001, C77-4 (Faculté d'Oenologie collection; Bordeaux, France), isolated from Château d'Yquem grapes in 1977, and SAS56, supplied by F. Faretra (Bari, Italy). The remaining strains tested (156) came from the several sites studied in Beaujolais (Gamay grapes) in 2004 (INRA-UMR Santé Végétale collection).

Culture on solid media

Penicillium strains were cultured in polystyrene Petri dishes containing 20 ml Malt Agar (MA) medium, Czapek (CZA) medium, Czapek Yeast Agar medium (CYA: CZA + 5 g/l Yeast Extract) or Grape Juice medium (GJ medium: 15 ml grape juice + 5 ml agar solution at 60 g/l). Media were inoculated with 20 µl of a spore suspension containing 1.10^7 conidia/ml of *P. expansum* and stored at 20 °C for 4 days before geosmin extraction.

Culture in grape juice

Juice obtained from crushed Cabernet Sauvignon, Gamay, and Sauvignon Blanc grapes was sterilized by filtration on a 0.45-µm pore polycarbonate membrane (Millipore Corporation, Bedford, MA, USA). The grape juice was then inoculated with 200 µl of a spore suspension containing 1.10^7 conidia/ml of *P. expansum*. The culture was stored at 20 °C for 3 days before geosmin extraction.

Percolation of grape juice on C_{18} silica and on a cation-exchange resin

100 ml C_{18} silica (0.06–0.20 mm; Alltech, Lexington, KY, USA) was rinsed in absolute ethanol 3 times then transferred into a glass column (20 mm × 400 mm) and washed with 3 volumes distilled water (3 ml/min). 2 volumes grape juice (200 ml) were passed through the column to remove colour, then rinsed with 5 volumes of distilled water. Non-polar compounds retained on the column were eluted with 3 volumes absolute ethanol. The ethanol fraction was discarded. The 500 ml of percolated water were evaporated under vacuum to 50 ml and this volume was reincorporated to the juice discoloured by percolation. This volume (250 ml) was then passed through a glass column (20 mm × 400 mm) containing 100 ml AG50W-X4 cation exchanger (Bio-Rad, Hercules, CA, USA), previously rinsed with deionized water (3 volumes), and percolated at 3 ml/min. Non specifically retained compounds were eluted with 5 volumes of deionized water. This fraction was mixed with the non-retained fraction and evaporated to the initial volume under vacuum at 45°C. This fraction was then sterilized by filtration and used as a base for liquid or solid grape-juice media, as described above. Ionized compounds were eluted with 2 volumes potassium chloride 3M.

Amino nitrogen (mainly consisting of ammonium and amino acids) was measured in the various fractions obtained during this purification. C_{18} silica does not fix amino acids (amino nitrogen values were similar before and after percolation) whereas cation-exchange resin (AG50W-X4) does. Moreover, the KCl eluate contained the maximum amino nitrogen concentration.

Sequential culture of *Botrytis cinerea* and *Penicillium expansum* on grape juice

For each *B. cinerea* strain, 20 µl of a spore suspension containing 1.10^7 conidia/ml were inoculated in a 100-ml Erlenmeyer flask containing 35 ml sterilized grape juice (La Guerche et al. 2005b). Cultures were maintained at ambient temperature (20 °C ± 2 °C). After 7 days, the mycelium was removed with

tweezers. Grape juice was sterile filtered (0.45 µm pore polycarbonate membrane; Millipore Corporation, Bedford, MA, USA), put in a sterile Erlenmeyer flask, and inoculated with 20 µl of a spore suspension of *P. expansum* containing 1.10^7 conidia/ml. 0.5 g/l NH_4^+ was added to compensate for nitrogen deficiency due to *B. cinerea* development. After 3 days' culture at ambient temperature, the mycelium was removed with tweezers and geosmin was extracted. As a test, geosmin was assayed for each culture of *B. cinerea* strain on GJ medium.

Ethanol precipitation of grape juice cultured with *B. cinerea*

50 ml grape juice were cultured for 8 days after inoculation of *B. cinerea* strains with [bot +] or [bot -] phenotype (1.10^7 conidia/ml). The samples were then sterile-filtered (0.45 µm pore polycarbonate membrane; Millipore Corporation, Bedford, MA, USA), treated with 5 volumes anhydrous ethyl alcohol, and kept at 4°C overnight (Brillouet et al. 1990). The ethanol solution (300 ml) was centrifuged (6000 rpm, 10 min) and the supernatant separated. Ethanol was removed from the supernatant by evaporation under vacuum at ambient temperature and the residual aqueous fraction was used as a culture medium after sterile filtration on polycarbonate membrane. The pellet was rinsed with ethyl alcohol and dissolved in distilled water. This fraction was dialysed against 4 l deionized water on a membrane (6000–8000 Da; Spectrapore, CA, USA) at 4°C overnight, then ultrafiltered in a 50 ml stirred ultrafiltration cell (Amicon; Millipore Corporation, Bedford, MA, USA). The cell was pressurized to 4 Bars using nitrogen (Air Liquide, Floirac, France). A polyethersulfone membrane with a 10,000 molecular-weight cutoff (NOVA 10K; Pall Corporation, New York, NY, USA) was located at the bottom of the cell. A magnetic stir bar, placed on the surface of the membrane, was used to simulate crossflow filtration. Ultrafiltration was conducted at ambient temperature (20–22 °C) for 24 h. After sterile filtration, this fraction was added to the grape juice medium.

Amino-acid and ammonium assay in grape juice

Two methods were used to assay nitrogen in grape juice. First, amino nitrogen (ammonium and amino acids) was assayed using the Ninhydrin Reagent Solution kit (Sigma-Aldrich; St Louis, MO, USA).

Free amino acids were analysed by high-performance liquid chromatography, according to (Hilbert et al. 2003), and the amino acids were identified and quantified as described by Rodriguez-Lovelle and Gaudillère (2002).

Geosmin extraction and quantification

Geosmin was extracted from Petri dish and Erlenmeyer flask cultures, analysed by gas chromatography–mass spectrometry analysis, and quantified as described by La Guerche et al. (2006).

Results and discussion

Representation in the vineyard of the *B. cinerea* strains inducing geosmin production by *P. expansum*

In 2004, a population of *B. cinerea* strains was collected from the centre of 120 matured rotten Gamay grape bunches, containing or not geosmin, and sampled from several vineyards in the Beaujolais region. The wines from these sites were severely affected by earthy off-odours due to geosmin (Stéphane La Guerche, unpublished data). 156 *B. cinerea* strains were isolated and characterized, then precultured on GJ medium to assess their capacity to induce geosmin synthesis by *P. expansum*. Twelve strains were also tested on CZA, CYA, and MA media, to evaluate their geosmin-producing potential.

Irrespective of the medium (GJ, CZA, CYA, and MA), no geosmin was quantified above the detection threshold (i.e. 5 ng/Petri dish) in the *B. cinerea* cultures. *P. expansum* (strain UMRSV01M206) did also not produce geosmin on media pre-cultured with 122 out of the 156 *B. cinerea* strains. The media of these strains (labelled [bot -]) induced less than 5 ng/l geosmin (Figure 1). In contrast, culture media of 34 *B. cinerea* strains (labelled [bot +]) did induce geosmin production by *P. expansum*, sometimes with high concentrations of geosmin: 185 ± 11 ng/l for *B. cinerea* UMRSV2BAT13B, 247 ± 15 ng/l for *B. cinerea* UMRSVBA5B, and 494 ± 30 ng/l for *B. cinerea* UMRSV2DB14B. Concentrations obtained with 26 strains out of 34 were over the olfactory

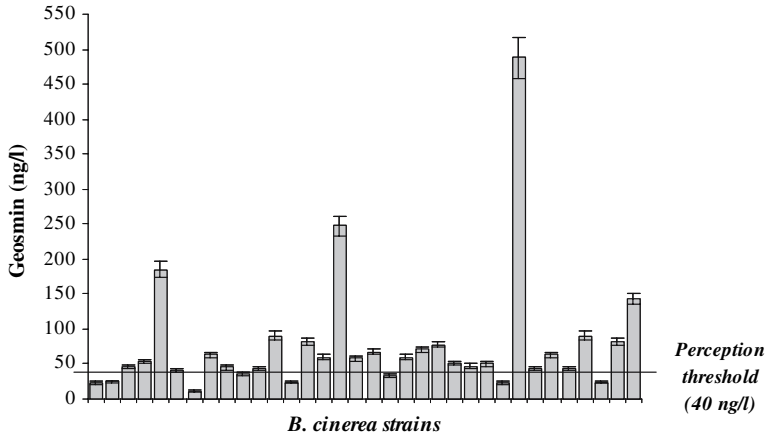

Fig 1 Geosmin production by *P. expansum* (strain UMRSV01M206) in grape juice pre-cultured with 34 *B. cinerea* strains among the 156 tested

perception threshold for this compound, i.e. 40 ng/l. Five of the *B. cinerea* strains that had induced high geosmin production by *P. expansum* UMRSV01M206 were also tested in association with 2 other *P. expansum* strains, resulting in similar geosmin levels to those obtained with *P. expansum* UMRSV01M206 (Table 1).

Our previous data were based on a collection of *B. cinerea* strains from all parts of grape bunches, not only the centre. Only 5% of these strains (3/60) were found capable of inducing geosmin synthesis by *P. expansum* (La Guerche et al. 2005b). Surprisingly, *B. cinerea* strains isolated from the centre of grape bunches from specific sites included a higher proportion of strains inducing geosmin production by *P. expansum*. These results concur with findings indicating that geosmin is localized in the skins of grapes inside the bunches (La Guerche 2004). They confirm that pre-culture with *B. cinerea* is necessary for geosmin production by *P. expansum* in grape juice, under usual conditions of temperature and humidity. Also, the significant proportion of *B. cinerea* [bot +] strains postulates that the presence of this species is necessary for geosmin synthesis by *P. expansum* on grapes.

In order to improve our knowledge of the way geosmin is produced, the study then focused on grape juice ammonium and amino-acid concentrations and their impact on geosmin production by *P. expansum*.

Influence of amine fraction removal on geosmin production by *P. expansum* on GJ medium

Preliminary studies established that *P. expansum* synthesized geosmin on CZA, CYA (Dionigi 1994, Mattheis and Roberts 1992), and MA media, but not healthy grape juice (La Guerche et al. 2005b). *P. expansum* culture on colourless, non-botrytized, grape juice obtained by C_{18} silica percolation did not produce geosmin at concentrations above 5 ng/Petri dish. Similar results were obtained when the juice was de-coloured using Charcoal (Decoloryl®; Laffort Oenologie, Floirac, France). Therefore, grape juice was percolated on a cation-exchange resin (AG50W-X4) to estimate the impact of amine fraction removal (amino acids and peptides were mainly retained on this resin) on geosmin production by *P. expansum*.

After percolation on AG50W-X4, the eluted grape juice was used as a base for various media and

Table 1 Geosmin production (ng/l) by 3 *P. expansum* strains in grape juice pre-cultured with 6 *B. cinerea* strains

P. expansum strains	*B. cinerea* strains					
	C77-4	UMRSVBA1B	UMRSVBA5B	UMRSV2BAT13B	UMRSV2DB14B	UMRSV01M103
UMRSV01M206	19 ± 1	91 ± 6	143 ± 9	185 ± 11	288 ± 18	494 ± 30
UMRSV01M102	23 ± 1	84 ± 5	114 ± 7	201 ± 12	290 ± 18	451 ± 28
UMRSV02VL115	20 ± 1	102 ± 6	154 ± 9	177 ± 11	267 ± 16	466 ± 28

cultured with 4 *P. expansum* strains: (1) eluted juice only, (2) eluted juice supplemented with ammonium (0.5 g/l), (3) eluted juice plus KCl eluate, and (4) eluted juice supplemented with ammonium plus KCl eluate. *P. expansum* grew on healthy grape juice medium but no geosmin was detected (Table 2). No *P. expansum* development was observed on medium in the absence of nitrogen, and no geosmin was detected. *P. expansum* growth was normal on the media containing ammonium. No geosmin was detected at concentrations above 5 ng/Petri dish on the other two media containing the eluate with the amine fraction of the grape juice, with or without ammonium [(3) and (4)]. However, on medium (2), geosmin was quantified at levels between 15 ± 1 and 22 ± 1 ng/Petri dish, indicating that percolating grape juice on a cation exchanger and supplementing it with ammonium raised the inhibition of geosmin synthesis by *P. expansum*. Supplementing KCl 3M on medium (2) did not modify geosmin production (Stéphane La Guerche, unpublished data). The following experiment confirmed the postulated inhibitory effect of an amino-acid mixture in grape juice on geosmin synthesis by *P. expansum*. A mixture of the main amino acids in grape juice (proline, arginine, tryptophan, and valine) was added at increasing concentrations (0.5, 1, and 2.5 g/l) to the eluted grape juice supplemented with ammonium [medium (2)]. Various *P. expansum* strains developed but no geosmin was detected at concentrations above 5 ng/Petri dish (Table 2).

Impact of nitrogen content on geosmin production by *P. expansum* on model media

Considering the above results, suggesting that the amine composition of grape juice had a major effect on geosmin production by *P. expansum*, the role of several nitrogen sources on various model media (CZA and CZN) was studied.

First, the sodium nitrate in CZA medium was replaced by 0.5 g/l ammonium or several amino acids, singly or in combination, to assess the impact of the type of nitrogen source in the culture medium on geosmin synthesis by *P. expansum* (strain UMRSV01M206).

Similar fungal growth was observed on all media. High geosmin production was detected, up to 38 ± 1 ng/Petri dish, in the medium containing ammonium (CZN medium) (Table 3). Moreover, a correlation was established between the ammonium concentration (0.1–1 g/l) and geosmin production after 3 days' *P. expansum* culture, suggesting that ammonium plays a leading part in inducing geosmin synthesis. A comparison of several amino-acid nitrogen sources revealed that glutamate led to high geosmin concentrations (169 ± 6 ng/Petri dish), while arginine, serine, and valine resulted in concentrations only half as high as those obtained with ammonium, approximately 19 ± 1 ng/Petri dish.

Similarly, when the nitrogen source consisted of a mixture of amino acids (Table 3), geosmin production was never over half the value observed with

Table 2 Geosmin production by 4 *P. expansum* strains on GJ medium after purification on a cation exchanger (AG50W-X4)

P. expansum strains	Geosmin concentration (ng/Petri dish)							
	Healthy grape juice	AG50W-X4 eluted juice + eluate	AG50W-X4 eluted juice	AG50W-X4 eluted juice + NH_4^+ [a]	AG50W-X4 eluted juice + NH_4^+ + eluate	AG50W-X4 eluted juice + NH_4^+ + AA[b]		
						0.5 g/l	1 g/l	2.5 g/l
UMRSV00S102	<5[c]	<5	ng	15 ± 1	<5	<5	<5	<5
UMRSV00S203	<5	<5	ng	18 ± 1	<5	<5	<5	<5
UMRSV00M101	<5	<5	ng	22 ± 1	<5	<5	<5	<5
UMRSV00M201	<5	<5	ng	19 ± 1	<5	<5	<5	<5

[a] $[NH_4^+]$: 0.5 g/l

[b] AA: proline+arginine+tryptophan+valine at 0.5, 1, and 2.5 g/l

[c] <5: below 5 ng/Petri dish, i.e. detection threshold; ng: no growth

Table 3 Geosmin production by *P. expansum* (strain UMRSV01M206) on CZA medium containing ammonium and/or amino acids (AA) at various concentrations

	Geosmin concentration (ng/Petri dish)			
	0.5 g/l N[b]	1 g/l N	2.5 g/l N	5 g/l N
NH_4^+ [a]	38 ± 1	71 ± 3	nt	nt
Glu	169 ± 6	nt	nt	nt
Arg	18 ± 1	nt	nt	nt
Ser	19 ± 1	nt	nt	nt
Val	14 ± 1	nt	nt	nt
mix 3AA 1	20 ± 1	nt	nt	nt
mix 3AA 2	<5[c]	nt	nt	nt
mix 4AA	<5	nt	nt	nt
mix 5AA 1	11 ± 1	nt	nt	nt
mix 5AA 2	<5	nt	nt	nt
mix 11AA	<5	nt	nt	nt
NH_4^++ Glu	191 ± 7	nt	nt	nt
NH_4^++ Arg	49 ± 2	72 ± 3	276 ± 10	21 ± 1
NH_4^++ Ser	<5	106 ± 4	123 ± 5	148 ± 6
NH_4^++ Val	<5	<5	<5	<5
NH_4^++ Trp	20 ± 1	24 ± 1	23 ± 1	19 ± 1
NH_4^++ Gly	34 ± 1	72 ± 3	89 ± 3	53 ± 2
NH_4^++ Pro	*52 ± 2*	*59 ± 2*	104 ± 4	52 ± 2
NH_4^++ His	45 ± 2	54 ± 2	207 ± 8	47 ± 2
NH_4^++ Met	<5	<5	<5	<5
NH_4^++ mix 3AA 1	15 ± 1	nt	nt	nt
NH_4^++ mix 3AA 2	<5	<5	<5	<5
NH_4^++ mix 4AA	8 ± 1	nt	nt	nt
NH_4^++ mix 5AA 1	19 ± 1	nt	nt	nt
NH_4^++ mix 5AA 2	<5	<5	<5	<5
NH_4^++ mix 11AA	<5	8 ± 1	10 ± 1	<5

[a] NH_4^+: Ammonium, Pro: proline, Arg: arginine, Trp: tryptophan, Val: valine, Glu: glutamate, Gln: glutamine, His: histidine, Gly: glycine, Ser: serine, Met: methionine, Leu: leucine, Lys: lysine, Thr: threonine, 3AA1: Arg+Ser+Val, 3AA2: Gly+His+Ser, 4AA: Pro+Arg+Trp+Val, 5AA1: Glu+Gln+Arg+Ser+Val, 5AA2: Pro+Arg+Trp+Val+Met, 11 AA: Pro+Arg+Trp+Val+Met+Gly+His+Ser+Leu+Lys+Thr

[b] 0.5 g/l N: 0.5 g/l nitrogen source (i.e. NH_4^+ and/or AA)

[c] <5: below 5 ng/Petri dish, i.e. detection threshold. nt: not tested

ammonium (i.e. 20 ± 1 ng/Petri dish). These results indicated that a mixture of amino acids resulted in lower geosmin synthesis levels than those measured on media containing ammonium or only one of the amino acids tested.

In a second series of experiments, CZN medium (CZA medium with 0.5 g/l ammonium) was supplemented with several amino acids, singly or mixed: proline, arginine, tryptophan, and valine [the main amino acids in grape juice (Flanzy 1998)], as well as glutamate, glutamine, methionine, glycine, histidine, serine, leucine, lysine, and threonine, at various concentrations (0.5, 1, 2.5, and 5 g/l).

Similar fungal growth was observed on all media. Except for 2 mixtures (3AA1: Arg+Ser+Val and 5AA1: Glu+Gln+Arg+Ser+Val), which resulted in up to 20 ± 1 ng/Petri dish, i.e. less than half that observed with ammonium (38 ± 1 ng/Petri dish), no geosmin was detected at concentrations above 10 ng/Petri dish on any medium containing a mixture of

amino acids and ammonium (Table 3). Significantly, the mixture containing the main grape amino acids (Pro+Arg+Trp+Val) in CZN medium did not induce geosmin synthesis at concentrations above 8 ng/Petri dish. Amino acids added separately had varying effects on geosmin production by *P. expansum* UMRSV01M206. Valine and methionine did not induce any geosmin production, while concentrations up to 2.5 g/l of each mixture containing ammonium and one amino acid, except serine, increased geosmin production (up to 276 ± 10 ng/Petri). In contrast, above this concentration, geosmin levels decreased (Table 3).

These results corroborate those cited above concerning the inhibitory impact of mixed amino acids on geosmin synthesis by *P. expansum* and illustrate the leading role played by ammonium. The absence of geosmin biosynthesis by *P. expansum* in grape juice with a high amino-acid content supports these observations.

Involvement of the *B. cinerea* nitrogen metabolism on geosmin production by *P. expansum*

As *B. cinerea* development on grapes leads to the degradation of amino acids, their concentrations in grape juice were measured in relation to the development time of *B. cinerea*, and a correlation was established between these concentrations and the ability of *P. expansum* to synthesize geosmin in *B. cinerea* pre-cultured grape juice.

As previously indicated, no geosmin was produced after up to 4 days *B. cinerea* pre-culture (Fig. 2) (La Guerche et al. 2005b), with amino-acid concentrations above 100 mg/l. Geosmin production increased following up to 7 or 8 days in culture, while amino-acid levels in the pre-cultured grape juice were below 100 mg/l. These results showed that geosmin synthesis by *P. expansum* was linked to the medium's deficiency in amino acids due to *Botrytis cinerea* development. Again, the inhibiting effect of amino acids was confirmed by the supplementation of grape juice pre-cultured with 3 *B. cinerea* [bot+] strains (SAS56, UMRSVM103, and UMRSV2DB14B) with a mixture of the main amino acids in grape juice (proline, arginine, tryptophan, and valine at 0.5, 1, and 2.5 g/l). No geosmin was quantified above 5 ng/l, irrespective of the *B. cinerea* strain.

To confirm that the *B. cinerea* metabolism was responsible for the previously observed changes in grape juice, numerous *B. cinerea* strains ([bot+] and [bot−] strains) were cultured in grape juice, and their amino-acid, ammonium, and mannitol contents were assayed. For all *B. cinerea* strains, the total amino-acid concentration in grape juice after 8 days' culture was 22–76 mg/l, i.e. under 10% of the initial level. Similar results were obtained for each amino acid and all the strains tested (Table 4). Ammonium levels varied from 19 to 69 mg/l during *B. cinerea* development, as compared to the 29 mg/l in healthy grape juice. Mannitol concentrations were between 40 and 90 mg/l in *B. cinerea* cultured grape juice, i.e. concentrations similar to those inducing geosmin production by *P. expansum* in healthy grape juice (La Guerche 2004). These results corroborate our previous studies highlighting the importance of *B. cinerea* in geosmin synthesis by *P. expansum*. This effect

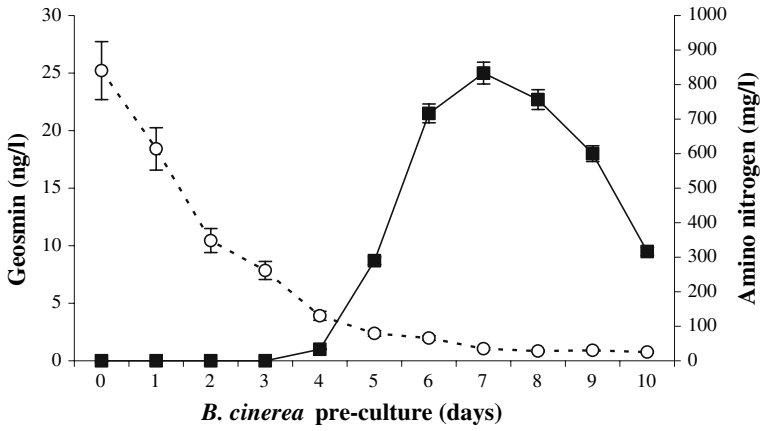

Fig 2 Impact of *B. cinerea* pre-culture (strain C77-4) in grape juice on amino nitrogen levels (○) and geosmin production by *P. expansum* UMRSV01M206 (■)

Table 4 Ammonium, amino-acid, and mannitol concentrations in healthy grape juice and grape juice cultured with several *B. cinerea* strains; geosmin production by *P. expansum* UMRSV01M206 on healthy and *B. cinerea* pre-cultured grape juice, without their ethanol precipitate or with [bot−] ethanol precipitate

	Concentrations (mg/l)			Geosmin production (ng/l)
	NH_4^+	Amino acids	Mannitol	
Healthy grape juice				
−ethanol precipitate	29	684	0	<5
+ethanol precipitate [bot -]	na[b]	na	na	<5
Pre-cultured grape juice				
[bot +] B. cinerea strains				
C77-4	19	56	50	23 ± 1
UMRSVBA5B	23	64	46	247 ± 15
UMRSV2BAT13B	55	59	76	185 ± 11
UMRSVBA7B	32	22	90	77 ± 5
SAS56	45	32	65	49 ± 3
SAS56−ethanol precipitate	na	na	na	39 ± 2
SAS56 + ethanol precipitate [bot−][a]	na	na	na	<5
UMRSV01M103	43	39	76	494 ± 30
UMRSV01M103−ethanol precipitate	na	na	na	197 ± 12
UMRSV01M103 + ethanol precipitate [bot−]	na	na	na	<5
UMRSV2DB14B	31	44	58	288 ± 18
UMRSV2DB14B − ethanol precipitate	na	na	na	227 ± 14
UMRSV2DB14B + ethanol precipitate [bot−]	na	na	na	<5
[bot−] B. cinerea strains				
UMRSV99VBC506	51	76	55	<5
UMRSV99VBC508	57	47	40	<5
UMRSV03BG103	69	43	76	<5
UMRSV03BG103 − ethanol precipitate	na	na	na	42 ± 2
UMRSV01M101	48	39	84	<5
UMRSV01M101 - ethanol precipitate	na	na	na	67 ± 4

[a] ethanol precipitate [bot−]: ethanol-precipitable fraction from *B. cinerea* [bot−] strain UMRSV01M101 culture

[b] <5: below 5 ng/l, i.e. detection threshold; na: not assayed

may be interpreted at least as resulting from its degradation of amino acids and production of mannitol. However, no significant difference was observed between the concentrations of any of these previous components in grape juices cultured with *B. cinerea* [bot+] or [bot−] strains (Table 4).

Evidence of the role of an ethanol-precipitable fraction produced by *B. cinerea* [bot−] strains on geosmin production by *P. expansum*

B. cinerea is known to produce polysaccharides, particularly *β*-1,3-glucan, during its development on grapes (Dubourdieu and Ribéreau-Gayon 1981). Among numerous parameters tested (La Guerche 2004), it was then investigated whether part of the difference between the two types of strains, [bot+] and [bot−], was due to an ethanol-precipitable fraction, generally considered as polysaccharide fraction (Brillouet et al. 1990). We demonstrated that, in grape juice, *B. cinerea* [bot−] strains synthesized an ethanol-precipitable fraction that inhibited geosmin production by *P. expansum*. To illustrate this, it was shown that the precipitate fraction (ethanol precipitate obtained from grape juice pre-cultured with the *B. cinerea* [bot−] strain UMRSV01M101) inhibited geosmin synthesis by *P. expansum* UMRSV01M206 when it was added to grape juice

pre-cultured with various *B. cinerea* [bot+] strains (SAS56, UMRSV01M103, and UMRSV2DB14B) (Table 4). Furthermore, when ethanol was removed from the supernatant obtained after ethanol precipitation of grape juice pre-cultured with *B. cinerea* [bot+] or [bot−] strains, geosmin was produced by *P. expansum* UMRSV01M206 in the resulting juice. Moreover, in healthy grape juice, ethanol precipitation had no effect on geosmin synthesis by *P. expansum*. This fraction is currently being characterized.

Conclusions

In the context of continuing research into the origin of geosmin on grapes, this article describes experiments aimed at elucidating the factors involved in geosmin production by *P. expansum* in grape juice and on crushed grapes pre-cultured with several *B. cinerea* strains. Analysis of the representativeness in the vineyard of the *B. cinerea* strains able to induce geosmin synthesis by *P. expansum* found that a significant proportion (22%) of the *B. cinerea* strains in the centre of grape bunches, labelled with the [bot+] phenotype, induced high geosmin production (concentrations up to 494 ± 30 ng/l). Then, an assessment of the effect of grape juice composition on geosmin production by *P. expansum* demonstrated the positive impact of the presence of ammonium, combined with amino-acid deficiencies. These parameters, linked to *B. cinerea* development, potentiate geosmin synthesis by *P. expansum* in grape juice.

Nevertheless, as concentrations of amino acids, mannitol, and ammonium in grape juices pre-cultured with *B. cinerea* [bot−] and [bot+] strains were very similar, the difference between these strains was certainly due to other factors. Experiments revealed that an ethanol-precipitable fraction, synthesized by the *B. cinerea* [bot−] strains, inhibited geosmin production by *P. expansum*. Further investigation is required to characterize this fraction and elucidate its effect on geosmin synthesis by *P. expansum*.

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