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A *Basidiomycete Fungus* responsible for fresh mushroom off-flavour in wines: *Crustomyces subabruptus*, (Bourdot & Galzin) Jülich 1978

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The authors dedicate this paper to the memory of Bernard Duhem, who passed away in December 2016.

ABSTRACT

Grape rot can cause organoleptic deviations in wines, including the aroma of fresh mushrooms; one of the molecules responsible for this is 1-octen-3-one. The bunches affected by this defect are often contaminated by *Botrytis cinerea;* however, they may also contain a whitish mycelium that cannot be attributed to *B. cinerea*. This additional fungal growth is the subject of this study. Several isolations of this fungus were carried out on grape bunches from different vineyards in the French Alsace and Champagne regions using a culture medium containing an anti-*Botrytis* fungicide (Boscalid). The sequencing of the ITS regions showed that most isolations corresponded to *Crustomyces subabruptus* (Bourdot & Galzin; Jülich 1978), an endophyte basidiomycete. Contamination tests carried out on bunches and grape juice with this fungus confirmed its responsibility for the appearance of fresh mushroom defects in wines, and showed that it has the capacity to produce large quantities of 1-octen-3-one in a wet and warm environment. The results of this study suggest that this basidiomycete is responsible for fresh mushroom aromas in wines.

KEYWORDS

wine quality, off-flavor, *Crustomyces subabruptus*, 1-octen-3-one, fungal rot

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INTRODUCTION

The main biological agent of bunch rot is *Botrytis cinerea*, which has well-known effects on wine quality (Bayonove, 1989; Dittrich, 1989; Steel *et al.,* 2013). The growth of other fungi on grapes, alone or in combination with *B. cinerea*, can also diminish the phytosanitary and organoleptic properties of wine made from infected grapes. These alterations relate to the capacity of these fungi to produce molecules which affect wine quality in small concentrations. It only takes a small number of infected grape bunches to contaminate the entire harvest of a vine plot. This is the case with the production of a mycotoxin, ochratoxin A, by *Aspergillus carbonarius* (Drouillard *et al.,* 2003; Sage *et al.,* 2002). Some off-flavours that alter wine quality in a negative way are explained by the capacity for fungi to produce compounds that are perceived in wine at very low concentrations. The olfactory perception thresholds of geosmin, which is responsible for earthy-musty off-odours, is close to 10 ng/L in water and 50 ng/L in red wine (La Guerche *et al.,* 2006). This compound is a product of the metabolism of *Penicillium expansum* in contaminated grapes modified by *B. cinerea* (Drouillard *et al.,* 2005; Guérin *et al.,* 2011; La Guerche *et al.,* 2006; Lempereur *et al.,* 2005).

Fresh-mushroom off-odours appeared increasingly in Alsace vineyards in the 1990s, with a peak in 2006 due to particular climatic conditions (Meistermann, 2006) causing the rapid development of *B. cinerea* and other fungi. The defect also occurred in the Champagne region in 2005 (Panigai *et al.,* 2009). Alsace and Champagne mainly produce white wines; i.e., wines made by pressing fresh grapes (of red and white varieties) without fermentation with solid parts. It is known from previous experience of the authors in the field, and confirmed by observations made by winegrowers in the study regions, that the aroma of fresh mushroom is discernible from grapes, but is often imperceptible from must after pressing. The defect usually arises after alcoholic or malolactic fermentation, after the first sulphiting, or even later, after filtration (Panigai *et al.,* 2009). The perception of the defect may vary in intensity during the storage of wines in vats. Oxidation-reduction conditions seem to play a role in these variations, which results in either a stronger revelation of the aroma or by a phenomenon which masks the defect. The aroma of mushroom is particularly detrimental to the quality of dry white wines and sparkling wines. In more powerful or sweet wines it is integrated into the aromatic complexity of the wines. The descriptor "undergrowth" is part of the aromatic palette of Pinot gris d'Alsace wines (CIVA, 2019).

Different molecules are responsible for fresh-mushroom aromas in wines (La Guerche *et al.,* 2006; Vacher *et al.,* 2008). 1-octen-3-one and 1-octen-3-ol are well-known metabolites of various ascomycetes and basidiomycetes (Tressl *et al.,* 1982). The concentrations of 1-octen-3-ol are almost lower than the olfactory perception thresholds for this compound, whereas 1-octen-3-one, and occasionally 1-nonen-3-one, are detected, in wines which have a mushroom flavour, at concentrations over their olfactory perception threshold and recognition threshold (Pons *et al.,* 2011). The olfactory perception threshold for 1-octene-3-one is around 3 ng/L in water, 30 ng/L in model solution and 70 ng/L in red wine (La Guerche *et al.,* 2006).

Very little research has been carried out on the fungal species responsible for fresh-mushroom off-odours in vine grape. The aim of this work, carried out between 2006 and 2015, was to isolate, identify and study the fungi present in the bunches with fresh mushroom aromas and to verify its involvement in the occurrence of the defect.

MATERIALS AND METHODS

1. Grapes and vegetative fragment sampling

The isolations were performed exclusively on grape samples from plots of vines known to have produced defective wines in the past. There were eight plots in Alsace spread out along the vineyard over a distance of 70 km for the most distant. They were all planted with Pinot gris, which is the grape variety to most frequently develop fresh mushroom aromas. In the Champagne region, the samples were taken from nine plots of Meunier, fresh mushroom aromas having been identified in the resulting wine in 2011. In these plots we looked for bunches characterised by a strong aroma of fresh mushrooms and the presence of white mycelium (Figure 1). These grapes were collected at the time of harvest, packed one by one into freezer bags, and kept for about two weeks at room temperature. Such closed containment favours basidiomycete growth.

The white mycelium was taken directly from the bunches, trying to avoid taking any areas contaminated with *Botrytis*. On bunches harvested in 2014, isolations were performed on stalk fragments, whose surface was disinfected before

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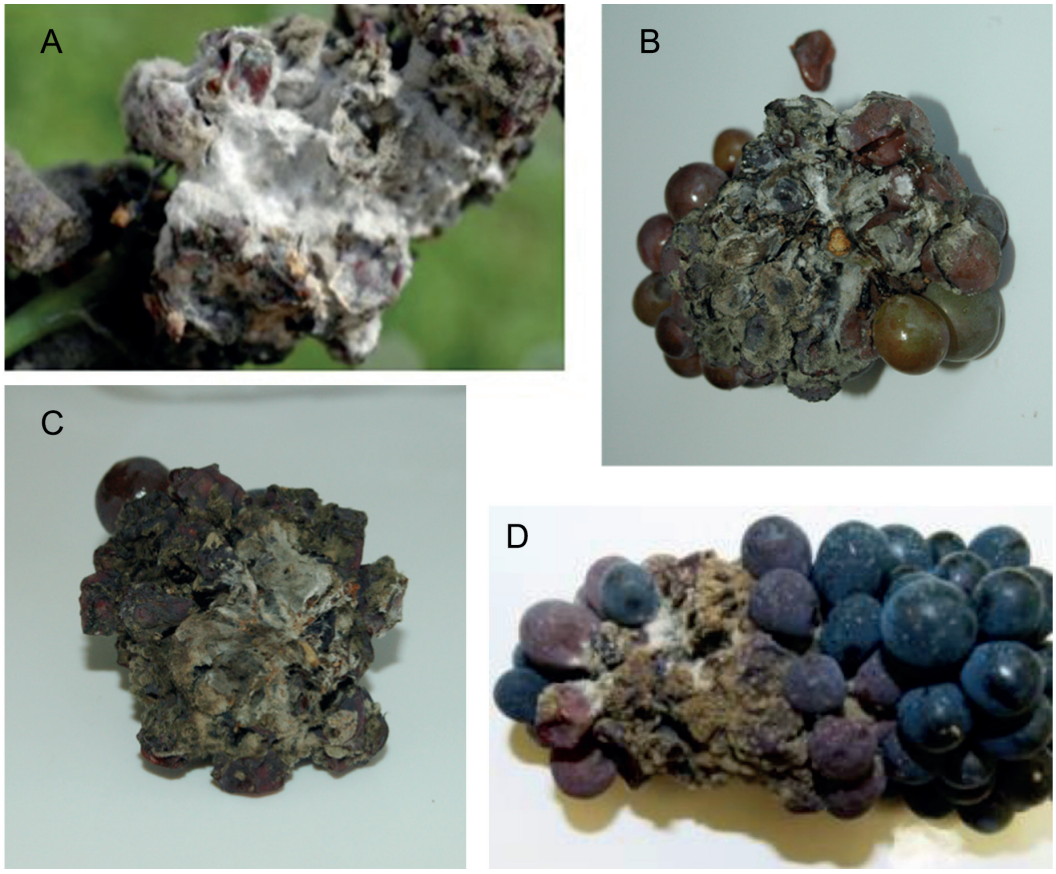


FIGURE 1. Grapes with fresh mushroom off-odour from some isolates.
A- Isolate 09-E6; B- Isolate 14-SchG9R1; C- Isolate 14-Wtz G4B2; D- Isolate 02RE14

cultivation by soaking the fragments for 1 min in a 3 % calcium hypochlorite solution. In order to verify the endophytic character of the fungus, in the spring of 2014, we took external scales from the buds at the time of budburst and placed them directly on the growing medium.

2. Method of isolating the fungus

Isolations were carried out on malt extract agar (MEA). The fungal growth was good but slow, and we noticed that the fresh mushroom aroma was more intense with this culture medium than with Czapek Yeast Agar. However, due to the slow growth of the basidiomycete, isolations were very often contaminated by *Botrytis cinerea* or different species of mould. In the first two years, only one isolation (06.DPG2) on MEA developed a strong fresh mushroom odour after one month of cultivation. In order to improve the effectiveness of the isolations, we compared seven commercial anti-*Botrytis* fungicides added to the MEA medium at a concentration of 10 mg/L for their influence on the growth in pure culture of *Botrytis cinerea* (strain isolated by us), *Penicillium sp.* (strain isolated by us) and *Crustomyces subabruptus* (strain 06.DPG2 isolated by us). The growth on triplicate petri dishes was monitored by carrying out diameter measurements (by photo cutting and weighing) and 1-octen-3-one analysis. As a result of this preliminary study, the active ingredient Boscalid (commercial name Cantus®) was retained due to its efficiency against *B. cinerea* and *Penicillium sp.* and its harmlessness in relation to *C. subabruptus*. The surface area of the mycelium after 27 days of growth in a petri dish on MEA medium with and without Boscalid were respectively: a) 59.4 ± 2.4 cm² and < 1 cm² for *B. cinerea,* b) 8.5 ± 0.3 cm² and < 1 cm² for *Penicillium* sp., and c) 57.9 ± 2.3 cm² and 53.6 ± 3.3 cm² for *C. subabruptus*. The presence of Boscalid in the culture medium did not affect the growth of *C. subabruptus*; only a moderate slowing was observed. This method increased the success rate of the isolations, although the mould inhibition was not perfect. After 3-4 weeks of cultivation, the isolates with a strong mushroom aroma (which is particularly pronounced when a hot microbiological loop encounters mycelium) were transplanted onto MEA medium for genetic analysis.

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3. Genetic analysis

DNA from strains isolated in Alsace in 2006, 2008, 2009 and 2010 was extracted from the mycelium of two-week-old fungal cultures grown in malt extract broth at 25 °C using a Nucleospin Plant II DNA extraction kit (Macherey Nagel, France) according to the manufacturer's specification. Fungal ITS1 – 5.8 S – ITS2 regions were then amplified with ITS 1 F (5 ' TCC GTA CGT GAA CCT GCG G 3 ') and ITS 4 R (5 ' TCC TCC GCT TAT TGA TAT GC 3 ') primers (Eurobio Ingen, France) on a TC 512 thermal cycler (Techne, United Kingdom). The PCR protocol consisted of an initial 5 min denaturation step followed by 35 cycles of 95 °C for 30 sec, 50 °C for 30 sec, 72 °C for 60 sec, and a final extension at 72 °C for 5 min. The amplicons were then restricted with Hae III, Hinf I and Mnl I restriction endonucleases (New England Biolabs, France). After electrophoresis on 1.5 % agarose gel in TBE buffer, restricted DNA fragments were visualised using Ethidium bromide and an E BOX VX2 Imaging System (Vilber Lourmat, France). Furthermore, the ITS region PCR products were purified using the Agencourt AMPure method (Beckman Coulter) and directly sequenced in the two directions, sense and anti-sense, using the Big Dye Sequencing kit according to the manufacturer's specifications (Applied Biosystems Inc.). The sequencing products were purified using the Agencourt CleanSEQ method (Beckman Coulter) and loaded onto an ABI PRISM® 3130 XL (Applera) capillary sequencer. The DNA sequences were analysed using the Staden Package. Heterozygous SNPs were identified as double pics on the chromatograms and coded according to international codes (nucleotide codes of the International Union of Biochemistry). Insertion/Deletions were easily identified by overlapping sequences.

4. Inoculation trials on grapes

In order to verify the responsibility of *C. subabruptus* for the development of the fresh mushroom off-flavor, the grapes and musts were inoculated with some of the isolated fungi.

Grape contaminations were performed on healthy bunches of Pinot gris grapes which were disinfected by soaking them first in physiological water containing 0.2 % (v/v) Tween 80 for 45 min and then in ethanol 95 % for 1 min. The bunches were placed in groups of three on netting in plastic boxes that contained a volume of sterile water or a saline solution depending on the desired relative humidity.

In the first trial, we compared the inoculation of bunches with *C. subabruptus* (isolate 08.E2) alone and in combination with *B. cinerea* (strain isolated by us) at three humidity levels. The contaminations were carried out by taking 0.5 x 0.5 cm pieces of mycelium from each of the two fungi in petri dishes. The bunches were contaminated by depositing a piece of mycelium within the bottom, middle and top parts of each bunch. *B. cinerea* was placed close to, but not in contact with, the deposit of *C. subabruptus*. Three different humidity levels were compared by placing salt solutions in the bottom of the box with a) 400 ml of water containing 160 g of sodium chloride per box; the measured hygrometry was between 79 and 85 %, b) 400 ml of water containing 140 g of sodium chloride per box; the measured hygrometry was around 89 %, c) 400 ml of water containing 80 g of potassium sulphate per box; the measured hygrometry was around 93 %.

In the second trial, we compared the inoculation of the bunches in the same way as previously described using two different *C. subabruptus* isolates (08.E2 and 09.W5) at two humidity levels: a) 79 % and c) 90 %.

The boxes were tightly sealed and kept at 25 °C for about 30 days. The temperature and humidity were monitored using an Ebro Ebi-2 sensor. After one month, the three bunches of grapes were crushed together using a Parapress laboratory press. The juices were clarified by centrifugation at 4800 rpm for 10 min and stabilised with 2 g/L of sodium benzoate and 70 mg/L sulphur dioxide (potassium bisulphite 15 % solution from AEB-France, Sigolsheim, France) before being sent for analysis. The dosage of sulfur dioxide was fixed according to the oenological practices in use on an altered harvest.

5. Contamination of musts

Must inoculations were obtained by placing a piece of *C. subabruptus* mycelium (2 x 4 cm taken from a petri dish) directly onto sterilised grape must (fresh juice or commercial grape juice) and by maintaining it at the surface of the liquid (Figure 2). The mycelium was harvested from 3-week-old petri dishes of *C. subabruptus* on MEA media in order to standardise the growth stage, and the must was sterilised by autoclaving (20 min, 90 °C). After three weeks of growth on must at 20 °C, the mycelium formed a compact mass, occupying the entire surface of the beaker, which could be easily removed from the must with a laboratory spatula.

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The must was first clarified with pleated paper filter to remove any residue and then clarification and stabilisation were carried out as previously described. Simultaneous must contamination was not carried out with *C. subabruptus* and *B. cinerea*.

6. Production of contaminated wines

The juices obtained from the contaminated grape and must were used as a source of contamination by adding them to commercial grape juice or a clarified must which we had obtained (not the same as that used for the growth of the fungus). The characteristics of the juices used were as follows:

- 12I101 and 13I102 commercial grape juice: 235 g/L sugar, 3.6 g/L total acidity expressed as sulfuric acid (H_2SO_4), pH 3.24
- 15B1 Pinot gris juice: 252 g/L sugar, 4.1 g/L total acidity expressed as sulfuric acid (H_2SO_4), pH 3.28
- 15B2 Riesling juice: 222 g/L sugar, 5.5 g/L total acidity expressed as sulfuric acid (H_2SO_4), pH 3.18

The additions were made in order to obtain pyramiding doses of 0, 20, 50, 100, 200 and 400 ng/L of 1-octen-3-one from the concentration determined in the contaminated must. The juices were fermented in a 1 or 5 L glass container (Figure 2) after active dry yeast addition at 0.2 g/L, strain OI 051 (Levulia® GC from AEB-France, Sigolsheim, France) or strain CIVC 8130 (Levuline® CHP from Oenofrance, Magenta, France). Fermentation was monitored by weighing the juice for CO_2 loss. When the weight had stabilised, the end of the fermentation was determined by measuring the concentration of glucose and fructose. The kinetics of alcoholic fermentation and the sugar content after fermentation were identical within the same trial. After fermentation, the wines were racked. The clear wines were stabilised with 80 mg/L of sulphur dioxide (potassium bisulphite 15 % solution from AEB-France or Sulfivin K150® from IOC, Epernay, France) and stored at 15 °C in half bottles or glass flasks. A sample was sent for analysis.

7. Analysis of volatile compounds

The analysis of volatile compounds was outsourced to two laboratories, Laboratory EXACT (Mâcon, France) and NYSEOS (Montpellier, France), which were selected from seven candidates after carrying out a double-blind inter-laboratory comparison of wines. Prior to analysis, the musts were clarified by centrifugation (3500 rpm, 5 min) to eliminate the deposits, which can interfere with the results (Dumoulin, 2011). Both laboratories used Solid Phase Microextration technique (SPME) combined with gas chromatography coupled to mass spectrometry (GC-MS). Methods for the determination of 1-octen-3-one were adapted from studies on wines (Culleré *et al.,* 2006), beers (Ochiai *et al.,* 2003), water (Cancho *et al.,* 2002) and stoppers (Ezquerro and Tena, 2005). 1-octen-3-ol was determined according to methods described in Boutou and Chatonnet (2007).

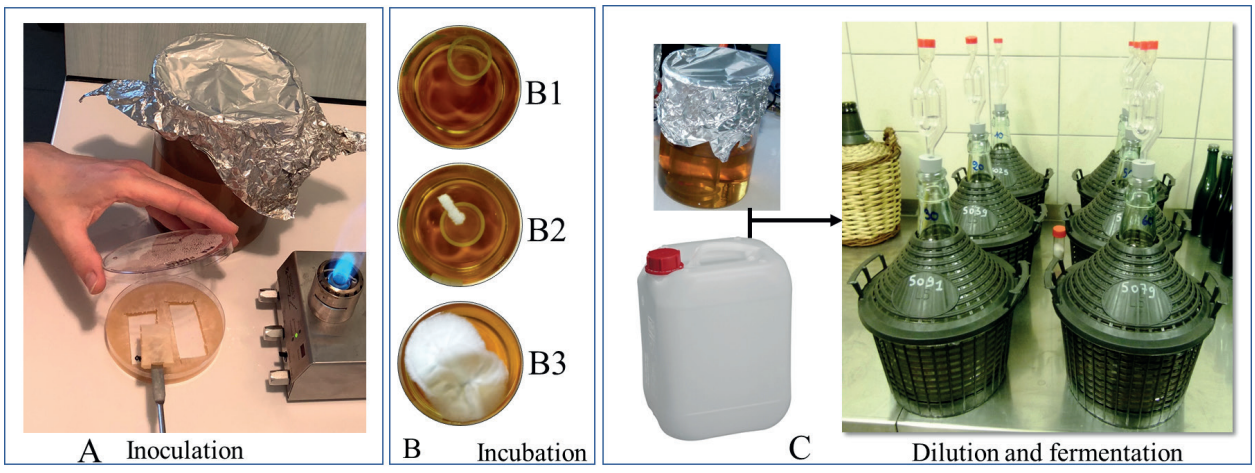


FIGURE 2. Contamination of musts and production of contaminated wines.

A- C. subabruptus inoculation on sterilised must;

B- Growth of C. subabruptus on must surface, control (B1), 1 week (B2), 3 weeks (B3);

C- Healthy must contamination and fermentation.

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Analysis by Nyseos was performed with a gas chromatograph Varian 3800 coupled with a Varian Saturn 2000 ion trap mass spectrometer. Volatile compounds were analysed using a DBWax column (30 m x 0.25 mm i.d., 0.5 µm film thickness, J&W, Folsom, CA, USA) with helium as the carrier gas at a constant flow of 1.0 ml/min. The oven temperature was programmed to increase from 60 °C to 150 °C at 3 °C/min, then from 150 °C to 245 °C at 15 °C/min. The SPME analysis was performed using 10 mL of sample placed in a 20 mL vial with 100 µg/L of 4-nonanol (internal standard). After 5 min of equilibration at 50 °C, the extraction was performed at the same temperature on a DVB/CAR/PDMS fibre 50/30 µm 2 cm (Supelco, France) for 30 min while stirring (250 rpm). The fibre was then desorbed in the GC injection port at 250 °C for 3 min. The mass spectrometer was operated in MRM mode with chemical ionisation using isobutane as the reagent gas. Non-resonant fragmentation of the m/z 127 and m/z 129 parent ions and subsequent analysis of the m/z 109 and m/z 111 daughter ions were carried out for 1-octen-3-one and 1-octen-3-ol respectively. The applied excitation amplitude was 25 V and the ionisation energy was 42.4 eV and 41.7 eV for 1-octen-3-one and 1-octen-3-ol respectively. Calibration was carried out using [$^{2/3}H_2$]-1-octen-3-one synthesised according to Lin *et al.* (1999). The limits of detection were 5 ng/L and 0.5 µg/L and the limits of quantification 10 ng/L and 1 µg/L for 1-octen-3-one and 1-octen-3-ol respectively.

Geosmin was analysed by gas chromatography–mass spectrometry as described by La Guerche *et al.* (2004) and quantified using its main characteristic ion, m/z 112. The m/z 59 was used for the internal standard (3-octanol). The limit of detection was 5 ng/L and the limit of quantification 10 ng/L.

The analyses of 1-octen-3-one by Laboratoire EXACT were conducted using a gas chromatograph Varian 3800 coupled with a Varian Saturn 2100 ion trap mass spectrometer equipped with a Combipal autosampler for SPME extraction. The analysis was carried out after *in situ* derivation with o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) as described by Culleré *et al.* (2006) and Ochiai *et al.* (2003). The 1-octen-3-one was extracted from 5 mL sample in the presence of 200 µL PFBHA at 10 g/L, 5 mL acidified HPLC water (pH 3.50 with H_2SO_4), 3 g NaCl and 50 µL internal standards (TCA and cyclohexanone at 10 µL). The headspace extraction was carried out using 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco) at 50 °C for one hour under regular agitation. The fiber was desorbed in the injector for 10 min at 260 °C. Separation was achieved on a DB-5 MS capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness; J&W, Folsom, CA, USA). Helium was used as the carrier gas with a constant column flow rate of 1 mL/min. The GC oven temperature was programmed to increase from 50 °C (after 1 min) to 75 °C at 2 °C/min, then to 280 °C at 25° C/min, at which it was held for 3.3 min. The mass spectrometer was operated in MRM mode as indicated by Culleré *et al.* (2006). Non-resonant fragmentation of the m/z 140 parent ion and subsequent analysis of the m/z (77 and 79) was carried out. The applied excitation amplitude was 50 V. The limit of detection was 10 ng/L and the limit of quantification was 20 ng/L. The linearity range runs from 20 to 1000 ng/L.

8. Sensory analysis and statistical analysis

A sensory tasting panel of 10 tasters (oenologists, vineyard technicians and wine growers) evaluated the wines in a tasting room after alcoholic fermentation. The wines were presented in black tasting glasses and in random order in sets of a maximum of six wines. The number of wines tasted per session was limited to 12. The intensity of the fresh mushroom aroma was quantified using a five-point intensity scale by olfaction without taking the wine in the mouth and by retro nasal route. The data were presented as mean ± standard deviation. They were statistically tested by analysis of variance (ANOVA) using XLSTAT Base 19.4 software (Addinsoft, France, 2017). Fisher's least significant difference (LSD, 5 % level) test was applied to determine significant differences between the treatments.

RESULTS

1. Isolations and identification

In Alsace, 261 bunches were harvested between 2006 and 2014, and 357 isolations were carried out. Twenty percent of the isolations had an identifiable mushroom odour. After subculturing, 24 pure isolations were obtained and sequenced. In 2014, isolations carried out on buds at the time of budburst enabled two other isolations to be carried out with mushroom aromas.

In Champagne, isolations carried out between 2005 and 2013 in bunches with mushroom aromas showed only the presence of *Botrytis cinerea*, *Penicillium* sp., *Clonostachys* sp.,

TABLE 1. Isolations of Crustomyces subabruptus in Alsace and Champagne.

Isolate	Year	Municipality, region	Organ	Stage	Sequence
06.DPG2	2006	Dorlisheim, Alsace	Grape	Harvest	NM02240
08.E2	2008	Dorlisheim, Alsace	Grape	Harvest	NM02241
09.E6	2009	Eguisheim, Alsace	Grape	Harvest	NM02244
09.W2	2009	Wintzenheim, Alsace	Grape	Harvest	NM02243
09.W5	2009	Wintzenheim, Alsace	Grape	Harvest	NM02242
10.Bois_Sch	2010	Scharrachbergheim, Alsace	Vine shoot	Harvest	NM02249
10.C4	2010	Eguisheim GC, Alsace	Grape	Harvest	NM02256
10.P1	2010	Eguisheim P, Alsace	Grape	Harvest	NM02257
10.P3	2010	Eguisheim P, Alsace	Grape	Harvest	NM02246
10.T6	2010	Turckheim, Alsace	Grape	Harvest	NM02255
10.T14	2010	Turckheim, Alsace	Grape	Harvest	NM02252
10.V4	2010	Voegtlinshoffen, Alsace	Grape	Harvest	NM02254
10.V8	2010	Voegtlinshoffen, Alsace	Grape	Harvest	NM02245
10.R1	2010	Ribeauvillé, Alsace	Grape	Harvest	NM02247
14.Wtz_G4B1	2014	Wintzenheim, Alsace	Grape	Harvest	GenBank MF 183946.1
14.Wtz_G4B2	2014	Wintzenheim, Alsace	Grape	Harvest	GenBank MF 183944.1
14 Sch_G9R1	2014	Scharrachbergheim, Alsace	Disinfected stalk	Harvest	GenBank MF 183942.1
14 Sch_G10R1	2014	Scharrachbergheim, Alsace	Disinfected stalk	Harvest	GenBank MF 183945.1
14 Tur_G1R	2014	Turkheim, Alsace	Disinfected stalk	Harvest	GenBank MF 183943.1
02RE14	2014	Reuil, Champagne	Grape	Harvest	02RE14
14.Sch_G4R1	2014	Scharrachbergheim, Alsace	Disinfected stalk	Harvest	(1)
14.Sch_G6R1	2014	Scharrachbergheim, Alsace	Disinfected stalk	Harvest	(1)
14 Sch_G7R2	2014	Scharrachbergheim, Alsace	Disinfected stalk	Harvest	(1)
14 Sch_G9R2	2014	Scharrachbergheim, Alsace	Disinfected stalk	Harvest	(1)
14 Tur_G3B	2014	Turkheim, Alsace	Grape	Harvest	(1)
14.RR7-3	2014	Ribeauvillé, Alsace	Bud	Budburst	(1)
14.RR10-2	2014	Ribeauvillé, Alsace	Bud	Budburst	(1)

(1) isolates belonging to the same restriction group as those from GenBank.

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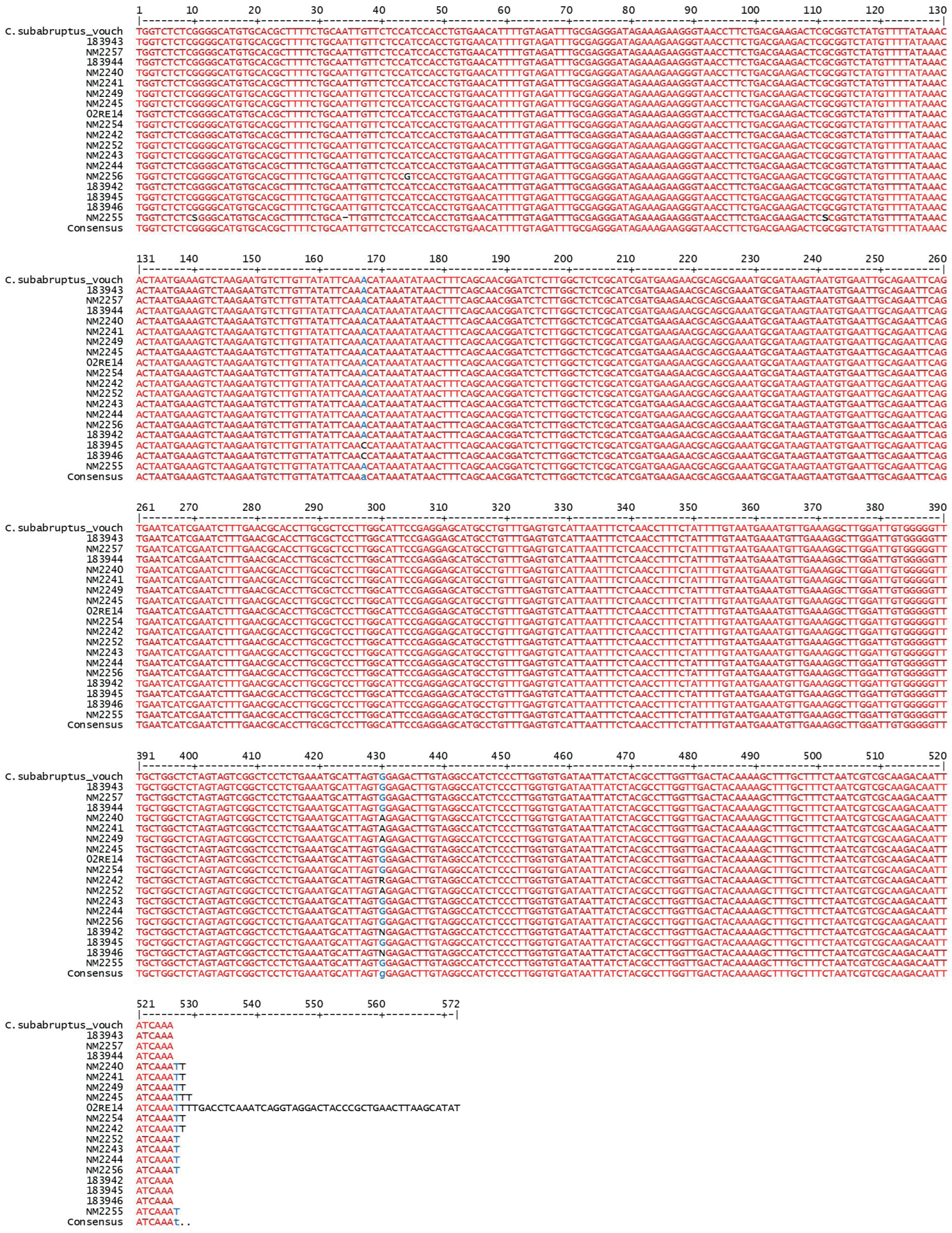


FIGURE 3. ITS region sequences of the strains isolated in Alsace and Champagne aligned with *Crustomyces subabruptus* voucher UC2022949 ITS sequence, according to "Multiple sequence alignment with hierarchical clustering" (Corpet, 1988)

Trichoderma sp., *Verticillium* sp. et *Trichotecium* sp. (Vacher *et al.,* 2008). The use of the culture medium with the addition of Boscalid on 55 samples in 2014 made it possible to achieve an isolation with a strong mushroom aroma.

A total of 25 isolations were submitted for genetic analysis (Table 1). These isolates originated from the eight plots studied in Alsace and the one plot in Champagne. Most of the isolations were made from bunches and some by placing a fragment of previously disinfected stalk on the growing medium. Isolate 10.Bois_Sch came from a piece of vine shoot imprisoned in a bunch with the characteristic white mycelium.

The sequencing of the ITS regions revealed that there was a very high degree of similarity between the first 18 strains in Table 1. The last 7 strains could unfortunately not be sequenced, but data from the restriction enzyme analysis showed that they belong to the same group as those deposited in the GenBank database. Microscopic observations showed the presence of the clamp connection characteristic of the mycelium of many species of Basidiomycetes, but it was only possible to identify the species after the first gene sequences had been deposited in GenBank by Rosenthal L.M. and Bruns T.D. (University of California, Berkeley) in July 2016. A very large majority of isolates belonged to *Crustomyces subabruptus* (Bourdot & Gakzin) Jülich 1978, with more than 99 % identity with *Crustomyces subabruptus* UC2022949 sequence (Figure 3).

All the strains isolated in Alsace gave the same restriction pattern of the ITS region using restriction endonucleases Hae III, Hinf I and Mnl I (Table 2).

2. Grapes inoculation with *C. subabruptus*

Healthy grapes were inoculated with *C. subabruptus* alone and in co-inoculation with *B. cinerea* after disinfection as previously described. When the bunches were contaminated by *C. subabruptus* alone, grapes were covered with the mycelium one month later, but the berries remained intact. The fungus alone was not able to degrade the skin of the berries; this only occurred in the presence of *B. cinerea*.

Analysis of the juice after the pressing of the three contaminated grapes (Table 3) showed geosmin contents lower or close to the limit of detection. These values are well below the olfactory perception thresholds for geosmin, which are 60-65 ng/L in white wine and 80-90 ng/L in red wine (Darriet *et al.,* 2000). 1-octen-3-ol concentrations were between 6 and 10 µg/L, comparable to those reported in rotten grapes (La Guerche *et al.,* 2006) and lower than olfactory perception thresholds (20 µg/L in model solution and 40 µg/L in red wine) (La Guerche *et al.,* 2006). No effect of humidity on the 1-octen-3-ol content was observed in this test.

In contrast, the observed levels of 1-octen-3-one were much higher than those indicated in the literature. La Guerche *et al.* (2006) measured maximum levels of 130 ng/L in the musts; they do not give results on the levels in the grapes. Levels of up to 400 ng/L were measured in the wines (Dauphin *et al.,* 2007). The dilution required for analysis is a source of error. Nevertheless, the production of 1-octen-3-one by this fungus was very high and was related to high fungal development on the clusters. The growth of the fungus was favoured by high humidity, as shown in the photographs in Table 3, and seemed to be accompanied by a higher production of 1-octen-3-one.

In the second trial, the bunches were contaminated with two different *C. subabpruptus* strains (Isolates 08.E2 and 09.W5) at two humidity levels. The levels of 1-octen-3-one in the juices obtained after pressing of the bunches (Table 4) were comparable to the previous test; i.e., 40-50 µg/L at the highest moisture content. The very high levels of 1-octen-3-one observed in these trials can be explained by the high contamination of the bunches, especially in the presence of high humidity. With a level of contamination comparable to that observed in the field the 1-octen-3-one content was 1.9 µg/L (Table 4, trial 10Hr1a at 79 % relative humidity).

3. Inoculation of musts with *C. subabruptus*

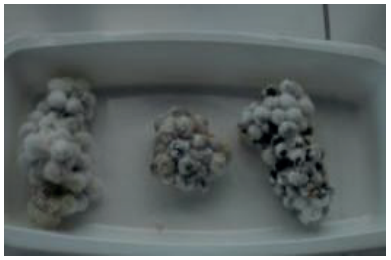
The contamination of grapes involves the risk of over-contamination with mould as it is impossible to perfectly disinfect the bunches.

TABLE 2. Restriction patterns of strain ITS region.

	Undigested amplicon	*Hae* III	*Hinf* I	*Mnl* I
Approximative fragment length (pb)	650	500 - 150	320 - 170 - 160	230 - 130 - 120

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TABLE 3. *C. subabpruptus* contamination on Pinot gris bunches, alone and in co-inoculation with *Botrytis cinerea*, at different relative humidities: impact on 1-octen-3-one, 1-octen-3-ol and geosmin production.

09Hr2a - Contamination of 2009 Pinot gris bunches with *C. subabruptus* (strain 08.E2).			
Relative humidity [a]	85 %	89 %	93 %
Condition of the bunches after one month			
	Analysis of the juices obtained after pressing [b]:		
1-octen-3-one (µg/L)	6.7	23.8	52.9
1-octen-3-ol (µg/L)	7.6	10.2	6.1
geosmin (ng/L)	< LOD	< LOD	< LOD
09Hr2b – Simultaneous contamination of 2009 Pinot gris bunches with *C. subabruptus* (strain 08.E2) and *Botrytis cinerea* (strain isolated by us).			
Relative humidity [a]	85 %	89 %	93 %
Condition of the bunches after one month			
	Analysis of the juices obtained after pressing [b]:		
1-octen-3-one (µg/L)	26.5	19.3	92.6
1-octen-3-ol (µg/L)	6.0	5.0	6.0
geosmin (ng/L)	5.4	< LOD	5.4

[a] Measured value during conservation in closed boxes, temperature = 22 ± 2 °C

[b] Determined by SPME and IT-MS/MS in direct analysis with a deuterated analogue for the determination of 1-octen-3-one

TABLE 4. *C. subabpruptu*s contamination of Pinot gris bunches with two different strains at different relative humidities: impact on 1-octen-3-one.

10Hr1a - Contamination of 2010 Pinot gris bunches with *C. subabruptus* (strain 08.E2).		
Relative humidity [a]	79 %	90 %
Condition of the bunches after one month		
	Analysis of the juices obtained after pressing [b]:	
1-octen-3-one (µg/L)	1.9	42.3

10Hr1b - Contamination of 2010 Pinot gris bunches with *C. subabruptus* (strain 09.W5).		
Relative humidity [a]	79 %	90 %
Condition of the bunches after one month		
	Analysis of the juices obtained after pressing [b]:	
1-octen-3-one (µg/L)	20.9	52.4

[a] Measured value during conservation in closed boxes, temperature = 22 ± 2 °C

[b] Determined by SPME selective preconcentration in cartridge derivatization and GC-MS-MS analysis.

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TABLE 5. Impact of *C. subabruptus* (strain 08.E2) growth on the surface of sterilised musts on 1-octen-3-one production when the mycelium covered the entire surface of the beaker (after 3 weeks).

Trial code	Must origin	1-octen-3-one (µg/L) [a]	
		Control	Must inoculation with *C. subabruptus*
15.B1	Alsace, Pinot gris must 2015	< LOD	5.59
15.B2	Alsace, Riesling must 2015	0.10	2.24
0.13.316	Champagne, commercial white grape juice	0.03	10.5

[a] Determined by SPME selective preconcentration in cartridge derivatisation and GC-MS-MS analysis.

In order to obtain juices contaminated exclusively with *C. subabruptus*, the fungus was grown on the surface of sterilised musts. After three weeks of growth at room temperature, the mycelium covered the entire surface of the beaker. This growth was accompanied by an enrichment of the must with 1-octen-3-one at levels at between 2 and 10 µg/L (Table 5).

4. Impact of must contamination on wine quality

In order to verify the impact of contamination on the quality of wines, the musts were vinified with juice contaminated by *C. subabruptus*. After alcoholic fermentation, the wines were tasted and their 1-octen-3-one content determined.

The contaminated musts obtained in Alsace in 2012 and 2013 by grape inoculation were added to a healthy must in increasing concentrations calculated by determining the 1-octen-3-one content of the initial must (around 20 µg/L). After alcoholic fermentation, the levels of 1-octen-3-one were below the expected concentrations and below the limit of quantification in the lowest additions (Table 6). Moreover, the levels are not correlated with the quantities added. In addition to the uncertainties of linked to the quantities, it has been shown that 1-octen-3-one is degraded by nonproliferating *S. cerevisiae* yeasts to 3-octanone by a specific enzymatic activity (enone-reductase) (Darriet *et al.,* 2002), which is a possible explanation for the results obtained in this study.

TABLE 6. Incidence of the addition of must containing 1-octen-3-one, obtained by grape inoculation by *C. subabruptus* (strain 08.E2), in a healthy grape juice on 1-octen-3-one content and sensorial quality after alcoholic fermentation.

Trial code	1-octen-3-one (ng/L) [a]		Intensity of fresh mushroom off-odour (Note5) [c]	
	Desired content [a]	Measured content in wine [b]	Olfaction [d]	Taste [d]
12I102	0	< 20	0.8 ± 0.9 d	1.2 ± 1.4 cd
	20	< 20	1.1 ± 0.9 d	0.9 ± 1.1 d
	50	< 20	2.5 ± 1.0 c	2.8 ± 0.9 b
	100	< 20	2.9 ± 1.2 c	1.7 ± 0.9 c
	200	80	3.7 ± 0.5 b	3.4 ± 0.7 ab
	400	173	4.5 ± 0.7 a	3.9 ± 0.7 a
13I101	0	< 20	1.1 ± 1.2 c	1.2 ± 1.1 c
	20	< 20	2.1 ± 1.0 bc	2.2 ± 1.5 bc
	50	< 20	2.3 ± 1.2 b	1.9 ± 1.0 c
	100	67	2.9 ± 0.9 ab	3.3 ± 1.3 a
	200	238	3.5 ± 0.8 a	3.1 ± 1.1 ab
	400	271	3.8 ± 1.7 a	3.6 ± 1.6 a

[a] Obtained by adding, in increasing doses, a contaminated must with a known concentration of 1-octene-3-one (12I102 = 21.4 µg/L and 13I101 = 18.0 µg/L).

[b] Determined by SPME selective preconcentration in cartridge derivatisation and GC-MS-MS analysis.

[c] Mean value corresponding to the tasting score given by 10 expert tasters.

[d] Means with the same letter are not significantly different at the 0.05 level.

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However, they show that this deterioration is not total. Sensory analysis showed that when the 1-octen-3-one content exceeded 60 ng/L, the wines had a significantly more intense fresh mushroom aroma. This content is compatible with the threshold of olfactory perception in the wines. The significant differences observed for the other wines are explained by the presence of other defects, such as musty notes, because the contamination of the grapes is never pure.

The same experiment was carried out in 2015 using must enriched with 1-octene-3-one by culture of *C. subabruptus* (strain 08.E2) on must. The 1-octen-3-one content of the two initial musts is indicated in Table 5. Contrary to the previous test, the levels of 1-octen-3-one measured in wines after alcoholic fermentation were 20 to 40 times higher than expected (Table 7). However, the analyses were carried out by the same laboratory and under the same operating conditions both on the musts used for the contaminations and in the wines after fermentation. Similarly, the calculations and dilutions were performed in the same way. The checks carried out make it possible to eliminate the hypothesis of a handling or analysis error. The possibility of a neogenesis of 1-octen-3-one from precursors present in must contaminated with *C. subabruptus* cannot be excluded.

The must of the second series (15.B2) was not free of defects. The control without the addition of contaminated must (15.B200) had a 1-octen-3-one content of 171 ng/L after alcoholic fermentation and the defect was identified by the tasters (compare with 15.B100). This defect was not perceptible before fermentation. The defect was clearly identified by tasters who detected intense fresh mushroom aromas in the contaminated wines. These results highlight the role of *C. subabruptus* in the development of fresh mushroom aromas.

DISCUSSION

In this study, the basidiomycete fungus *Crustomyces subabruptus* (Bourdot & Galzin) Jülich 1978 was identified on several occasions in bunches with aromatic defects of the "fresh mushroom" type in Alsace and Champagne. It is a species of toothed crust fungus from the Cystostereaceae family and the Polyporales order (also known as *Odontia subabrupta* and as *Cystostereum pini-canadense*). Cystostereaceae members are often found on rotting coniferous and angiosperm wood, are probably saprobic, and are widespread especially in temperate regions (Cannon and Kirk, 2007). They are widely distributed in Europe and identified as a latent endophyte in *Fagus sylvatica* in Italy and Greece (Bernicchia *et al.,* 2007; Dimou *et al.,* 2002), in *Quercus* sp. in Russia (Spirin, 2002)

TABLE 7. Incidence of the addition of must containing 1-octen-3-one obtained by must inoculation by C. subabruptus (strain 08.E2) in a healthy grape juice on 1-octen-3-one content and sensorial quality after alcoholic fermentation.

Trial code	1-octen-3-one (ng/L) [a]		Intensity of fresh mushroom off-odour (Note5) [c]
	Desired content [a]	Measured content in wine [b]	Olfaction [d]
15.B100	0	< 20	0.6 ± 0.5 c
15.B101	20	962	3.0 ± 0.7 b
15.B102	50	2 515	3.4 ± 0.5 b
15.B103	100	3 461	4.4 ± 0.5 a
15.B104	200	7 474	4.4 ± 0.5 a
15.B200	0	171	1.8 ± 0.8 d
15.B201	20	446	2.8 ± 0.4 c
15.B202	50	1 255	3.4 ± 0.5 bc
15.B203	100	2 172	4.0 ± 0.7 ab
15.B204	200	5 139	4.4 ± 0.5 a

[a] Obtained by adding, in increasing doses, a contaminated must with a known concentration of 1-octene-3-one (15.B1 = 5.59 µg/L and 15.B2 = 2.24 µg/L).

[b] Determined by SPME selective preconcentration in cartridge derivatization and GC-MS-MS analysis.

[c] Mean value corresponding to the tasting score given by 10 expert tasters.

[d] Means with the same letter are not significantly different at the 0.05 level.

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and in *Pseudotsuga menziesii* in Oregon (Daniels *et al.,* 2018). Several sequences from wood rotting specimens and as endophytes of *Ferula* sp from China have been deposited in GenBank.

In the present study, the isolations made in different locations and over several years on bunches, stalks, and most likely in buds seem to indicate that *C. subabruptus* is also an endophytic fungus of vine. The spontaneous development of *C. subabruptus* was sometimes observed on control disinfected grapes without inoculation and in boxes with high relative humidity, which again confirms the endophyte character of this fungus. Most often it goes unnoticed, but climatic conditions allowing, it can develop in the grape bunches. As the fungus is endophyte, it preferentially grows inside bunches affected by grey rot, because the environment is confined and the humidity conditions are more favourable.

The contaminations carried out on the healthy bunches and grape juice with pure cultures of *C. subabruptus* showed that this fungus has the particularity of producing high quantities of 1-octen-3-one, of the order of a few tens of µg/L in musts. Meanwhile, the production of 1-octene-3-ol and geosmin was very low. The levels were below the olfactory perception thresholds. The determination of 1-octene-3-one in musts presents some difficulties. The presence of solid particles at the time of analysis leads to very significant increases in the levels of 1-octen-3-one, which suggests that this molecule is endogenously produced (Dumoulin, 2011). The clarification of the must by centrifugation before analysis limits this source of variation. But despite these imprecisions, it is clear that high quantities of 1-octen-3-one were produced by *C. subabruptus*. This is confirmed by the analyses carried out on the wines after alcoholic fermentation, which are more precise. The production of 1-octen-3-one seems to be higher when hygrometry is high. This would explain the observations made in Alsace during the 2008 harvest where, despite the presence of fungus in the bunches, there were no defects in the wines; the fungus had developed during a rainy episode before the harvest, but afterwards, the hot and dry weather slowed down its growth and the production of the molecules responsible for the defect.

In the present study, contamination tests on grapes and grape musts confirmed the role of this fungus in the development of olfactory defects of the "fresh mushroom" type in relation to its ability to produce large quantities of 1-octen-3-one. *C. subabruptus* was not able to degrade the skins of grape berries. In pure culture, it developed on their surface without damaging the berries. Berry damage was observed when the fungus is co-inoculated with *B. cinerea*. Although this could not be demonstrated by our results, it is possible to admit that the alteration of the skins favours the contamination of the juices extracted at the time of pressing in the vinification of white wine.

The results of this study suggest that the development of the fresh mushroom aroma in wines cannot be explained by a simple enrichment of 1-octen-3-one in must and from must to wine, as in the case of the formation of mouldy-earthy flavours with geosmin (La Guerche *et al.,* 2006). During alcoholic fermentation, 1-octen-3-one can be degraded by nonproliferating *S. cerevisiae* yeasts to 3-octanone by specific enzymatic activity (enone-reductase) (Darriet *et al.,* 2002). According to our results, this deterioration does not seem to be total. Higher levels of 1-octene-3-one were measured in wines after alcoholic fermentation than those present in the must used for doping, which implies that the occurrence of neogenesis of 1-octen-3-one from precursors present in must contaminated with *C. subabruptus*. The existence of precursors would explain why the defect is generally imperceptible on must and appears only after alcoholic fermentation, or even after the first sulphiting. It is also possible that 1-octen-3-one may be difficult to perceive in musts due to its combination or adsorption with other molecules. Eight-carbon (C8) volatiles, such as 1-octen-3-ol and 1-octen-3-one, have been reported to be formed from linoleic acid with a number of intermediate compounds (Assaf *et al.,* 1997; Brodhun and Feussner, 2011; Tressl *et al.,* 1982; Wurzenberger and Grosch, 1982; Wurzenberger and Grosch, 1984). These molecules are found almost ubiquitously among fungi, and they are the characteristic of the fungal aroma (Combet *et al.,* 2006).

There is no doubt that the presence of *C. subabruptus* in the bunches and 1-octene-3-one in the wines are one of the causes of the development of fresh mushroom like defects in the wines. But the mechanisms leading from one to the other are still unclear.

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