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Pervasive fungicide resistance in *Botrytis* from strawberry in Norway: Identification of the grey mould pathogen and mutations

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Abstract

Control of grey mould, caused by *Botrytis* spp., is a major challenge in open field strawberry production. *Botrytis* was isolated from plant parts collected from 19 perennial strawberry fields with suspected fungicide resistance in the Agder region of Norway in 2016. Resistance to boscalid, pyraclostrobin and fenhexamid was high and found in 89.1%, 86.0% and 65.4% of conidia samples, respectively. Multiple fungicide resistance was common; 69.6% of conidia samples exhibited resistance to three or more fungicides. *Botrytis* group S and *B. cinerea sensu stricto* isolates were obtained from 19 and 16 fields, respectively. The *sdhB*, *cytb*, *erg27* and *mrr1* genes of a selection of isolates were examined for the presence of mutations known to confer fungicide resistance to boscalid, pyraclostrobin, fenhexamid and pyrimethanil plus fludioxonil, respectively. Allele-specific PCR assays were developed for efficient detection of resistance-conferring mutations in *cytb*. Among *B. cinerea* isolates, 84.7%, 86.3% and 61.3% had resistance-conferring mutations in *sdhB*, *cytb* and *erg27*, respectively. A triplet deletion in *mrr1*, resulting in ΔL497, commonly associated with the multidrug resistance phenotype MDR1h, was detected in 29.2% of *Botrytis* group S isolates. High frequencies of resistance to several fungicides were also detected in *Botrytis* from both imported and domestically produced strawberry transplants. Fungicide resistance frequencies were not different among fields grouped by level of grey mould problem assessed by growers, indicating factors other than fungicide resistance contributed to control failure, a fact that has important implications for future management of grey mould.

KEYWORDS
Botrytis species complex, latent infection, multidrug resistance (MDR)

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1 | INTRODUCTION

Grey mould, caused by *Botrytis* spp., is the most important disease and the largest contributing factor to economic loss in Norwegian open field production of strawberry (*Fragaria* × *ananassa*). The combination of cool temperatures and often numerous precipitation events during the growing season favours disease development, and conventional growers rely heavily on fungicides with single-site modes of action. Currently, single-site fungicides from the following groups are available for grey mould management in strawberry in Norway: anilinopyrimidines (APs), ketoreductase inhibitors (KRIs), phenylpyrroles (PPs), succinate dehydrogenase inhibitors (SDHIs) and quinone outside inhibitors (QoIs). Dicarboximides (DCs) were formerly available but the last one was withdrawn from the market in 2018.

For several of the fungicides, resistance mechanisms are known, and target-site modifications are well studied. Mutations at various sites in the *succinate dehydrogenase subunit B* (*sdhB*) gene confer moderate to high resistance to the respiratory inhibitor boscalid (SDHI). The substitutions H272Y and H272R are most common in SdhB, but H272L/V, P225T/F/L and N230I have also been shown to confer resistance to boscalid (Amiri et al., 2014; Hu, Fernández-Ortuño, et al., 2016; Konstantinou et al., 2015). Mutations can vary in terms of cross-resistance between different SDHIs. For example, the H272Y/R substitutions confer resistance to boscalid while simultaneously rendering *Botrytis cinerea* sensitive or hypersensitive to another SDHI, fluopyram (Lalève et al., 2014; Veloukas et al., 2013). In cytochrome b, the G143A substitution confers high resistance to the respiratory inhibitor pyraclostrobin and other QoIs, but it has yet to be found in isolates with a specific 1205 bp intron, called Bcbi-143/144 or bi2, that immediately follows the codon 143 (Banno et al., 2009). The presence of this intron, or lack thereof, in a population can indicate the degree to which development of high resistance due to the G143A substitution is possible (Grasso et al., 2006). In 3-ketoreductase (Erg27) four substitutions have been shown to confer high resistance to fenhexamid (F412S/I/V/C), and several other substitutions at different positions in Erg27 confer moderate resistance (Fillinger et al., 2008; Grabke et al., 2013). Natural fenhexamid resistance in *Botrytis pseudocinerea* is mainly attributed to a different resistance mechanism, that is, detoxification involving a cytochrome P450 monooxygenase (Debieu & Leroux, 2015).

The mechanism conferring resistance to APs is not fully elucidated, but potential targets include proteins involved in methionine biosynthesis (Fritz et al., 1997) or mitochondrial function (Mosbach et al., 2017). The mechanism responsible for sporadically detected specific resistance to the PPs also remains unknown; however, multidrug resistance (MDR) based on overexpression of efflux transporters has been shown to confer a degree of resistance to both APs and PPs in *Botrytis* (Kretschmer et al., 2009). Specifically, the MDR1 and MDR1h phenotypes involving overexpression of ABC transporter B (AtrB) have been detected in *Botrytis* from strawberry and shown to confer resistance to cyprodinil (AP) and fludioxonil (PP) at a level with consequences for field control (Fernández-Ortuño et al., 2014; Leroch et al., 2013; Leroux & Walker, 2013; Li et al., 2014b).

B. cinerea appears on FRAC's list of pathogens with high risk of fungicide resistance development (FRAC, 2019). Aspects of the pathogen's biology, including profuse conidial production, genetic variability and a polycyclic life cycle, contribute to its ability to develop fungicide resistance faster than many other fungal pathogens (Leroux et al., 2002; Veloukas et al., 2014). Although *B. cinerea* is a key species in strawberry, *Botrytis* in this host is regarded as a species complex (Walker, 2016). In addition to *B. cinerea*, *B. pseudocinerea*, *B. fragariae*, *B. mali*, *B. caroliniana* and a novel clade, *Botrytis* group S, have all been identified in strawberry (Dowling & Schnabel, 2017; Fekete et al., 2012; Fernández-Ortuño et al., 2012; Leroch et al., 2013; Plesken et al., 2015; Rupp et al., 2017). Inherent differences in *Botrytis* species/groups can affect capacity to develop fungicide resistance, so species identification is relevant for fungicide resistance management (Plesken et al., 2015).

A further potential challenge in grey mould control and fungicide resistance management in strawberry production comes from planting material. Fungicide-resistant *Botrytis* has been demonstrated to enter strawberry production systems as latent infections in planting material (Oliveira et al., 2017; Weber & Entrop, 2017).

Open field strawberry growers in Norway have experienced disease control failures and suffered huge economic losses due to grey mould in recent years. The 2016 growing season was particularly difficult; conducive weather conditions for grey mould were presumably exacerbated by loss of fungicide efficacy. Yield losses due to grey mould were large throughout the main open field production areas of the country, but especially in the southernmost region of the country, Agder, and there were examples of complete crop failure.

In 2015, legislation in Norway was enacted to allow for the importation of strawberry transplants. Prior to this, growers relied exclusively on domestically produced transplants. As strawberry production ranks first in fruit and berry production in Norway, the fungicide resistance situation for *Botrytis* is important to investigate. In this study, we (a) characterized and ascertained the range of fungicide resistance in *Botrytis* from strawberry in the Agder region of Norway; (b) developed and employed two new allele-specific PCR assays to detect resistance-conferring mutations in *cytb*; (c) tested for known mutations in *sdhB*, *erg27* and *mrr1* that are relevant for fungicide resistance; (d) identified *Botrytis* species/groups of field isolates; (e) examined fungicide resistance in relation to grey mould control problems experienced by growers in Agder in 2016; and (f) screened *Botrytis* isolates from imported and domestically produced strawberry transplants for fungicide resistance.

2 | MATERIALS AND METHODS

2.1 | Sample collection and *Botrytis* isolation

In late summer 2016, plant samples were collected from 20 strawberry fields in Agder, Norway. One field had strawberry production in a high tunnel and the rest were open field production. Ten plants

were selected at random from each field and collected for incubation in plastic boxes with moist paper to maintain water-saturated air at ambient temperature for 2–7 days to stimulate *Botrytis* sporulation. *Botrytis* conidia were obtained from 19 of the 20 fields. Grower assessments of the degree of grey mould problem in their own fields for the season were also recorded as extreme, major, moderate or low. In 2018 and 2019, samples of imported and domestically produced strawberry transplants were collected and frozen at approximately −20°C, until they were thawed and incubated as previously described. Conidia were isolated from sporulating *Botrytis* on strawberry transplants, and single-spore isolates were made by excising germinating conidia from water agar. This study includes analyses of both single-spore isolates and conidia samples.

2.2 | Fungicides

The following fungicides were used as product formulations in testing for fungicide resistance (product, company; fungicide group abbreviation): boscalid (Cantus, BASF; SDHI), fenhexamid (Teldor WG 50, Bayer; KRI), fludioxonil (Geoxe 50 WG, Syngenta; PP), fluopyram (Luna Privilege, Bayer; SDHI), iprodione (Rovral 75 WG, BASF; DC), pyraclostrobin (Comet Pro, BASF; QoI) and pyrimethanil (Scala, BASF; AP).

2.3 | Testing conidia samples from strawberry fields

Conidia samples obtained directly from plant material (not single-spore isolates) were examined for fungicide resistance using a spore germination test adapted from Weber and Hahn (2011). Briefly, 15 μl of suspensions of 10^4–10^5 conidia per ml in autoclaved, distilled water were pipetted onto agar plates amended with discriminatory doses of fungicides (Table 1). Plates were incubated in darkness for approximately 13–15 h at 20 ± 1°C. Ten germ tubes per conidia sample per fungicide concentration were then measured using a light microscope, and resistance categories were assigned based on germ tube length in relation to controls. Resistance categories for the spore germination test were ss for highly sensitive, s for less sensitive, mR for moderately resistant and R for resistant.

2.4 | Testing *Botrytis* isolates from strawberry transplants

In 2018, a total of 138 single-spore isolates of *Botrytis* were obtained from imported and domestically produced strawberry transplants and tested for fungicide resistance using the spore germination test as described above. In 2019, a total of 227 single-spore isolates of *Botrytis* from strawberry transplants were obtained and screened for fungicide resistance with a mycelial growth assay adapted from Schnabel et al. (2015). Fungicide products were mixed into liquid medium to create stock solutions. For the assay, agar growth media were amended with the stock solutions and transferred to 24-well cell culture plates (Nunclon Delta Surface; Thermo Scientific), 1.5 ml medium per well (Table 2). Conidia from sporulating single-spore isolates of *Botrytis* were transferred using sterile toothpicks to a point at the centre of the wells, and the plates were incubated in darkness at 20°C for 4 days. Diameter of mycelial growth in relation to well diameter was assessed to assign resistance category: sensitive (S, no growth), low resistant (LR, less than 20%), moderately resistant (MR, more than 20% and less than 50%), resistant (R, more than 50%). Each isolate was tested once.

2.5 | *Botrytis* DNA extraction and analysis

After testing conidia samples (not single-spore isolates) collected from Agder in 2016 with the spore germination test, the same conidia

TABLE 1 Growth media and fungicide active ingredients in the spore germination test used to analyse resistance in *Botrytis* from strawberry

Fungicide active ingredient	FRAC code	Growth medium	Discriminatory concentration (mg/L)[a]	
Boscalid	7	Yeast extract agar	1	50
Fenhexamid	17	Malt extract agar	1	50
Fludioxonil	12	Malt extract agar	0.1	10
Fluopyram[b]	7	Yeast extract agar	1	10
Iprodione[c]	2	Malt extract agar	5	50
Pyraclostrobin[d]	11	Malt extract agar	0.1	10
Pyrimethanil	9	Sucrose agar	1	25

[a]Low and high concentration of fungicide active ingredient used in the test.

[b]Only for single-spore isolates collected from strawberry transplants in 2018.

[c]Only for conidia samples collected from strawberry fields in Agder in 2016.

[d]Plates used for testing pyraclostrobin were amended with salicylhydroxamic acid (SHAM) dissolved in methanol for alternative oxidase inhibition (Weber & Hahn, 2011) such that the final concentration of SHAM in the medium was 98–99 mg/L.

TABLE 2 Growth media and fungicide active ingredients in the mycelial growth assay used to analyse resistance in single-spore isolates of *Botrytis* from strawberry transplants collected in 2019

Plate	Row	Fungicide active ingredient	Discriminatory concentration (mg/L)	Growth medium
A	A	Control	–	Czapek-Dox agar
	B	Pyrimethanil	4	Czapek-Dox agar
	C	Boscalid	75	Yeast bacto acetate agar
	D	Fluopyram	10	Yeast bacto acetate agar
B	A	Fenhexamid	50	Malt extract agar
	B	Fludioxonil	0.5	Malt extract agar
	C	Pyraclostrobin + SHAM[a]	10 + 100	Malt extract agar

[a]Salicylhydroxamic acid dissolved in methanol and added to the growth medium for alternative oxidase inhibition (Schnabel et al., 2015).

samples were used to generate single-spore isolates that were used for molecular analyses. Sporulating *Botrytis* mycelia from agar plates were ground in liquid nitrogen using a mortar and pestle. Genomic DNA was isolated from frozen tissue using the DNeasy Plant Mini Kit (Qiagen), as described by the manufacturer. All PCRs were performed in a total volume of 25 μl using 1 U Platinum *Taq* DNA polymerase (Invitrogen). Primers were designed using Primer3Plus (Untergasser et al., 2007). All primers and PCR conditions used in this study are presented in Table 3. In general, PCR products were sequenced in both directions with the same primers as used for amplification at Eurofins Genomics. The forward and reverse sequences were assembled into contigs to create consensus sequences, and quality trimmed using the CLC Main Workbench (Qiagen). For species identification and characterization of mutations, the consensus sequences were aligned with various reference sequences retrieved from the NCBI nucleotide database (Table S1). Sequences from this study that have been deposited in GenBank appear in Table S2 with accession numbers.

2.6 | *Botrytis* identification and construction of phylogenetic trees

B. pseudocinerea isolates were identified by a distinctive 24 bp deletion in the homologue of the *B. cinerea* gene *BC1G_07159* as described by Plesken et al. (2015). *Botrytis* group S isolates are associated with two regions of a transcription factor-encoding gene, *multidrug resistance regulator 1* (*mrr1*), covering 18 and 21 bp indels. These regions were characterized using primer pairs BcinN-in-F/BcinN-in-R (Plesken et al., 2015) and Mrr1-spez-F/Mrr1-Pira (Leroch et al., 2013). For some isolates, a larger region of *mrr1* was amplified and sequenced using primers Bcin-IN-F and Mrr1-Pira. The MDR1h genotype, characterized by a triplet deletion in *mrr1* resulting in ΔL497, was further identified by digesting the Mrr1-spez-F/Mrr1-Pira PCR-product with *Hpy*CH4V as previously described (Leroch et al., 2013). Mating type was determined as described by De Miccolis Angelini et al. (2016). Individual phylogenetic trees were constructed for regions of the *glyceraldehyde-3-phosphate dehydrogenase* (*g3pdh)* gene and the *necrosis- and ethylene-inducing protein* (*nep2*) gene (107 and 114 isolates, respectively) using the neighbour-joining method and the Jukes–Cantor substitution model (Figure S1). All sequences within a gene were trimmed to the same length, and all trees were inferred with 1000 bootstrap replicates. Various *g3pdh* and *nep2* reference sequences used for analysis were obtained from the NCBI nucleotide database (Table S1).

2.7 | Detection of mutations

Mutations in *sdhB*, *cytb* and *erg27* genes are known to confer resistance to boscalid (SDHI), pyraclostrobin (QoI) and fenhexamid (HA), respectively, so these genes were included in analysis of target-site mutations. Mutations in the *sdhB* gene were identified by amplification of the gene using primers IpBcBeg and IpBcEnd2 (Leroux et al., 2010), followed by sequencing.

Two new allele-specific PCR (AS-PCR) assays were developed to characterize codon 143 in *cytb* (Figure 1). In the first assay, primers Qo_G143A_F and Qo_universal_R amplify a 262 bp fragment only if a mutation that leads to the G143A substitution is present. In the second assay, primers Qo_wt_F and Qo_universal_R amplify a 262 bp fragment only from isolates carrying the wild-type allele resulting in G143. This assay is also designed to identify the 1205 bp intron, the presence of which will increase the size of the resulting fragment to 1468 bp. The specificities of the AS-PCR assays were validated using the cleavable amplified polymorphic sequences (CAPS) test developed by Leroux et al. (2010) or by sequencing using primers Qo13ext and Qo14ext (Leroux et al., 2010).

F412 and T63 in Erg27 were characterized using a multiplex AS-PCR assay described by Grabke et al. (2013), or by sequencing using primers T63_F and F412_R. A novel allele-specific primer (F412V_int) compatible with the aforementioned multiplex AS-PCR assay was designed to identify the mutation F412V, reported by Esterio et al. (2011) (Table 3).

2.8 | Statistics

R v. 4.1.0 (2021–05–18) was used for statistical analysis of resistance data and grey mould problem level data. Resistance

TABLE 3 Primers and PCR conditions

Target	Primer	Sequence (5′–3′)	Annealing temperature (°C)	Extension time (s)	Reference
nep2	NEP2forD	TTGCCTTCTCAAAATCATTACAGC	55	90	Staats et al. (2007)
	NEP2revD	TCTAGAAAGTAGCCTTCGCAAGAT	55	90	Staats et al. (2007)
g3pdh	G3PDHfor+	ATTGACATCGTCGCTGTCAACGA	55	90	Staats et al. (2005)
	g3pdh_R2	GAGTGGTTGTCACCGTTCATGTCAG	55	90	This study
BC1G_07159	g2944_137_F	GCAGATGAGGCGGATGATAG	55	30	Plesken et al. (2015)
	g2944_273_R	TCCACCCAAGCATCATCTTC	55	30	Plesken et al. (2015)
mat1-1	MAT1.1	AAGCTTCGATGACCCTTTGA	60	90	De Miccolis Angelini et al. (2016)
	MAT1.1295	GATCGTGGAGCCGAGATAAT	60	90	De Miccolis Angelini et al. (2016)
mat1-2	hmg.sp.162	GTGGAGATGGTGGTGGAGTT	60	90	De Miccolis Angelini et al. (2016)
	hmg.sp.1119	GAAAATGGGTACCGCATCAC	60	90	De Miccolis Angelini et al. (2016)
mrr1	Mrr1-spez-F	TATCGGTCTTGCAGTCCGC	56	45	Leroch et al. (2013)
	Mrr1-Pira	CCACCACAATCTTGGATCATTGGGATCAGAACCTGC	56	45	Leroch et al. (2013)
	BcinN-in-F	GCGACCTCATCGTTCTTTCAC	55	45	Plesken et al. (2015)
	BcinN-in-R	GGCTCTCGATGAGCTGTTTC	55	45	Plesken et al. (2015)
sdhB	IpBcBeg	CCACTCCTCCATAATGGCTGCTCTCCGC	60	60	Leroux et al. (2010)
	IpBcEnd2	CTCATCAAGCCCCCTCATTGATATC	60	60	Leroux et al. (2010)
cytb	Qo_G143A_F	CGGGCAAATGTCACTGTGAGC	64	90	This study
	Qo_wt_F	CGGGCAAATGTCACTGTGAGG	64	90	This study
	Qo-universal_R	TCCGTAGGTTTCCTGCTGAT	64	90	This study
	Qo13ext	GGTATAACCCGACGGGGTTATAGAATAG	55	90	Leroux et al. (2010)
	Qo14ext	AACCATCTCCATCCACCATACCTACAAA	55	90	Leroux et al. (2010)
erg27	F412_F	GACATTACGTTCTCGCACACG	63	45	Grabke et al. (2013)
	F412_int	CTTCCCATCCATCTTACAAGGTAGAA	63	45	Grabke et al. (2013)
	F412_R	CAACCAGGAACTTCGGTTCG	63[a]	45[b]	Grabke et al. (2013)
	F412S_int	CTTCCCATCCATCTTACAAGGTAGG	63	45	Grabke et al. (2013)
	F412I_int	CTTCCCATCCATCTTACAAGGTAGAT	63	45	Grabke et al. (2013)
	F412C_int	CTTCCCATCCATCTTACAAGGTAGCA	63	45	Grabke et al. (2013)
	T63_F	TGGGAGACAAGTGAGAGCCAG	63[a]	45[b]	Grabke et al. (2013)
	T63_int	CACCTCTGAAGACACGATTCACA	63	45	Grabke et al. (2013)
	T63_R	CGCCTTCAGACCCTTCCTTC	63	45	Grabke et al. (2013)
	F412V_int	CTTCCCATCCATCTTACAAGGTAGAC	63	45	This study

Note: Initial denaturation at 95°C for 5 min followed by 35 cycles of amplification (denaturation at 95°C for 30 s, annealing at the indicated temperature for 30 s, extension at 72°C for the indicated time), and final elongation at 72°C for 7 min.

[a]When used for sequencing, 60°C.

[b]When used for sequencing, 90 s.

frequencies per fungicide per field from the spore germination test were calculated and these observations were grouped according to grey mould problem level (low, moderate, major or extreme) as assessed by the growers. The data were analysed using one-way analysis of variance (ANOVA) including the following tests for the assumptions of homogeneity of variance, normality of residuals and independence of residuals: Levene's test, the Durbin–Watson test and the Shapiro–Wilk normality test, respectively. In cases where residuals were not normally distributed, the Kruskal–Wallis *H* test was used. R v. 4.1.2 (2021-11-01) was used to analyse mating type data with the Pearson's chi-squared test.

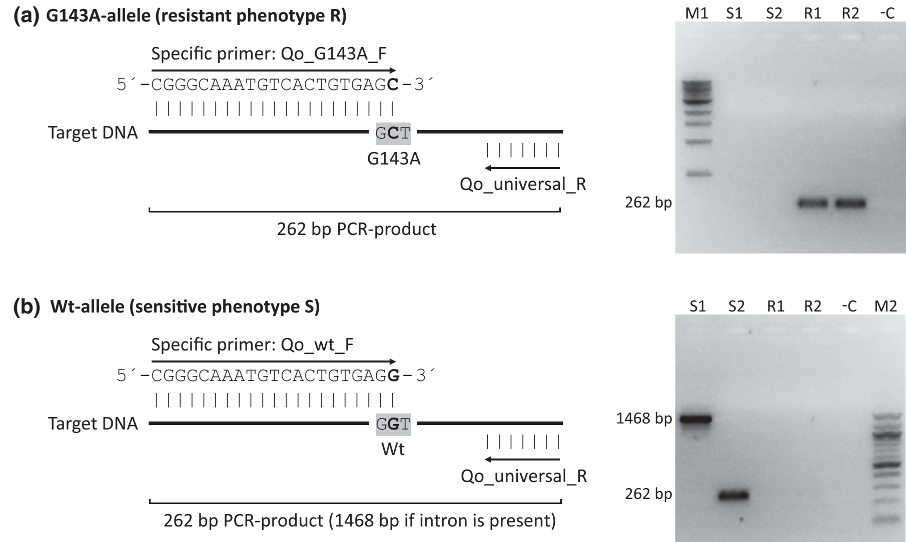


FIGURE 1 Schematic representation and demonstration of the allele-specific PCR strategy for detecting mutations in codon 143 of the *cytb* gene of *Botrytis*. (a) The 3′ of primer Qo_G143A_F is designed to be specific for the mutant allele that leads to a G143A substitution, which is associated with resistance to pyraclostrobin. A 262 bp fragment is only amplified when the mutant allele is present (R1 and R2). (b) The 3′ of primer Qo_wt_F is specific for the wild-type allele. The resulting PCR fragment is 262 bp (S2), or 1468 bp if a 1205 bp intron is present (S1). The negative control (−C) is water. M1 and M2 are 1 kb and 100 bp DNA ladders (New England Biolabs), respectively. R1 and R2 are resistant isolates, S1 a sensitive isolate with intron and S2 a sensitive isolate without intron. Wt denotes wild type

TABLE 4 Fungicide resistance in conidia samples of *Botrytis* from 19 strawberry fields in Agder, Norway in 2016

	Fungicide resistance (% isolates in each category)					
Resistance category[a]	Boscalid	Fenhexamid	Fludioxonil	Iprodione	Pyraclostrobin	Pyrimethanil
R	89.1	65.4	–	2.6	86.0	24.3
mR	–	–	1.9	29.0	–	52.1
s	10.9	27.6	28.0	56.6	7.6	21.6
ss	0.0	7.0	70.1	11.8	6.4	2.0
No. conidia samples tested	156	156	157	152	157	148

[a]Resistance categories for spore germination test: R (resistant), mR (moderately resistant), s (less sensitive) and ss (highly sensitive), according to Weber and Hahn (2011). "–" indicates the category was not part of the test for the specific fungicide.

3 | RESULTS

3.1 | Fungicide resistance in field samples

Botrytis conidia samples isolated from strawberry plants collected in 2016 were tested for fungicide resistance using a spore germination test for six fungicides: boscalid, fenhexamid, fludioxonil, iprodione, pyraclostrobin and pyrimethanil. Results revealed high frequencies of resistance (R) to multiple fungicides (Table 4), particularly to boscalid, fenhexamid and pyraclostrobin. Pyrimethanil resistance was detected in 13 of 19 fields, while resistance to iprodione was only detected in two fields. Samples less sensitive (s) and moderately resistant (mR) to fludioxonil were detected in 13 of 19 fields. Multiple fungicide resistance was assessed for 141 of the *Botrytis* conidia samples (Table 5). Three samples, 2.1%, were resistant to all five fungicides, and 67% of all samples were resistant to boscalid, fenhexamid and pyraclostrobin. Only 5% of the samples were not resistant.

3.2 | *Botrytis* species, groups and mating types

Single-spore isolates generated from conidia samples from strawberry in Agder in 2016 were identified based on sequencing the *g3pdh* and *nep2* genes. Further characterization into groups was based on the presence of specific indels in the *mrr1* gene as described by Plesken et al. (2015), and our material included a unique isolate, *Botrytis* A for Agder, Norway (Table 6). *Botrytis* group S isolates were detected in 19 fields and *B. cinerea sensu stricto* in 17 fields. Three single-spore *Botrytis* isolates were identified as *B. pseudocinerea* based on the specific PCR assay for this species (Plesken et al., 2015) and *g3pdh* and *nep2* sequence data. The three *B. pseudocinerea* isolates came from different fields. Isolates of *B. pseudocinerea* are expected to have an 18 bp indel in *mrr1*, but one of the three *B. pseudocinerea* isolates had, in addition, the 21 bp indel that is typical for *Botrytis* group S. Phylogenetic trees were constructed based on *g3pdh* and *nep2* sequence data (Figure S1). Mating type was

TABLE 5 Multiple fungicide resistance in a selection of *Botrytis* conidia samples from 19 strawberry fields in Agder, Norway in 2016

Phenotype[a]	Boscalid	Fenhexamid	Iprodione	Pyraclostrobin	Pyrimethanil	% samples (n = 141)
5R	×	×	×	×	×	2.1
4R	×	×		×	×	20.6
	×	×	×	×		0.7
3R	×	×		×		44.0
	×			×	×	1.4
	×	×			×	0.7
2R	×			×		21.3
		×		×		0.7
1R	×					2.1
				×		0.7
		×				0.7
0R						5.0

[a]Number of fungicides (0–5) to which there was resistance (R) in conidia samples in a spore germination test that had a category R for five fungicides. There was no category R for fludioxonil in this test, so it was not included in the table.

TABLE 6 Molecular identification and mating type of single-spore isolates of *Botrytis cinerea* from strawberry fields in Agder, Norway in 2016

		mrr1 indels[b]		Mating type			
B. cinerea[a]	Isolates	18 bp	21 bp	MAT1-1	MAT1-2	MAT1-1/1-2	NA[e]
Botrytis group S	73	×	×	38	29	2[d]	4
Botrytis group X	1	×		1	0	0	0
Botrytis A[c]	1		×	0	1	0	0
B. cinerea sensu stricto	44			17	26	0	1

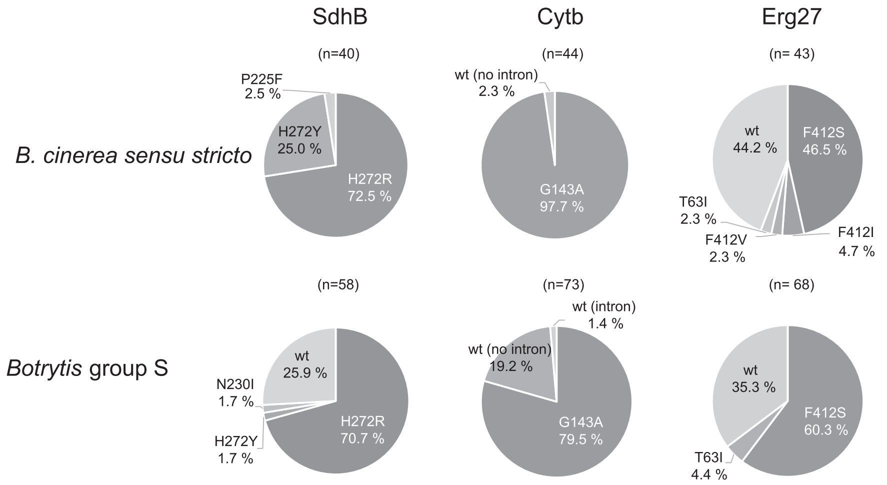
[a]Identified as *B. cinerea* based on sequence data for *g3pdh* and *nep2*.
[b]Identified as described by Plesken et al. (2015).
[c]Unique for this study.
[d]Weak PCR product for one of these isolates.
[e]Mating type not determined.

determined for single-spore isolates identified as *B. cinerea* (Table 6). Despite the limited number of isolates examined per field, there were instances of both mating types from *Botrytis* group S and *B. cinerea sensu stricto* being detected in the same field. The Pearson's chi-squared test indicated no significant difference from a 1:1 ratio of the two mating types for *Botrytis* group S and *B. cinerea sensu stricto* ($\chi^2 = 3.09$, $df = 1$, $p = 0.08$). Of three *B. pseudocinerea* isolates, two were MAT1-1 and one was MAT1-2.

3.3 | Mutations conferring resistance

Target-site mutations in the *sdhB*, *cytb* and *erg27* genes that are known to confer resistance to boscalid, pyraclostrobin and other QoIs and fenhexamid, respectively, were identified in the single-spore isolates from 2016. Frequencies of target-site mutations varied somewhat between *B. cinerea sensu stricto* and *Botrytis* group S (Figure 2). The single-spore isolate identified as *Botrytis* group X lacked substitutions known to confer fungicide resistance in SdhB, Cytb and Erg27 and had the intron known to preclude G143A. The *Botrytis* A isolate had the H272R and G143A substitutions that confer resistance to boscalid and pyraclostrobin but lacked those known to confer resistance to fenhexamid. Two of the *B. pseudocinerea* isolates lacked mutations that are known to confer fungicide resistance in the *sdhB*, *cytb* and *erg27* genes and had the intron known to preclude the G143A substitution. The third *B. pseudocinerea* isolate did not have the intron known to preclude G143A, but also lacked mutations in the *sdhB*, *cytb* and *erg27* genes. *Botrytis* group S isolates were further analysed for the presence of the triplet deletion resulting in ΔL497 in Mrr1 that is associated with the MDR1h phenotype, and this was detected in 29.2% of isolates. A selection of single-spore isolates from 2016 were also examined for accumulation of resistance-conferring mutations (Figure 3).

FIGURE 2 Proportions of substitutions that are known to confer resistance to boscalid, pyraclostrobin and fenhexamid, in SdhB, Cytb and Erg27, respectively, for isolates of *Botrytis cinerea sensu stricto* and *Botrytis* group S. Isolates lacking changes are designated as wild type (wt), and, in addition for Cytb, intron or no intron for detection of a specific 1205 bp intron known to preclude a mutation leading to G143A and resistance



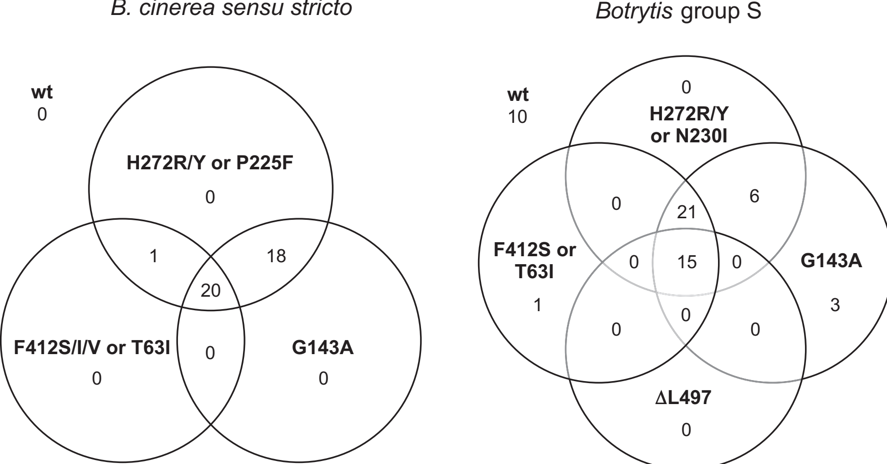


FIGURE 3 Accumulation of substitutions known to confer fungicide resistance in a selection of 39 *Botrytis cinerea sensu stricto* and 56 *Botrytis* group S isolates. Substitutions in SdhB (H272R/Y, N230I, P225F), Cytb (G143A), Erg27 (F412S/I/V, T63I) and Mrr1 (L497 deletion) confer resistance to boscalid, pyraclostrobin, fenhexamid, and both fludioxonil and cyprodinil, respectively. Isolates for which these changes were not detected are designated as wt, wild type

3.4 | Grower evaluation of grey mould problem

We did not observe differences in fungicide resistance frequencies between the grey mould problem level groups low, moderate, major or extreme (Table S3), but there was a trend towards higher frequencies of resistance to pyrimethanil as well as moderate resistance and reduced sensitivity to fludioxonil in the extreme group (Figure 4).

3.5 | Resistant *Botrytis* from strawberry transplants

A total of 365 single-spore *Botrytis* isolates from strawberry transplants collected in 2018 and 2019 were analysed, and resistance was detected for all fungicides tested in *Botrytis* from both imported and domestically produced strawberry transplants (Table 7). Fungicide resistance frequencies were generally higher in *Botrytis* from imported strawberry transplants compared with domestic strawberry transplants.

4 | DISCUSSION

Fungicide resistance was suspected to have played a role in the extensive grey mould control failures experienced in Agder in 2016, and there was concern regarding latent *Botrytis* infections entering fields with planting material, particularly after importation of strawberry transplants from Europe to Norway began in 2015. This study documents fungicide resistance in the grey mould pathogen in strawberry through in vitro and molecular analysis. In addition, it shows growers face the challenge of resistance entering fields with latent infections in planting material, but that crop failures in 2016 should not be entirely attributed to loss of fungicide efficacy.

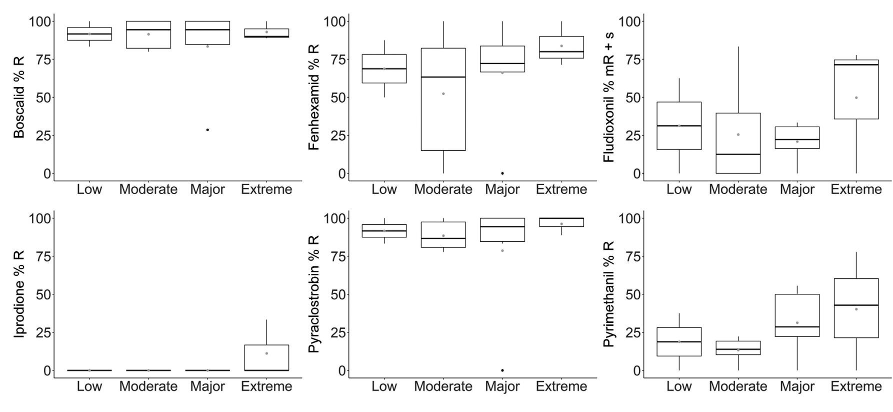


FIGURE 4 Fungicide resistance frequencies from *Botrytis* conidia samples from strawberry fields and the growers' perception of grey mould problem. Results from 17 fields (16 in the case of pyrimethanil) in Agder are grouped according to grower assessments of level of grey mould problem (low, moderate, major or extreme) in their fields for the 2016 season. Bold horizontal bars indicate median values, grey dots indicate means, black dots are outliers and hinges are placed at the 1st and 3rd quantiles. Fungicide resistance was measured by the spore germination test. Values are for percentage of resistant (R) conidia samples from the germination test except for fludioxonil, which is percentage of moderately resistant (mR) plus less sensitive (s)

TABLE 7 Fungicide resistance frequency means and standard error (*SE*) for 365 single-spore *Botrytis* isolates from samples of imported and domestically produced strawberry transplants

			Boscalid	Fenhexamid	Fludioxonil	Fluopyram	Pyraclostrobin	Pyrimethanil
Year	Source[a]	Value	% R	% R	% s	% R	% R	% R
2018[b]	Import (5)	Mean	88.7	29.9	16.7	45.5[c]	98.8	20.7
		SE	4.6	1.7	5.2	10.1[c]	1.3	6.2
	Norway (5)	Mean	28.7	8.6	1.7	4.4	31.6	5.7
		SE	16.6	5.7	1.7	2.9	15.5	2.7
2019[d]	Import (4)	Mean	15.0	37.5	42.8	0.0	100	22.7
		SE	8.9	17.9	12.8	0.0	0.0	10.9
	Norway (8)	Mean	3.2	37.9	18.8	1.3	35.6	12.6
		SE	1.9	9.2	6.2	1.3	9.9	4.6

[a]Number of transplant samples analysed is given in parentheses, and number of single-spore isolates analysed per transplant sample ranged from 8 to 20.

[b]Spore germination test, with resistance categories resistant (R), moderately resistant (mR), less sensitive (s) and highly sensitive (ss), according to Weber and Hahn (2011). For fludioxonil, s in the spore germination test is comparable with R in the mycelial growth assay (Fernández-Ortuño et al., 2014).

[c]Only four imported transplant samples were analysed for fluopyram in 2018.

[d]Mycelial growth assay, with categories resistant (R), moderately resistance (MR), low resistant (LR) and sensitive (S), according to Schnabel et al. (2015).

We found high frequencies of fungicide resistance to boscalid, pyraclostrobin and fenhexamid as well as high levels of multiple fungicide resistance in the spore germination test. Even though resistance testing of samples from Agder obtained in 2016 was not done with single-spore isolates, we found comparable results through detection of mutations known to confer fungicide resistance in the *cytb*, *sdhB*, *erg27* and *mrr1* genes in single-spore isolates made from the original conidia samples. The mutations were detected using sequencing and an arsenal of previously established tests, as well as new allele-specific PCR assays developed in this study for the detection of the G143A substitution in Cytb and an intron known to preclude the mutation resulting in the substitution. Based on these results, products containing boscalid, fenhexamid and pyraclostrobin would not have been expected to adequately control the *Botrytis* population during the 2016 season in Agder. High frequencies of resistance to these fungicides have also been reported in other parts of the world (Amiri et al., 2013; Kanetis et al., 2017; Weber et al., 2015).

The most common mutations detected in *sdhB* were at codon 272, of which the majority resulted in H272R, followed by H272Y, both of which confer resistance to boscalid and sensitivity to fluopyram (Veloukas et al., 2013; Weber et al., 2015). However, we also detected N230I and P225F, which confer resistance to both boscalid and fluopyram (Veloukas et al., 2013; Weber et al., 2015).

The isolate with N230I came from a field established with domestically produced strawberry transplants, indicating that mutations conferring resistance to fluopyram emerged independently in Norway and could have been selected for through use of boscalid prior to the introduction of fluopyram. This information, along with resistance data for specific substitutions detected in Cytb, has practical implications for a recently approved product containing the SDHI fluopyram and the QoI trifloxystrobin. Cross-resistance is well established within FRAC 11 QoI fungicides (FRAC, 2021), and this study has shown that QoI resistance in *Botrytis* in strawberry is widespread in the Agder region of Norway. The effectiveness of trifloxystrobin as a resistance-delaying mixing partner for fluopyram is therefore doubtful, a sentiment shared by Oliveira et al. (2017) and Weber et al. (2015). Our data show resistance to fluopyram and trifloxystrobin existed in the *Botrytis* population in Norway before the new product was introduced, so conservative and prudent use of this product is necessary.

The spore germination test also detected resistance to pyrimethanil and moderate resistance and reduced sensitivity to fludioxonil in *Botrytis* conidia samples. Based on pyrimethanil's cross-resistance with cyprodinil, another AP fungicide (Hilber & Schüepp, 1996), our findings indicate that the efficacy of the product containing cyprodinil and fludioxonil could have been affected in Agder in 2016. Previous studies have indicated that moderate resistance and reduced sensitivity to fludioxonil can be conferred by the efflux mechanism associated with multidrug resistance (MDR), particularly MDR1 and MDR1h (Kretschmer et al., 2009; Leroch et al., 2013; Weber & Hahn, 2011). As MDR1h resistance is associated with *Botrytis* group S, we tested these isolates for the presence of the characteristic 3-bp deletion in *mrr1* (Leroch et al., 2013), and it was detected in 29.2% of group S isolates. Pyrimethanil resistance can also be affected by MDR, but conidia samples that were resistant in the spore germination test could have target-site mutations in genes that have yet to be identified (Kretschmer et al., 2009). The MDR1h genotype was present together with several other mutations conferring resistance to fungicides with other modes of action, and this could be consistent with the pattern of stepwise accumulation of resistance mutations described by Li et al. (2014a).

We identified both *Botrytis* group S and *B. cinerea sensu stricto*, often coexisting. Phylogenetic trees of *g3pdh* and *nep2* gene sequences did not indicate distinct monophyletic groups for these, which is consistent with other studies (Leroch et al., 2013; Yin et al., 2016). Even if *Botrytis* group S and *B. cinerea sensu stricto* are distinct subpopulations, mating type data indicate they each have the potential to undergo sexual reproduction in Norway, and the resulting recombination may provide a source of variation that can contribute to emergence and selection of fungicide resistance. The scarcity of *B. pseudocinerea* isolates in this study is consistent with the observed general inability of this species to develop fungicide resistance and the theory that it is outcompeted by *B. cinerea* in the presence of selective pressure exerted by fungicide treatments (Plesken et al., 2015).

Open field strawberry production in Norway faces unique challenges in terms of geography and climate, yet high resistance frequencies could be explained by mutations similar to what has been found in other parts of the world. The high degree of multiple fungicide resistance in this study presents a challenge for grey mould control. Application of single-site fungicides is the main method of chemical control, and rotating single-site fungicides can contribute to maintaining resistance and selecting for more multiple fungicide resistance (Hu, Cox, et al., 2016). In the USA, multisite fungicides are recommended in fungicide rotations as a technique to address challenges posed by fungicide resistance (Hu et al., 2018), but growers in Norway do not have access to these multisite products. Only a low percentage of conidia samples were resistant to iprodione, but as this fungicide is no longer approved, its low frequency of resistance in the population cannot be exploited.

In addition to reliance on single-site fungicides, other aspects of open field strawberry production in Norway can exacerbate the resistance problem. Our screening of imported and domestically produced strawberry transplants shows that growers may start with latent *Botrytis* infections that exceed risk thresholds for development of fungicide resistance established by Schnabel et al. (2015). In general, resistance frequencies were higher in *Botrytis* from imported transplant samples than in domestically produced samples. Therefore, growers should be aware that choosing imported transplants may entail a greater risk of chemical control failure caused by fungicide resistance. Importation of fungicide-resistant *Botrytis* populations may also threaten efficacy of fungicides not yet approved in Norway, as the EU often registers fungicides for use before the authorities in Norway. The detection of resistance to fluopyram in these data represents a further risk for resistance development to the previously mentioned new product containing fluopyram and trifloxystrobin. In addition to entering the field with planting material, resistance also builds up over consecutive seasons. In perennial strawberry production, plants typically remain in the field for two to three years, and *Botrytis* overwinters, serving as an inoculum source for the following growing season (Strømeng et al., 2009).

Remarkably, there were no significant differences in fungicide resistance frequencies among fields for which different levels of grey mould problems were reported in Agder in 2016. Fungicide resistance is an undeniable problem in *Botrytis* in strawberry in Norway, but there must have been other factors contributing to grey mould control failures when low grey mould problems were coincident with high resistance frequencies and vice versa. One of the fields with low grey mould problems was the field with the high tunnel (Field 16, Table S4). The weather conditions of the 2016 season would obviously not have had the same consequences for grey mould control in the tunnel field as for the open fields, thus rendering the potential effects of fungicide efficacy failure less severe. The resistance frequencies for the tunnel field were high for all fungicides tested apart from iprodione, yet the problem with grey mould was considered low. Conversely, there was also an example of a field for which the grey mould problem was evaluated to be major despite having the lowest resistance frequencies of the sampled fields (Field 6, Table S4). Another field with extreme grey mould problems had high resistance

frequencies, but also high, wide rows with dense foliage due to a high rate of fertilizer application (data not included), making the canopy conducive to grey mould development (Field 9, Table S4). Regardless of fungicide resistance frequencies in the grey mould pathogen, this shows that strawberry growers must include integrated control measures. The use of high tunnels (Nes et al., 2017; Xiao et al., 2001), planting density (Legard et al., 2000), as well as amount and type of nitrogen fertilization (Walter et al., 2008), are examples of cultural methods that may prevent development of conditions conducive to grey mould and can contribute to disease control in situations where fungicide choices are limited and efficacy is compromised by resistance. There is potential for grey mould control, even in the face of pervasive fungicide resistance.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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