Norwegian University of Life Sciences
Faculty of Biosciences (BIOVIT)
Department of Plant Sciences

Philosophiae Doctor (PhD)
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Fungicide resistance and resistance dynamics in *Botrytis* populations in Norway

Fungicidresistens og resistensdynamikk i *Botrytis*-populasjoner i Norge

Katherine Ann Gredvig Nielsen

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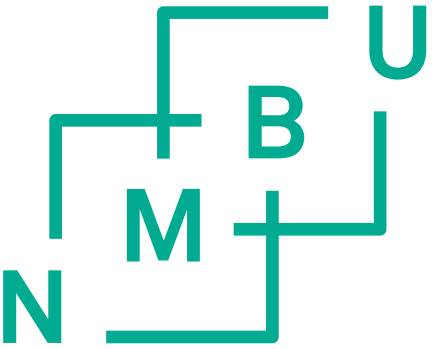

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Nielsen, K. A. G., Skårn, M. N., Strømeng, G. M., Brurberg, M. B., Stensvand, A. Pervasive fungicide resistance in *Botrytis* from strawberry in Norway: Identification of the grey mould pathogen and mutations. (Accepted, Plant Pathology)

Paper II:
Nielsen, K. A. G., Skårn, M. N., Talgø, V., Pettersson, M., Fløistad, I. S., Strømeng, G. M., Brurberg, M. B., Stensvand, A. Fungicide-resistant *Botrytis* in forest nurseries may impact disease control in Norway spruce. (Manuscript)

Paper III:
Nielsen, K. A. G., Strømeng, G. M. Brurberg, M. B., Stensvand, A. Fenhexamid resistance and fitness dynamics in *Botrytis* in strawberry and raspberry fields in Norway. (Manuscript)

Paper IV:
Gahatraj, B., Nielsen, K. A. G., Le, V. H., Sønsteby, A., Stensvand, A. Steam thermotherapy strongly reduces *Botrytis* in strawberry transplants without negative effects on plant growth and yield. (Manuscript)

Abstract

Grey mould, caused by *Botrytis* spp., causes serious losses in agriculturally and economically important crops worldwide. Growers in Norway have experienced grey mould control problems, and fungicide resistance in the pathogen was suspected. *Botrytis* is notorious for developing fungicide resistance, and growers' main control method is application of single-site fungicides which are inherently at risk for resistance development. The aim of this work was to quantify resistance in the grey mould pathogen, identify *Botrytis* species, and gain a better understanding of resistance dynamics of *Botrytis* in strawberry (*Fragaria* × *ananassa*), raspberry (*Rubus idaeus*), and Norway spruce (*Picea abies*).

Results from *in vitro* resistance testing revealed high levels of resistance to boscalid, fenhexamid, and pyraclostrobin in *Botrytis* isolates obtained from strawberry fields in Agder, Norway in 2016. Detection of mutations in the respective target-genes (*sdhB*, *erg27*, and *cytb*) corroborated results from resistance testing. Results also showed mutations leading to the N230I and P225F substitutions in SdhB were present, indicating the genetic potential for resistance to the SDHI fluopyram existed in *Botrytis* in strawberry field populations before the fungicide was approved for use in Norway. *In vitro* resistance testing of *Botrytis* isolates from Norway spruce showed a moderate level of resistance to fenhexamid and high resistance to thiophanate-methyl, both commonly used fungicides for grey mould control in forest nurseries. Detection of mutations in the *erg27* and *tubA* genes largely corroborated these results.

Testing of *Botrytis* from both imported and domestically produced strawberry transplant samples revealed a trend for higher levels of resistance in *Botrytis* from imported samples, but there were several examples of high resistance from domestic sources as well. Our results confirmed transplants as an important source of fungicide-resistant inoculum in strawberry production. Fungicide-resistant *Botrytis* was obtained from the air, surfaces, and weeds at forest nursery facilities. Weeds in particular can serve as reservoirs where *Botrytis* can continue to produce inoculum and infect new rounds of production if not removed.

The majority of *Botrytis* isolates from strawberry, Norway spruce, and forest nursery facilities were *Botrytis cinerea*. Only a few isolates obtained from strawberry were *Botrytis pseudocinerea*, a known member of the *Botrytis* species complex in this host. *Botrytis pseudocinerea* was also isolated from Norway spruce, which has not previously been documented, and a pathogenicity test confirmed its ability to infect Norway spruce seedlings. *Botrytis prunorum* was identified for the first time in northern Europe in samples obtained from the air in a forest nursery.

Results from an experimental strawberry field gave a strong indication of potential fitness cost associated with fungicide resistance. Monitoring results from commercial raspberry fields, however, detected overall minimal changes in fenhexamid resistance frequencies when growers discontinued use. This was likely due to other fungicides selecting for multiple fungicide resistance genotypes. Multiple fungicide resistance was observed often in resistance testing results in this study and represents a serious threat to the viability of chemical control as an effective grey mould disease control method in the future. The technique of using steam thermotherapy to reduce *Botrytis* infections in strawberry transplants was effective and has potential applications in other production systems. Cultural and other non-chemical methods will be important for future grey mould control. Examples of strawberry fields where low grey mould problems were reported despite high resistance show control of grey mould is possible.

Norsk sammendrag

Gråskimmel, forårsaket av sopper innen slekten *Botrytis*, er kjent for å forårsake betydelige avlingstap i viktige plantekulturer verden over. Dyrkere i Norge har hatt utfordringer med å bekjempe gråskimmel, og det har vært pekt på at fungicidresistens hos patogenet kan være en årsak. *Botrytis* er kjent for å utvikle resistens mot fungicider. Dyrkere er avhengige av fungicider med god virkning mot soppen. Målet med dette arbeidet var å undersøke resistens hos gråskimmelsoppen, identifisere *Botrytis*-arter, og utforske resistensdynamikk hos *Botrytis* i jordbær (*Fragaria × ananassa*), bringebær (*Rubus idaeus*), og gran (*Picea abies*).

Resultater fra *in vitro* resistenstesting viste høye nivåer a resistens mot virkestoffene boskalid, fenheksamid, og pyraklostrobin hos *Botrytis* i prøver fra jordbærfelt i Agder, Norge, i 2016. Det ble også påvist mutasjoner i mål-genene til virkestoffene (*sdhB*, *erg27*, og *cytb*) som var i samsvar med resultatene fra *in vitro* testingen. Testene viste også at N230I- og P225-substitusjonene i SdhB proteinet var til stede allerede da, noe som betyr at det genetiske bakgrunnen for resistens mot fluopyram var til stede i feltpopulasjoner i jordbær allerede før dette virkestoffet ble godkjent for bruk i Norge. Et moderat nivå av resistens og høyt nivå av resistens mot henholdsvis fenheksamid og tiofanatmetyl ble påvist hos gråskimmel fra gran. Påvisning av mutasjoner i *erg27* og *tubA* genene var for det meste i samsvar med resistenstestresultater.

Testing av gråskimmel fra importerte og norskproduserte småplanteprøver av jordbær viste en tendens til mer resistens i gråskimmel fra importerte prøver, men det var flere eksempler på høy resistens i gråskimmel fra norsk-produserte planter også. Undersøkelsene våre bekrefter at småplanter er en viktig kilde til fungicidresistent smitte i jordbærproduksjon. Det ble også påvist fungicidresistens hos gråskimmel fra luft, overflater, og ugras i planteskoler. Spesielt ugras kan være en vertsplante der Botrytis kan overleve og fortsette å produsere smitte som kan infisere nye hold dersom den ikke blir fjernet.

De fleste isolatene fra jordbær, gran, og planteskoler var *Botrytis cinerea*. Noen få isolater fra jordbær var *Botrytis pseudocinerea*, som er kjent for å være en del av *Botrytis*-artskomplekset i jordbær. *Botrytis pseudocinerea* ble også isolert fra gran,

noe som er ikke dokumentert før. I smitteforsøk viste denne soppen evne til å infisere frøplanter av gran. *Botrytis prunorum* ble identifisert for første gang i Norden, fra luft i en planteskole.

Resultater fra et forsøksfelt med jordbær ga en sterk indikasjon at det er mulig «fitness cost» tilknyttet fungicidresistens. Resultater fra overvåkning av kommersielle bringebærfelt, derimot, viste generelt lite endring i resistens mot fenheksamid etter at dyrkerne sluttet å bruke fungicidet. Dette kan trolig knyttes til seleksjon av multiresistente genotyper som følge av bruk av andre fungicider. Multiresistens ble observert ofte i dette arbeidet med testing av resistens, og representerer en trussel mot effektiv bekjemping av gråskimmel ved hjelp av kjemiske midler. Behandling med vanndamp for å redusere *Botrytis* i småplanter av jordbær hadde god effekt og det finnes muligheter for å anvende denne metoden også i andre produksjonssystemer. Kulturtiltak og andre ikke-kjemiske metoder er viktig for fremtidig bekjempelse av gråskimmel. Eksempler fra jordbærfelt der dyrker rapporterte liten grad av gråskimmelangrep til tross for stor grad av fungicidresistens viser at kontroll av gråskimmel er mulig.

1 Introduction

1.1 *Botrytis*

1.1.1 Biology

Botrytis is a genus including phytopathogenic fungi known for their variation and potential to cause serious losses in crops and horticultural products (Elad et al., 2016b). Although traditionally considered a classical necrotroph, recent evidence indicates that some species, including *B. cinerea*, can behave as endophytes without negative consequences for their hosts (van Kan et al., 2014; Sowley et al., 2010; Rajaguru & Shaw, 2010; Shaw et al., 2016). The majority of *Botrytis* species produce copious amounts of conidia (macroconidia) forming in botryoidal clusters on branched conidiophores that are dispersed via air and can also be spread by splashing of water droplets (Carisse, 2016; Jarvis, 1977). Conidia need a period of wetness to germinate and infect susceptible plant tissue, where it can then remain latent until conditions for further growth and development are optimal (Jarvis, 1962). The asexual stage of the *Botrytis* life cycle is polycyclic. The optimum growth temperature for mycelium is approximately 20°C to 22°C, and mycelium can grow, spores can germinate, and sclerotia can germinate down to 0°C, 0°C, and 3°C, respectively (Jarvis, 1977). *Botrytis* overwinters in plant material as mycelium or as hardened, melanized survival structures called sclerotia which can then germinate in the spring, producing fresh crops of conidia over several weeks (Carisse, 2016; Strømeng et al., 2009). The sexual stage of the life cycle is seldom observed (Dewey & Grant-Downton, 2016), but microconidia have been demonstrated to fertilise sclerotia, from which apothecia generate and produce ascospores (Fukumori et al., 2004). In *B. cinerea*, sexual reproduction is mainly heterothallic and possible between compatible mating types, MAT1-1 and MAT1-2 (De Miccolis Angelini et al., 2016; Faretra et al., 1988). *Botrytis* is also known to have a parasexual cycle whereby hyphae fuse in a process called anastomosis, allowing the transfer of nuclei between strains to create a heterokaryon (Jarvis, 1977).

1.1.2 Taxonomy and species complex

Botrytis taxonomy is currently in a state of flux, largely owing to the use of genetic tools and an emphasis on the phylogenetic approach to species relatedness (Garfinkel, 2021; Walker, 2016). A major shift has occurred in the last two decades. Historically, morphology and host specificity played a key roles in *Botrytis* species delineation (Garfinkel, 2021; Giraud et al., 2008; Jarvis, 1977). Taylor et al. (2000) thoroughly explicated phylogenetic species recognition as an operational species concept, in contrast to a theoretical concept, also coining the term Genealogical Concordance Phylogenetic Species Recognition (GCPSR). GCPSR is an extension of phylogenetic species recognition that employs using the comparison of multiple gene genealogies in determining the limit of species, and Taylor et al. (2000) gave several examples of how this approach for fungi outperformed both traditional morphological species recognition and biological species recognition based reproductive isolation determined through mating tests.

ITS rDNA sequence data that is useful for some fungal species identification can be used to identify the *Botrytis* genus but is not sufficient alone for further species identification (Holst-Jensen et al., 1998; Hyde et al., 2014; Walker, 2016). Sequences of the three housekeeping genes glyceraldehyde 3-phospate dehydrogenase (*g3pdh*), heat shock protein 60 (*hsp60*), and DNA-dependent RNA polymerase subunit II (*rbp2*) were used to generate a classification of the *Botrytis* genus that was largely consistent with the existing classification that was based mostly on morphology (Staats et al., 2005). Sequences from the necrosis and ethylene-inducing protein genes (*nep1* and *nep2*) were also used to generate comparable classifications, and, although positive selection was found for *nep1*, a subsequent gene replacement study found no connection with virulence (Staats et al., 2007a; Staats et al., 2007b). Hyde et al. (2014) recommend using sequences from *nep1* and *nep2* when higher resolution is required within the *Botrytis* genus.

The establishment of these five genes as useful for species delineation laid the groundwork for the proliferation of *Botrytis* species identification which has taken place the past decade (Garfinkel, 2021). Just since the year this PhD project began, six new *Botrytis* species have been described using gene sequences from among the five first used by Staats and colleagues (Garfinkel et al., 2017; He et al., 2020; Harper et al., 2019; Rupp et al., 2017a; Zhong et al., 2019; Prasannath et al., 2021). The phylogenetic approach is useful, but both Walker (2016) and Garfinkel (2021) stress the

importance of not abandoning the ecological, morphological, and biological approaches.

The phylogenetic approach was key in the identification of *B. pseudocinerea*, which is indistinguishable from *B. cinerea* morphologically, and a biological approach, mating tests, were able to corroborate the phylogenetic findings (Walker et al., 2011). *B. pseudocinerea* has since been identified in several hosts (Azevedo et al., 2020; Fekete et al., 2012; Johnston et al., 2014; Li et al., 2015; Muñoz et al., 2016; Plesken et al., 2015; Saito et al., 2014b; Wessels et al., 2013) and has contributed to the concept of a *Botrytis* species complex (Walker, 2016). The composition of the *Botrytis* species complex in a host has implications for disease control as species can have different abilities to develop fungicide resistance (Hu et al., 2019; Notsu et al., 2021; Plesken et al., 2015; Walker et al., 2011). Alternatively, a species may have "natural" resistance, as in the case of *B. pseudocinerea* (Debieu & Leroux, 2015).

1.1.3 Hosts

Botrytis is known to cause grey mould and a multitude of other rots, blights, and spots in host plants belonging to at least 596 genera, and although several *Botrytis* species are host specific, the notorious *B. cinerea* can infect the majority of these (Elad et al., 2016a). Many host plants to *Botrytis* are of agricultural or economic value, so *Botrytis* has been studied extensively and *B. cinerea* was ranked number two in a top-ten list of plant pathogens of scientific and economic importance (Dean et al., 2012). *Botrytis* is problematic in several hosts in Norway, and this work focuses on three of them: strawberry (*Fragaria* × *ananassa*), raspberry (*Rubus idaeus*), and Norway spruce (*Picea abies*).

Grey mould is the most important disease and the largest cause of economic loss in strawberry production in Norway. Growers mainly operate with perennial open field matted row or plasticulture production systems, meaning the crop is exposed to cool temperatures and frequent precipitation during typical Norwegian summers. Losses due to grey mould have been extensive; the 2016 season was a particularly devastating year. Plants are infected during flowering, and the fungus can then remain latent in the floral parts until favourable conditions permit further growth and development, and *Botrytis* can also spread via direct contact with infected plant material (Jarvis, 1962). Strawberry production ranks highest of berry production in Norway with a production area of about 1440 ha in 2020 (Revdal et al., 2021).

Grey mould in raspberry production is also a serious problem for growers in Norway. Open field production is common, meaning plants are fully exposed to cool and often wet summers. Infection biology of *Botrytis* in raspberry has not been as well-understood as in strawberry. Jarvis (1962) suggested that latent infection in floral parts may play a role, as in strawberry, however recent studies indicate the key infection period may be during fruit development (Kozhar and Peever, 2018; Kozhar & Peever, 2021). Raspberry production ranks second in berry production in Norway, with a production area of approximately 700 ha in 2020 (Revdal et al., 2021).

Grey mould is an especially challenging disease in production of Norway spruce seedlings in forest nurseries. Seedlings are at risk for mould development during cold storage, where *Botrytis* from infected material can continue to grow and spread amongst tightly packed seedlings at low temperatures (Lilja et al., 2010; Petäistö, 2006; Unestam & Beyer-Ericson, 1980). Seedlings can also be at risk for grey mould development during short-day treatment where stressed plants and high humidity are conducive to fungal infection (Lilja et al., 2010). Younger seedlings of Norway spruce have been found to be more susceptible to infection (Petäistö et al., 2004). Norway spruce comprised 97% of production of plants delivered for use by forest nurseries in Norway in 2020, amounting to over 41 million plants (skogfroverket.no).

Fig. 1 *Botrytis pseudocinerea* on a Norway spruce (*Picea abies*) seedling. (Photo: Erling Fløistad)

1.1.4 Grey mould disease control

According to Norwegian law, growers are required to employ the principles of integrated pest management (IPM) when making disease control decisions (Forskrift om plantevernmidler 2015). Barzman et al. (2015) made a thorough presentation of the eight IPM principles that are to inform all stages leading up to and after the implementation of disease control measures. Methods for controlling disease can be grouped into the following categories: cultural control, host resistance, physical control, biological control, and chemical control. The following subsections will address each category and its relevance for control of grey mould.

Cultural control involves crop management measures that aim to avoid creating conditions conducive for disease development, and as such fall under IPM Principles 1, Prevention and Suppression, 4 Non-chemical methods, and can contribute to 6, Reduced pesticide use (Barzman et al., 2015). Grey mould thrives in a dense, humid plant canopy with ample access too lush host material (Elad, 2016). Placing berry production under high tunnels helps reduce surface wetness and moisture (Nes et al., 2017; Xiao et al., 2001). Reducing planting density (Legard et al., 2000) and controlling amount and type of nitrogen fertilizer (Walter et al., 2008) in strawberry can reduce canopy density and help reduce humidity within the canopy. Application of calcium has also been shown to be beneficial against grey mould in strawberry (Walter et al., 2008). Sanitation measures that reduce inoculum in the immediate vicinity of the production area can help reduce disease pressure. Removing dead plant material in which *Botrytis* has overwintered helps to remove sources of inoculum for the subsequent production seasons in both strawberry and raspberry (Jarvis, 1962; Strømeng et al., 2009). Continuing to remove dead and infected plant material during the growing season, such as removing suckers in *Rubus* spp. also limits inoculum production (Walter et al., 1997). Removing dead and infected plant material, including weeds, can reduce grey mould problems in cold storage in Norway spruce production (Lilja et al., 2010; Petäistö, 2006; Unestam and Beyer-Ericson, 1980). Cultural practices can contribute to reduction of inoculum and prevention of conditions that favour disease spread, but it must be noted that these measures do not necessarily control *Botrytis* at an acceptable level, although they can still be beneficial as part of an integrated approach (Daugaard, 1999).

Like cultural control, host resistance is relevant for IPM Principles 1, Prevention and Suppression, and can contribute to 6, Reduced pesticide use (Barzman et al., 2015).

Choosing more resistant cultivars can help reduce problems with grey mould (Legard et al., 2000). Although Daugaard (1999) expressed a definite lack of optimism for the future of breeding for resistance to *Botrytis*, progress has been made. Relevant quantitative trait loci (QTLs) in chickpea and a wild relative of tomato have been found to be related to resistance to *Botrytis* and can be used in further breeding for resistance (Anuradha et al., 2011; Finkers et al., 2007). Gene *H* in raspberry has been confirmed to be associated with resistance to cane botrytis (Graham et al., 2006), and increased resistance to *Botrytis* has been conferred to strawberry via Agrobacterium-mediated transformation (Schestibratov & Dolgov, 2005). Very recently, a transgenic approach using an upregulated gene from tomato has been demonstrated to confer resistance to *B. cinerea* (Gong et al., 2021). In Norway, the political stance on gene editing and genetic modification in plant breeding has been conservative, so it remains to be seen whether this will be an option for producing resistant cultivars suited for Norwegian growing conditions in the future (Eriksson et al., 2018).

Using physical means to control grey mould incorporates IPM principle 4, Non-chemical methods, and can contribute to 6, Reduced pesticide use (Barzman et al., 2015). Thermotherapy, specifically steam thermotherapy, can help reduce latent infections in strawberry plants, thus reducing latent inoculum entering the field (Zuniga & Peres, 2017). Heat treatment has also been demonstrated to deactivate *Botrytis* conidia (Lichter et al., 2003; Marquenie et al., 2002). The use of UV-C (254 nm) irradiation followed by a dark period in tunnel production in strawberry has shown promising preliminary results, and the authors suggest the treatment could be complimented with biocontrol organisms (Janisiewicz et al., 2016). In studies relating to post-harvest control, both UV-C and heat have potential for *Botrytis* control through direct mechanisms on the pathogen and inducing resistance in the host (Charles et al., 2008).

Choosing biological control, or biocontrol, incorporates IPM principle 4, Non-chemical methods, and can contribute to 6, Reduced pesticide use (Barzman et al., 2015). The use of microorganisms to control *Botrytis* was already in practice when Daugaard (1999) wrote a review of non-chemical control methods over 20 years ago; both *Gliocladium roseum* and *Trichoderma* were mentioned specifically. Biocontrol works through direct mechanisms including parasitism, antibiosis, and competition, and it can also work though the indirect method of systemic plant resistance activation (Poveda et al., 2020). In a recent review, *Bacillus*, *Pseudomonas*, *Aureobasidium,* and *Trichoderma* were singled out as important biocontrol organisms

that work through direct mechanisms (Poveda et al., 2020). *Clonostachys rosea* has also been demonstrated to have effect against grey mould under field conditions in strawberry (Cota et al., 2008). Examples of microorganisms found to trigger systemic immune responses in *B. cinerea* hosts include *Streptomyces* sp. in chickpea and *Fusarium* in pepper (Díaz et al., 2005; Veloso and Díaz, 2013; Vijayabharathi et al., 2018). In addition, host-induced gene silencing is being explored as a biocontrol method (Xiong et al., 2019). In Norway, the product Serenade® ASO containing *Bacillus amyloliquefaciens* is approved for fungal disease control in both conventional and organic production.

IPM has a great deal of focus on chemical control: Principle 5, Pesticide selection and Principle 6, Reduced pesticide use. In many production systems, it is difficult to achieve adequate grey mould control without using fungicides (Fillinger and Walker, 2016). When fungicide used is deemed necessary, IPM principles dictate that the chemistry should be selective so as to minimize harmful side effects (Barzman et al., 2015). There are several fungicides on the market which have effect against *Botrytis*, however the propensity of *Botrytis*, particularly *B. cinerea*, to develop resistance to fungicides make chemical control challenging (Hahn, 2014). Furthermore, isolates increasingly exhibit multiple fungicide resistance, which, combined with fewer fungicides to choose from, makes Principle 7, Anti-resistance strategies, absolutely necessary if chemical control options are to remain effective (Rupp et al., 2017b).

1.2 Fungicide resistance

Fungicide resistance arose as an issue in the 1970's with the use of systemic single-site fungicides, prompting cooperation to address the problem that led to the formation of the Fungicide Resistance Action Committee (FRAC) (Hollomon, 2015a). Despite efforts to manage the situation it remains a major challenge. Companies face large economic hurdles in both practical development of new chemistries and the process to get new products registered. This has led to few new products becoming available while older products lose approval, and resistance rises against some of the key fungicides that are left (Lucas et al., 2015).

1.2.1 Resistance evolution and fitness

FRAC defines fungicide resistance as a sensitivity reduction to a specific fungicide that is both acquired and heritable (frac.info). Genetic changes or variation that confer

fungicide resistance can arise or be present in a pathogen population via de novo mutations, standing variation, intrinsic resistance and pleotropic co-option, or interspecific transfer (Hawkins et al., 2018). Fungicides exert strong selection pressure on the pathogen population, increasing the frequency of the resistance-conferring mutations in the population (Lucas et al., 2015). Resistant isolates may lose their selective advantage in the absence of fungicide pressure if the resistance-conferring mutations also have negative effects on factors such as survival, reproduction, or metabolism (De Miccolis Angelini et al., 2015). The degree to which this fitness cost hinders resistant isolates in competition with sensitive isolates affects the rate at which resistance increases in the pathogen population (Mikaberidze & McDonald, 2015).

1.2.2 Resistance mechanisms

Many different mechanisms can confer resistance to a pesticide: behavioural resistance, physical barriers, efflux, intracellular sequestration, molecular sequestration, detoxification via enhanced activity or overexpression, target overexpression, target modification, compensation, and protection against cytotoxic effects (Barrès et al., 2016). Target modification is particularly relevant for fungicides used to control *Botrytis* (Brent & Hollomon, 2007a), but efflux mechanisms conferring multidrug resistance (MDR) have also been identified (Kretschmer et al., 2009).

1.2.3 Modes of action

The mode of action (MoA) of a fungicide refers to the specific way in which it disrupts vital processes in the fungal pathogen. Some fungicides are very specific, affecting only one target protein and are referred to as single-site fungicides, while others have a range of different effects and are referred to as multisite fungicides (Lucas et al., 2015). Single-site fungicides exert disruptive selection pressure on the pathogen population, generally resulting in a high level of qualitative resistance when resistance-conferring mutations occur in the target-gene while multisite fungicides exert directional selection pressure which can be detected as a quantitative shift in level of resistance (De Miccolis Angelini et al., 2015). Mutations that occur in target-site genes can confer resistance to other fungicides belonging to the same chemical group in what is known as cross-resistance (Barrès et al., 2016; Hollomon, 2015a).

1.2.4 Fungicides for grey mould control in Norway

Most fungicides approved for grey mould control in Norway are single-site fungicides. The following list is arranged according to fungicide group name to which they belong, and corresponding abbreviations and codes are from the FRAC Code List© (FRAC, 2021). The following list includes fungicides that are currently approved. Two additional fungicides, which are included in this study, recently lost approval. Iprodione, a dicarboximide (FRAC 2), lost approval in 2019, and thiophanate-methyl, a methyl benzimidazole carbamate (MBC, FRAC 1), lost approval in 2021.

Anilinopyrimidines (AP, FRAC 9) available for grey mould control include cyprodinil (in a mixture with fludioxonil) and pyrimethanil. AP fungicides are proposed to disrupt methionine biosynthesis, but the exact target remains unidentified (Fritz et al., 1997). A more recent study suggested a mitochondrial function may be involved (Mosbach et al., 2017). Cross-resistance has been demonstrated among AP fungicides (Hilber and Schüepp, 1996). Cyprodinil resistance is included in both the MDR1 and MDR1h phenotypes, which involve the efflux mechanism associated with overexpression of ABC transporter B (AtrB) (Kretschmer et al., 2009; Leroch et al., 2013).

FRAC designates cuprous oxide (M 01) as a chemical with multisite activity for which resistance is not known. It is permitted for grey mould control in forest nurseries.

Fenhexamid is a ketoreductase inhibitor (KRI, FRAC 17) in the hydroxyanilide chemical group which targets 3-ketoreductase, C4- demethylation in sterol biosynthesis in membranes. Mutations in the 3-ketoreductase gene (*erg27*) in *Botrytis* have been found to lead to substitutions conferring high resistance, the most common of which include F412S, -I, -V, and -C (Amiri and Peres, 2014; Fillinger et al., 2008; Grabke et al., 2013; Saito et al., 2014a). In *B. pseudocinerea*, a lower level of resistance is likely conferred by a cytochrome P450 monooxygenase (Debieu & Leroux, 2015).

Fludioxonil is a phenylpyrrole (PP, FRAC 12) available for control in a mixture with cyprodinil. FRAC designated fludioxonil's MoA as signal transduction, but a specific target site is not yet identified (Leroch et al., 2013; Kilani & Fillinger, 2016). Fludioxonil is included in the MDR1 and MDR1h phenotypes, and resistance has been detected at a level demonstrated to be relevant for practical control (Fernández-

Ortuño et al., 2014; Leroch et al., 2013; Leroux & Walker, 2013; Li et al., 2014). MDR1h is associated with a triplet deletion in the gene encoding *multidrug resistance regulator 1* (*mrr1*) that leads to ΔL497 (Leroch et al., 2013), and several other mutations in mrr1 are associated with MDR1 (Kretschmer, 2009). Recently, isolates with high resistance to fludioxonil were found to have both mutations in *mrr1* leading to ΔL/V497 and mutations in the *Botrytis cinerea OS-1* (*Bos-1*) gene leading to the substitutions F127S, L267V, and I365N, the former and latter of which are also associated with dicarboximide resistance (Dowling et al., 2021). The study lends support to the target of fludioxonil being related to the high-osmolarity glycerol mitogen-activated protein kinase pathway (Dowling et al., 2021).

Quinone outside inhibitors (QoI, FRAC 11) affect respiration and there is cross-resistance within the group. There is not, however, cross-resistance with the recently established subgroup 11A for G143A mutants (frac.info). The two QoI (FRAC 11) fungicides approved for grey mould control are both available as mixtures, pyraclostrobin (with boscalid) and trifloxystrobin (with fluopyram). The product containing trifloxystrobin with fluopyram is only approved in strawberry. The mutation leading to the G143A substitution in the *cytochrome b* (*cytb*) gene affords high resistance to QoI fungicides (Leroux et al., 2010). In some isolates, a specific 1205 bp intron, Bcbi-143/144 or bi2, follows codon 143 and presumably interferes with having functional cytochrome *b* if it cooccurs with the G143A substitution (Banno et al., 2009). In several non-*Botrytis* species for which this intron has been detected, other mutations in *cytb* are common but they confer a lower level of QoI resistance than the G143A substitution (Grasso et al., 2006, Sierotzki et al., 2007).

Two succinate dehydrogenase inhibitor (SDHI, FRAC 7) fungicides are available in mixtures, boscalid (with pyraclostrobin) and fluopyram (with trifloxystrobin). Mutations in the *succinate dehydrogenase subunit B* (*sdhB)* gene are associated with resistance to boscalid and commonly lead to the H272Y and H272R substitutions but H272L, -V, P225T, -F, -L, and N230I have also been detected (Hu et al., 2016b; Konstantinou et al., 2015; Yin et al., 2011). As a group, SDHI fungicides exhibit incomplete cross-resistance. For example, the H272R substitution in SdhB confers resistance to boscalid but makes isolates sensitive to fluopyram (negative cross-resistance) while the P225F and N230I substitutions confer resistance to both boscalid and fluopyram (positive cross-resistance) (Lalève et al., 2014; Veloukas et al., 2013; Weber et al., 2015).

1.2.5 Monitoring fungicide resistance in *Botrytis*

Monitoring fungicide resistance is essential for management and should happen at field level to ensure appropriate anti-resistance strategies are chosen (Schnabel et al., 2015). Fungicide resistance is typically monitored through the use of bioassays or molecular methods (Barrès et al., 2016; Hollomon and Ishii, 2015; Schnabel et al., 2015). Bioassays employing a series of fungicide concentrations and sensitive reference strains can be used to obtain EC^{50} values, effective concentration required to impair growth by 50%, minimal inhibitory concentration (MIC), and resistances factors (RF) or levels (RL) (Fillinger and Walker, 2016). Monitoring EC^{50} values over time allow shifts in levels of resistance in the pathogen population to be detected. Data from EC^{50} values can be used to select discriminatory concentrations of fungicides to use in bioassays that can provide faster results about resistance frequencies to several fungicides in specific fields (Fernández-Ortuño et al., 2014; Schnabel et al., 2015).
Several molecular methods have been employed for detecting resistance in *Botrytis*: TaqMan® probe for single nucleotide polymorphism (SNP) identification (Billard et al., 2012), primer-induce restriction enzyme analysis PCR (PIRA-PCR) (Veloukas et al., 2011), and allele-specific PCR (AS-PCR) (Grabke et al., 2013; De Miccolis Angelini et al., 2014; Yin et al., 2011). Sequencing technology also provides the possibilities of multiplexing and pooling samples but requires specialized equipment and bioinformatics analysis (Barrès et al., 2016). Some techniques, such a loop-mediated isothermal amplification (LAMP) and portable nanopore sequencing have the potential for more in field application in the future (Donoso & Valenzuela, 2018). Multiplexed PCR amplicon sequencing (AmpSeq) is a method with potential for use in fungicide resistance monitoring (Kisselstein et al., 2017). Multiplexing several primer pairs per sample allows for the simultaneous generation of sequence data for several purposes, for example mutations conferring resistance and genetics studies, as has been done in work with malaria (LaVerriere et al., 2021).

Bioassays give information about resistance phenotypes *in vitro*. The most accurate assessment would be *in planta* assays that incorporate pathogen-host interaction (Corwin et al., 2016), but these tests are prohibitively resource demanding and therefore often used to test a selection of isolates (Fernández-Ortuño et al., 2014). Molecular identification of resistance genotypes has the advantage of being able to detect emerging fungicide resistance earlier (Barrès et al., 2016; Hollomon and Ishii, 2015), but methods that do not include sequencing could miss novel mutations that

confer resistance, and resistance phenotypes may be affected by genes not under investigation (Barrès et al., 2016; Hollomon and Ishii, 2015). Heteroplasmy, as found for *cytb* in *B. cinerea*, can also complicate certain molecular analyses (Ishii et al., 2009; Hollomon and Ishii, 2015).

1.2.6 Risk

FRAC lists four *Botrytis* species, including *B. allii*, *B. cinerea*, *B. elliptica*, and *B. squamosa*, as high risk for the development of fungicide resistance on their Pathogen Risk List© (FRAC, 2019). The risk of fungicide resistance development is comprised of a combination of factors relating to pathogen biology, fungicide characteristics, and agronomical practices (Brent and Hollomon, 2007b). Characteristics in the pathogen which contribute to increasing intrinsic risk for resistance development include short generation time, a polycyclic lifecycle, production of large amounts of spores, the potential for dispersal, and genetic variation (Atwell et al., 2015; Leroux et al., 2002; Lucas et al., 2015; Veloukas et al., 2014). Single-site fungicides have higher risk of resistance development than multisite, and in their work using trait-based risk assessment to make risk predictions, Grimmer et al. (2015) found that high-complexity molecules had higher risk due to more specific binding. Agronomical practices in production systems can have a significant impact on the risk of developing fungicide resistance. Such factors include how often a fungicide is applied, the degree to which the fungicide is applied exclusively or in alternation or mixture with other fungicides, how much of the pathogen population is exposed to the fungicide, how isolated the pathogen population is, and how well integrated measures reduce disease pressure in the field (Brent and Hollomon, 2007b).

1.2.7 Resistance management

Botrytis cinerea has very high intrinsic risk for resistance development, and chemical control of grey mould in Norway almost exclusively includes single-site fungicides, so agronomic risk factors, how disease is managed and growers actually use fungicides, must be in focus for management. The following paragraphs in this section include some common management strategies.

A major tenet of resistance management is avoiding exclusive use of a single fungicide (Brent and Hollomon, 2007a). In general, mixtures appear to exert less selection pressure than alternations, assuming the mixing partner is effective (Elderfield et al.,

2018; van den Bosch et al., 2014; van den Bosch et al., 2015). It has been pointed out, however, that mixtures and alternations will be of little help when multiple fungicide resistance is already present (Hu et al., 2016a; Rupp et al., 2017b).

Fungicide dose can affect selection for resistance; however the issue of dose is a "debate" (van den Bosch et al., 2011). Brent and Hollomon (2007a) convey that FRAC states recommended doses shall be followed to avoid resistance development, but then go on to point out that some evidence indicates lower doses could reduce selection for major gene resistance. Experimental and modelling studies indicate that increases in dose increase selection for resistance (van den Bosch et al., 2014). A recent study demonstrated how different doses selected for different mutations conferring resistance to a SDHI fungicide in *Zymoseptoria tritici* (Gutiérrez-Alonso et al., 2017). It is not known, however, if lower doses may select for quantitative resistance, or how dose affects resistance emergence (Brent and Hollomon, 2007a; van den Bosch et al., 2011; van den Bosch et al., 2015). Studies of nanoparticles and applications for pathogen control have also been on the rise. Nanoparticles can have an effect on pathogens alone or in combination, or serve as a delivery system for fungicides, potentially reducing the doses required for control (Espevig et al., 2019; Kutawa et al., 2021; Malandrakis et al., 2020)

Correct timing of fungicide treatment is important for resistance management. FRAC argues that waiting for the pathogen populations to reach a threshold before fungicides are applied increases selection since then there is a larger population than there would have been with an earlier, preventative treatment (Brent and Hollomon, 2007a). Yet, there is no evidence as to whether preventative or curative fungicide application reduces selection; pathogen, weather, and crop cultivar will all affect whether timing leads to increased or decreased selection for resistance (van den Bosch et al., 2014). *Botrytis* infects strawberry during flowering so crop phenology determines the optimum timing for fungicide treatment for control, and correct timing can reduce the need for further applications, thus contributing to reduced resistance selection (Mertely et al., 2002).

In general, any reduction of fungicide applications both slows selection and allows resistance-associated fitness costs, if present, to reduce the resistant proportion of the population (Brent and Hollomon, 2007a). Finally, non-chemical disease control methods must be employed to reduce disease pressure and fungicide input (Jørgensen et al., 2017). Any tactics used that reduce disease pressure using non-

chemical means also limit the resistant proportion of the population and limit selection, therefore contributing to resistance management (Brent and Hollomon, 2007b).

2 Objectives

The main objective of this study was to analyse and gain better understanding of the dynamics of the fungicide resistance problem in the grey mould pathogen in strawberry, raspberry, and Norway spruce and explore ways to address disease control and resistance management.

Grey mould is a considerable challenge in open field strawberry production and an especially difficult season in 2016 prompted an investigation to see if fungicide resistance was a contributing factor in the devastating losses experienced in Agder, a southern region in Norway. Grey mould has also been a challenging problem for forest nursery production of Norway spruce seedlings despite the use of fungicides for control. The first objective was to characterise resistance in the grey mould pathogen in strawberry and Norway spruce using both *in vitro* and molecular methods. Bioassays with discriminatory concentrations were used to obtain resistance frequencies and molecular methods were used to identify mutations conferring resistance in known target genes (Papers I & II).

Grey mould in strawberry and Norway spruce has been assumed to be caused by *B. cinerea* in Norway, however recent developments in *Botrytis* taxonomy indicate that assumptions could be incorrect. Fungicide resistance varies between *Botrytis* species, so correct identification of the *Botrytis* species complex is relevant for disease control. The second objective was to identify the composition of the *Botrytis* species complexes in both strawberry and Norway spruce (Papers I & II).

Resistance is selected for in a pathogen through fungicide application during the season, but there are other ways for resistant *Botrytis* to enter a production system. *Botrytis* infections are known to exist in strawberry transplant material, and with the legalization in 2015 of strawberry transplant importation, there was some concern regarding resistance in the latent pathogen entering the country. There is potential for inoculum reservoirs in forest nursery facilities, and *Botrytis* that remains in the facilities between seasons has the potential to infect seedlings in subsequent rounds of production. The third objective was to obtain *Botrytis* from these sources and test for fungicide resistance (Papers I & II).

Resistant *Botrytis* entering the field with planting material is detrimental in terms of increasing disease pressure, and it simultaneously threatens the ability to use chemical control to address increased disease pressure when it is also resistant to fungicides. Steam thermotherapy approaches have been demonstrated to reduce pathogens in strawberry transplants. The fourth objective was to test the use of steam thermotherapy treatment to reduce latent infections in strawberry transplants, test *Botrytis* from treated and untreated plants for fungicides resistance, and determine whether sclerotia, hardy survival structures, generated from fungicide-resistant *Botrytis* isolates could survive the treatment (Paper IV).

Raspberry growers were facing fenhexamid resistance problems in their fields, to the extent that some had stopped using the fungicide. With no prospects of new chemical control options, there was interest to see if there was fitness cost associated with fenhexamid resistance that could be exploited to reverse resistance enough to include fenhexamid in fungicide rotations again. The fifth objective was to assess if there was a fitness cost in *Botrytis* associated with fenhexamid resistance (Paper III).

3 Materials and Methods

3.1 *Botrytis* isolates

The following is a brief list of samples from which *Botrytis* isolates were obtained. More detailed information on sample collection and *Botrytis* isolation and storage is described in the respective papers.

Strawberry plants were collected from 20 commercial fields in Agder, Norway in late summer 2016. A total of 157 *Botrytis* conidia samples were obtained from 19 of the fields and used for resistance testing, and then single-spore isolates were made and used for molecular analysis (Paper I).

In 2018, we received five samples each of imported and Norwegian-produced strawberry transplants, and in 2019 we received four imported samples and eight samples produced in Norway. In all, 365 *Botrytis* isolates were obtained from the samples. Isolates were tested for fungicide resistance (Paper I).

Both symptomatic and asymptomatic Norway spruce seedlings were collected from eight Norwegian nurseries between 2013 and 2019, and 53 *Botrytis* isolates were obtained. Isolates were tested for fungicide resistance and used for molecular analysis (Paper II).

On visits to three different forest nurseries in the period from November 2018 to June 2019, a total of 68 *Botrytis* isolates were obtained from the air with open agar plates and surfaces by using cotton swabs. Isolates were tested for fungicide resistance and used for molecular analysis (Paper II).

Plant material was sampled from an experimental perennial strawberry field at the Norwegian University of Life Sciences (NMBU) in Ås Norway. Samples were collected eight times from the end to May 2018 to June 2020, and a total of 208 isolates were obtained and tested for fungicide resistance (Paper III).

Norwegian Agricultural Extension Service advisors collected samples pre-season (overwintered canes) and post-season (fruit) in six commercial perennial raspberry

fields from spring 2018 to spring 2021. Five of the raspberry fields were located in Vestland county and one in Viken county. A total of 624 *Botrytis* isolates were obtained from these samples and tested for fungicide resistance. (Paper III).

Five samples of imported strawberry transplants cv. Soprano, Favori, Murano, Falco, and Sonsation were used for steam thermotherapy experiments. In total, 62 *Botrytis* isolates were obtained from the samples, including from controls and transplants that had undergone steam thermotherapy treatment (Paper IV).

3.2 Fungicide resistance testing

Testing methods are described in detail in the respective Papers and briefly summarized in this section.

Botrytis isolates (not single-spore) collected in Agder in 2016 (Paper I) and *Botrytis* isolates from strawberry transplants obtained in 2018 (Paper I) were tested for fungicide resistance using a spore germination test (Weber and Hahn, 2011; Weber et al., 2015). Conidia suspensions were pipetted onto agar plates containing discriminatory concentrations of fungicides, two concentrations and a control per fungicide. Lengths of germ tubes from germinating conidia were measured using a light microscope and measurements were used to make calculations to determine resistance categories: ss for highly sensitive, s for less sensitive, mR for moderately resistant, and R for highly resistant.

A mycelial growth assay adapted from Fernández-Ortuño et al. (2014; Schnabel et al., 2015) was used for all other resistance testing of *Botrytis* isolates (Papers I, II, III, & IV). Agar growth media was amended with fungicides and pipetted into 24-well cell culture plates (Nunclon™ Delta Surface, Thermo Scientific). Conidia were transferred to the center of the wells with sterile toothpicks and diametric growth in relation to well diameter was used to determine resistance category: sensitive (S, no growth), low resistant (LR, less than 20%), moderately resistant (MR, more than 20% and less than 50%), or resistant (R, more than 50%).

3.3 Molecular characterization of *Botrytis*

The details of DNA extraction, PCR, and subsequent analyses are described in Papers I and II; brief summaries follow.

Sequence data from *g3pdh* and *nep2* genes were used to generate phylogenetic trees and identify species of *Botrytis* isolates obtained from strawberry, Norway spruce,

and from the air and surfaces of forest nursery facilities (Papers I & II). A PCR assay was also used for *B. pseudocinerea* identification in Paper I (Plesken et al., 2015). *Botrytis* group S isolates were identified by detecting characteristic 18 bp (Paper I) and 21 bp (Papers I & II) indels in the transcription factor-encoding gene multidrug resistance regulator 1 (*mrr1*) (Leroch et al., 2013; Plesken et al., 2015). Mating type was determined according to the method described by De Miccolis Angelini et al. (2016).

New allele-specific PCR (AS-PCR) assays were developed to identify the *cytochrome b* gene (*cytb*) mutation leading to the G143A substitution and a 1205 bp intron known to occur in some wild type (wt) alleles (Paper I). AS-PCR and sequencing were used to detect mutations relevant for resistance to pyraclostrobin and other QoIs, fenhexamid, fludioxonil and cyprodinil, boscalid, and thiophanate-methyl in *cytb*, 3-ketoreductase (*erg27*), *mrr1,* succinate dehydrogenase subunit B (*sdhB*), and beta-tubulin (*tubA,*), respectively (Banno et al., 2008; Grabke et al., 2013; Leroch et al., 2013; Leroux et al., 2010) (Papers I & II, except *tubA* Paper II only).

3.4 Pathogenicity test

Norway spruce seedlings were germinated in sterile conditions and transferred to glass test tubes with growth medium. Seedlings were inoculated with *Botrytis* isolates identified as *B. cinerea*, *Botrytis* group S, *B. prunorum*, and *B. pseudocinerea.* Results were registered and specimens photographed 17 days post inoculation (Paper II).

3.5 Fitness parameter tests

A total of 92 *Botrytis* isolates obtained from six commercial raspberry fields in the spring of 2020 and 2021 were tested for conidia production on PDA plates. Half of the isolates were resistant to fenhexamid and half sensitive, based on results from the mycelial growth assay. Three-dimensional fungal growth was measured with a photometric microplate absorbance reader (Bioscreen C° Pro™, Oy Growth Curves, Finland) as described by Medina et al. (2012) to determine mycelial growth rate for a selection of 73 of the above isolates (Paper III).

3.6 Steam thermotherapy treatment

Imported strawberry transplant samples of seasonal flowering cvs. Falco, Sonsation, and Soprano and of everbearing cvs. Favori and Murano were subjected to steam thermotherapy treatment. The treatment program included steam pre-treatment for one hour at 37°C, followed by a break for one hour in ambient conditions, and then a two-hour or four-hour steam treatment at 44°C. Plants were assessed after treatment for potential effects on growth and yield. Plant material from both treatments and untreated plant material was incubated and *Botrytis* incidence scored. Four *Botrytis* isolates with characterized genotypes (from Paper I) were used to produce sclerotia that were then subjected to the four-hour steam thermotherapy program. Following treatment, sclerotia were transferred to growth medium, incubated, and scored for germination (Paper IV).

3.7 Statistics

Data was analysed by one- or two-way analysis of variance (ANOVA) in R. Versions of R and follow-up analyses are specified in the respective papers (Papers I, III, & IV).

4 Main results and discussion

Detailed results are presented and discussed in each Paper. Principal findings from the Papers are combined and presented here and discussed in context of grey mould disease control and fungicide resistance management.

4.1 High levels of fungicide resistance and multiple fungicide resistance in the grey mould pathogen (Papers I, II, III, & IV)

Results from the spore germination test and the mycelial growth assay revealed high frequencies of resistance to several fungicides in the grey mould pathogen. Resistance detected in strawberry from Agder in 2016 (Paper I) was high for several fungicides; 89.1%, 65.4%, and 86.0% of conidia samples were resistant to boscalid, fenhexamid, and pyraclostrobin, respectively. Resistance was detected for pyrimethanil (24.3%) and moderate resistance and reduced sensitivity (1.9% and 28.0%) were detected for fludioxonil. Although collected at the end of the growing season, high resistance frequencies indicate these fungicides would not have been expected to control the *Botrytis* population and suggest resistance could have played a role in the 2016 losses.

Among all 125 isolates collected from Norway spruce and surface and air at forest nursery facilities, 8.8%, 33.6%, 17.6%, 36.0%, 13.6%, and 50.4% were resistant to boscalid, fenhexamid, fludioxonil, pyraclostrobin, pyrimethanil, and thiophanate-methyl, respectively (Paper II). Fenhexamid, fludioxonil, and cyprodinil (which exhibits cross-resistance with pyrimethanil) are currently available for use, and approval for thiophanate-methyl expired this year. Resistance frequencies in our data indicated possible efficacy problems with the single-site fungicides that are currently approved in forest nurseries.

Botrytis from six commercial raspberry fields exhibited high resistance to several fungicides at the beginning and end of the three-year monitoring period (Paper III). In the 2021 pre-season sampling, the ranges of resistance to boscalid, fenhexamid, fludioxonil, pyraclostrobin, and pyrimethanil were: 20.0-100%, 20.0-100%, 20.0-71.4%, 0-80.0%, and 20.0-90.0%, respectively. For most fields, resistance levels

indicated potential efficacy problems for all available fungicides, including boscalid with pyraclostrobin, fenhexamid, and fludioxonil with cyprodinil.

Fungicide resistance in *Botrytis* from strawberry transplants exhibited varying, but often high resistance to several fungicides (Papers I & IV). For tests conducted in 2018 to 2020, ranges of means for resistance to boscalid, fenhexamid, fludioxonil, fluopyram, pyraclostrobin, and pyrimethanil from all samples were: 3.2-88.7%, 8.6-37.9%, 1.7-42.8%, 0-45.5%, 31.6-100%, and 5.7-35.5%, respectively.

Multiple fungicide resistance was prevalent in the resistance data (Papers I, II, III & IV). Although different tests were used with different combinations of fungicides, extracting certain data points from the different studies can give a general indication of the degree of multiple fungicide resistance we found in the grey mould pathogen across different production systems and inoculum sources. In the results from strawberry in Agder, 23.4% of conidia samples were resistant to four or more fungicides and 67% of samples were resistant to at least boscalid, fenhexamid, and pyraclostrobin (Paper I). In isolates from Norway spruce and air, surfaces, and weeds in forest nurseries, 5.7%, 37.5%, 25.05, and 50%, respectively, were resistant to at least four fungicides (Paper II). In *Botrytis* from commercial raspberry fields obtained pre-season 2021, resistance to four or more fungicides ranged from 20.0% to 70.0% with a mean of 41.6% for the six fields (Paper III). In 62 isolates recovered from both untreated and steam thermotherapy-treated strawberry transplants, 16.1% were resistant to four or more fungicides (Paper IV).

Detection of mutations in the *cytb*, *erg27*, *mrr1*, *sdhB*, and *tubA* genes largely corroborated the findings of the resistance tests and provided additional useful information for disease control and resistance management (Papers I & II). A product containing fluopyram and trifloxystrobin was approved for grey mould control in strawberry in 2017. The results from Agder in 2016 (Paper I) show there was already a high frequency of the G143A substitution in *cytb* which is known to confer resistance to QoI fungicides including trifloxystrobin. Due to negative cross-resistance associated with the particular mutations detected in *sdhB* most of the population would be expected to be sensitive to the SDHI fluopyram. However, the N230I and P225F substitutions which would be expected to confer resistance to both boscalid and fluopyram were also detected. Thus, before the introduction of the product, the QoI mixing partner trifloxystrobin was expected to have little effect and resistance to the SDHI fluopyram was already present in the population. The data

indicate growers must adopt conservative use of the new product containing trifloxystrobin and fluopyram to delay loss of efficacy.

Findings of high resistance combined with widespread multiple fungicide resistance have serious implications for both grey mould disease control and fungicide resistance management. In terms of chemical control options, strawberry and raspberry growers have only a limited selection of single-site fungicides and no multisite fungicides. When chemical control is deemed necessary to achieve adequate disease control, growers are forced to choose fungicides which may have compromised efficacy and which will contribute to maintaining and selecting for multiple resistant genotypes in the pathogen population.

Data from the comparison of fungicide resistance frequencies to strawberry grower evaluations of grey mould problems in their fields for 2016 provides some hope (Paper I). We were not able to find correlation between degree of grey mould problem and resistance. Low grey mould problems were reported for fields with high resistance frequencies and *vice versa*. Additional field information communicated by the advisory services lend credence to the conclusion that control failures in 2016 had other contributing factors besides fungicide resistance in the grey mould pathogen. Some growers managed without major problems despite high resistance, so control is possible.

4.2 Identification of the *Botrytis* species complex (Papers I & II)

Among *Botrytis* isolates obtained from strawberry (Paper I), 119 were identified as *B. cinerea*, including 73 *Botrytis* group S isolates identified by detection of characteristic indels in the *mrr1* gene. Three isolates were identified as *B. pseudocinerea*. Low recovery of *B. pseudocinerea* in production systems with fungicide input is consistent with findings that this species is less likely to develop fungicide resistance than *B. cinerea* (Plesken et al., 2015).

Two *B. pseudocinerea* isolates were recovered from Norway spruce seedlings, and this species has not previously been reported in Norway spruce (Paper II). *B. pseudocinerea* isolates were also recovered from surfaces in forest nursery facilities, and one such isolate was used in a pathogenicity test with Norway spruce seedlings grown under sterile conditions. All six seedlings inoculated with the *B. pseudocinerea*

isolate had necrotic tissue and sporulation when registered 17 days post inoculation. Our results show that *B. pseudocinerea* can infect Norway spruce seedlings at this developmental stage, and is thus part of the *Botrytis* species complex in Norway spruce. Two isolates recovered from the air in a forest nursery greenhouse in June were identified as *B. prunorum*, making this the first recorded identification of the species in Northern Europe (Paper II). One of the isolates caused some symptoms on Norway spruce seedlings in the pathogenicity test, but in general caused less severe symptoms than the other isolates and sporulation was only observed on one of the seedlings. Whether *B. prunorum* should be considered part of the *Botrytis* species complex in Norway spruce remains to be determined.

4.3 Sources and control of fungicide-resistant *Botrytis* inoculum (Papers I, II, & IV)

Fungicide-resistant *Botrytis* was recovered from strawberry transplants (Papers I & IV). Latent infections in planting material represent an important source of primary inoculum for the development of grey mould in the field. Our results indicated that in many cases the levels of fungicide resistance present in this inoculum were already at risk for resistance development, meaning growers can have a resistance problem before they start spraying. In addition, resistance to fluopyram was detected in some imported material collected only a year after fluopyram was approved for use in Norway, meaning resistance in latent inoculum can also contribute to speeding up selection for resistance.

Reducing sources of inoculum is an important preventative tactic in integrated disease control. *Botrytis* recovered from the strawberry transplants was greatly reduced by steam thermotherapy treatment (Paper I). This included reduction of fungicide-resistant latent infections, meaning steam thermotherapy is a promising disease control technique that both reduces disease pressure through inoculum reduction and contributes to longevity of fungicides that are currently available. Steam thermotherapy had a comparable effect on *Botrytis* sclerotia. Sclerotia from the *B. pseudocinerea* isolate survived. Although this cannot be considered representative for the species in general due to high genetic variability in *Botrytis,* the survival of *B. pseudocinerea* is promising in terms of resistant management. *Botrytis pseudocinerea* is less adept at developing fungicide resistance than *B. cinerea* and will therefore more likely be controlled by chemical means.

In forest nursery facilities, fungicide-resistant *Botrytis* isolates were recovered from the air, surfaces, and weeds (Paper II). *Botrytis* can survive in weeds and produce inoculum during subsequent growing seasons if not removed from the vicinity of the seedlings. Sanitation measures can reduce the amount of inoculum in areas where seedlings are processed before cold storage, where *Botrytis* can continue to grow at cold temperatures between tightly packed plants.

4.4 Fitness cost associated with fungicide-resistant *Botrytis* in the field (Paper III)

Results from the experimental perennial strawberry field show the frequency of fenhexamid resistance increased from 3.4% to 90.0% in about a month with the combination of repeated fenhexamid applications and repeated distribution of fenhexamid-resistant inoculum in the field. Following this initial increase, resistance monitoring showed a steady decline in fenhexamid resistance, with two final readings of 5.3% and 6.9% after approximately two years. Results from testing isolates for resistance to boscalid, fludioxonil, fluopyram, pyraclostrobin, pyrimethanil, and thiophanate-methyl showed multiple fungicide resistance also increased from the first to second sampling. By the final sampling, multiple fungicide resistance had fallen below initial levels, and isolates without resistance had increased to 75.0%. Although this was only one field with no control or divided plots, it is a promising preliminary indication that fungicide resistance in *Botrytis* can be reversed under Norwegian field conditions in the absence of selection pressure exerted by fungicides.

Changes in fungicide resistance in the six commercial perennial raspberry fields were less drastic. Results from three years of monitoring showed increases in fenhexamid resistance in three fields and decreases in three, and final resistance frequencies for fenhexamid were 50% or higher for five of six fields. Data from resistance testing for other fungicides at the start and end of the experiment revealed high degrees of multiple fungicide resistance. The majority of isolates resistant to fenhexamid were also resistant to both boscalid and pyraclostrobin, and growers used the product containing boscalid and pyraclostrobin during the three-year monitoring period. We believe the best explanation for our results is that use of the product containing boscalid and pyraclostrobin contributed to maintaining fenhexamid resistance in the population through “selection by association” (Hu et al., 2016a). Differences were not

detected between fenhexamid-resistant and -sensitive isolates for either conidia production or mycelial growth rate, but there are several other factors that can affect survival and competition in the field.

5 Conclusions and future perspectives

Fungicide resistance in *Botrytis* threatens grey mould disease control. We detected resistance to all single-site fungicides currently permitted for use in Norway. In addition, growers have a limited number of fungicides to choose from. Experts tried to alert policy makers in Europe years ago, yet the trend of disappearing products with grim prospects for replacements continues, forcing growers to depend on fewer chemistries and exacerbating the resistance problem (Bielza et al., 2008). Multiple fungicide resistance further complicates the resistance problem. The results from Paper III showed that reducing the use of one high risk fungicide did not necessarily help resistance levels go down when there was multiple fungicide resistance.

Hope should not be placed on the discovery and approval of new fungicides with new modes of action. Creating new products is a huge expense for chemical companies, and the registration process can be daunting. The Norwegian market cannot promise large returns on this investment, and this affects the availability of diverse chemistries for control. Even if more modes of action were available for *Botrytis* control, there is a good chance *Botrytis* would become resistant to those too (Corkley et al.; 2021, Hollomon, 2015a). Focus should be placed on building up an arsenal of non-chemical control methods to complement fungicides in an IPM approach to disease control. Findings of fungicide resistance in various inoculum sources in Papers I, II, and IV show that efforts to reduce inoculum also reduces resistance. Steam thermotherapy is effective for *Botrytis* in strawberry. Perhaps it could also help to reduce *Botrytis* in Norway spruce seedlings before entering cold storage.

Continued monitoring of fungicide resistance is also important, both so growers know which products should be expected to work and to track changes in the pathogen population for resistance management purposes. In our data, fluopyram resistance had not yet reached high levels or was not detected in some locations. There is data on mutations known to confer fluopyram resistance, and given incomplete cross-resistance among SDHI fungicides, it would be beneficial to employ molecular methods to monitor for SDHI resistance in the future. One method which could be well-suited for *Botrytis* is AmpSeq. It provides the opportunity to obtain sequence data for known target-genes, house-keeping genes used for species identification, and genes for other genetic studies all in the same analysis.

Finally, resistance management is not disease control, and IPM must not lose sight of production and profitability that will ultimately determine growers' control decisions (Hollomon, 2015b). Research must be focused on continuing to develop effective and practical control measures in cooperation with the growers who will use them.

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7 Papers

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

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Paper I

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

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Keywords

Latent infection, multidrug resistance (MDR), *Botrytis* species complex

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 full 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 and commonly associated with the multidrug resistance phenotype MDR1h, was detected in 29.2 % of *Botrytis* group S isolates. High levels 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 which has important implications for future management of grey mould.

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* Duch.). 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 et al., 2016b; 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 *B. cinerea* sensitive or hypersensitive to another SDHI, fluopyram (Laleve 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 *B. pseudocinerea* is mainly attributed to a different resistance mechanism, i.e., detoxification involving a cytochrome P450 monooxygenase (Debieu and 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 and Walker, 2013; Li et al., 2014b).

Botrytis 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 conidia production, genetic variability, and a polycyclic life cycle, contribute to its ability to develop fungicide resistance faster than 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 and 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 and 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 (i) characterised and ascertained the range of fungicide resistance in *Botrytis* from strawberry in the Agder region of Norway; (ii) developed and employed two new allele specific PCR assays to detect resistance conferring mutations in *cytb*; (iii) tested for known target-site mutations in *sdhB*, *erg27*, and *mrr1* that are relevant for fungicide resistance; (iv) identified *Botrytis* species/groups of field isolates; (v) examined fungicide resistance in relation to grey mould control problems experienced by growers in Agder in 2016; and (vi) screened *Botrytis* isolates from imported and domestically produced strawberry transplants for fungicide resistance.

Materials and methods

Sample collection and Botrytis *isolation*

In late summer 2016, plant samples were collected from 20 strawberry fields in Agder, Norway (Figure 1). 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 two to seven 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 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.

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).

Testing conidia samples from strawberry fields

Conidia samples 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 hours 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 included: ss for highly sensitive, s for less sensitive, mR for moderately resistant, and R for highly resistant.

Testing Botrytis *isolates from strawberry transplants*

In 2018, a total of 138 single-spore isolates of *Botrytis* were collected 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 collected and screened for fungicide resistance with a mycelial growth assay adapted from Schnabel et al. (2015). Fungicide products were mixed into liquid media to create stock solutions. For the assay, agar growth media were amended with 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 four 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.

Botrytis *DNA extraction and analysis*

Single-spore isolates were made from conidia samples from strawberry plants collected from Agder in 2016 that were used for the spore germination test. 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 PCR reactions were performed in a total volume of 25 µl using 1 unit of 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 characterisation of mutations, the consensus sequences were aligned with various reference sequences retrieved from the NCBI nucleotide database (Supporting Information Table 1). Sequences from this study that have been deposited in GenBank® appear in Supporting Information Table 2 with accession numbers.

Botrytis *identification and construction of phylogenetic trees*

Botrytis 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 covering 18 and 21 bp indels of multidrug resistance regulator 1 (*mrr1*), a transcription factor-encoding gene. These regions were characterised 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, characterised by a

triplet deletion in *mrr1* resulting in ΔL497, was further identified by digesting the Mrr1-spez-F/Mrr1-Pira PCR-product with HpyH4V as previously described (Leroch et al., 2013). Mating type was determined as by De Miccolis Angelini (2016). Individual phylogenetic trees were constructed for regions of the *glyceraldehyde-3-phosphate dehydrogenase* (*g3pdh)* gene and the *necrosis- and ethylene-inducing protein* (*nep2*), (107 and 114 isolates, respectively) using the neighbour-joining method and the Jukes Cantor substitution model (Supporting Information Figure 1). 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 (Supporting Information Table 1).

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 characterise codon 143 in *cytb* (Figure 2). 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 wt 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 characterised 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).

Statistics

R version 4.1.0 (2021-05-18) was used for statistical analysis. Resistance 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.

Results

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 levels of full 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, pyraclostrobin, and fenhexamid. Only 5 % of the samples were fully sensitive.

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 characterisation 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 (Supporting Information Figure 1). Mating type was 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. Of three *B. pseudocinerea* isolates, two were MAT1-1 and one was MAT1-2.

Mutations conferring resistance

Target-site mutations in the *sdhB*, *cytb* and *erg27* genes which 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 3). 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 mutations (Figure 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 (Supporting Information Table 3), 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 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.

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 though *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.

We found high levels of fungicide resistance to boscalid, pyraclostrobin, and fenhexamid as well as high levels of multiple fungicide resistance in the spore germination test. Detection of mutations known to confer fungicide resistance in the *cytb*, *sdhB*, *erg27*, and *mrr1* genes supported these findings. 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, pyraclostrobin, and fenhexamid would not have been expected to adequately control the *Botrytis* population during the 2016 season in Agder. High levels 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, 2020), and this study has shown that QoI resistance in *Botrytis* in strawberry is high and widespread in the Agder region of Norway. The effectiveness of trifloxystrobin as a resistance-delaying mixing partner for fluopyram is therefore dubious, a sentiment shared by Oliveira et al. (2017) and Weber et al. (2015). Our data shows 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 and Schüepp, 1996), our findings indicate that the efficacy of the product containing cyprodinil and fludioxonil could have been suboptimal 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 and 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 fully 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 indicates 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 levels of resistance 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 et al., 2016a). In the U.S., 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 fully 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). Detection of resistance to fluopyram in this 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, Supporting Information Table 4). 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, Supporting Information Table 4). Another field with extreme grey mould problems had high resistance, but also high, wide rows with dense foliage due to a high rate of fertiliser application (data not included), making the canopy conducive to grey mould development (Field 9, Supporting Information Table 4). Regardless of fungicide resistance level 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 fertilisation (Walter et al., 2008), are examples of cultural methods which 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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Data Availability Statement

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

Conflict of Interest

The authors have no conflict of interest to declare.

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Supporting Information legends

Supporting Information Table 1. *Botrytis* reference sequences from GenBank® used in this study.

Supporting Information Table 2. Molecular results for *Botrytis* isolates included in this study, including accession numbers for sequences that have been submitted to GenBank®.

Supporting Information Table 3. Results of statistical analysis in R version 4.1.0 to test for differences in expected fungicide resistance frequencies among problem level groups (low, moderate, major, extreme).

Supporting Information Table 4. Fungicide resistance in *Botrytis* conidia samples from strawberry fields in Agder, Norway in 2016, based on a spore germination test[a].

Supporting Information Figure 1. Neighbour-joining phylogenetic trees of *nep2* and *g3pdh* sequences. The trees show the relationship between *Botrytis* species isolated from strawberry in Norway and other known species (see Supporting Information Table 1). Bootstrap percentages (n=1000) are indicated on the branches, and only branches with > 50 % bootstrap support are shown. Isolates identified as *Botrytis* group X, *Botrytis* A and *B. pseudocinerea* are green, red, and blue, respectively, whereas species used as references are grey.

Figure legends

Figure 1. Strawberry plant material was collected from 20 fields in Agder, Norway in 2016. The map template is by ©Kartverket (geonorge.no), licensed under CC BY 4.0.

Figure 2. 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 wt 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.

Figure 3. 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 4. Accumulation of substitutions known to confer fungicide resistance in a selection of 39 *B. 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.

Figure 5. 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 percent fully resistant conidia samples from the germination test except for fludioxonil, which is percent moderately resistant (mR) plus less sensitive (s).

Table 1. Growth media and fungicide active ingredients in the spore germination test used to analyse resistance in conidia samples and single-spore isolates of *Botrytis* from strawberry.

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

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

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

[c] Plates used for testing pyraclostrobin were amended with salicylhydroxamic acid (SHAM) dissolved in methanol for alternative oxidase inhibition (Weber and Hahn, 2011) such that the final concentration of SHAM in the media 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).

Table 3. Primers and polymerase chain reaction conditions.[a]

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[b]	45[c]	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

[a] Initial denaturation at 95˚C for 5 minutes followed by 35 cycles of amplification (denaturation at 95˚C for 30 seconds, annealing at the indicated temperature for 30 seconds, extension at 72˚C for the indicated time), and final elongation at 72˚C for 7 minutes.

[b] 60˚C

[c] 90s when T63_F and F412_R were used for sequencing purposes

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

Resistance category[a]	Fungicide resistance (% isolates in each category)					
	Boscalid	Fenhexamid	Fludioxonil	Iprodione	Pyraclostrobin	Pyrimethanil
R	89.1	65.4	-[b]	2.6	86.0	24.3
mR	-[b]	-[b]	1.9	29.0	-[b]	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 (fully resistant), mR (moderately resistant), s (less sensitive), ss (highly sensitive), according to Weber and Hahn (2011)
[b] Hyphen indicates the category was not part of the test for the specific fungicide.

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	X	X	X	X	X	2.1
4R	X	X		X	X	20.6
	X	X	X	X		0.7
3R	X	X		X		44.0
	X			X	X	1.4
	X	X			X	0.7
2R	X			X		21.3
		X		X		0.7
1R	X					2.1
				X		0.7
		X				0.7
0R						5.0

[a] Phenotype denotes number of fungicides (0-5) where there was full 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
Botrytis group S	73	X	X	38	29	2[d]	4
Botrytis group X	1	X		1	0	0	0
Botrytis A[c]	1		X	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

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
2018[a]	% R	% R	% s	% R	% R	% R
Import (5)[d]						
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) [d]						
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[b]	% R	% R	% R	% R	% R	% R
Import (4) [d]						
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) [d]						
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] Spore germination test, including resistance categories: fully resistant (R), moderately resistant (mR), less sensitive (s), highly sensitive (ss), according to Weber and Hahn (2011).

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

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

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

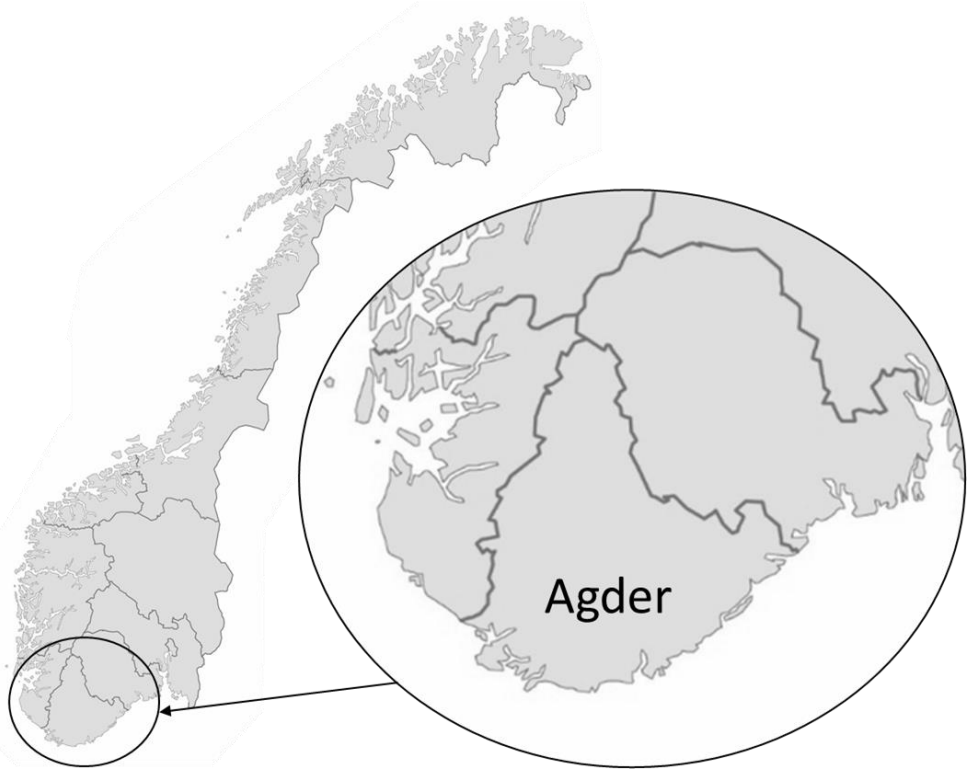


Figure 1. Strawberry plant material was collected from 20 fields in Agder, Norway in 2016. The map template is by ©Kartverket (geonorge.no), licensed under CC BY 4.0.

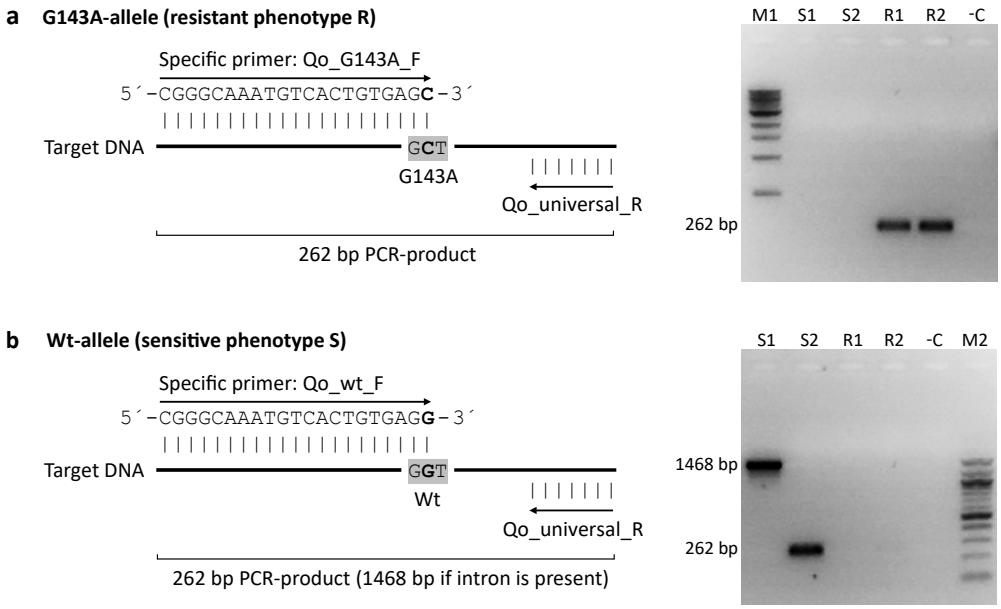


Figure 2. Schematic representation and demonstration of the allele-specific PCR strategy for detecting mutations in codon 143 of the cytb gene of Botrytis. (A) The 3' of primer Qo_G143A_F is designed to be specific for the mutant allele that leads to a G143A substitution, which is associated with resistance 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 wt 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.

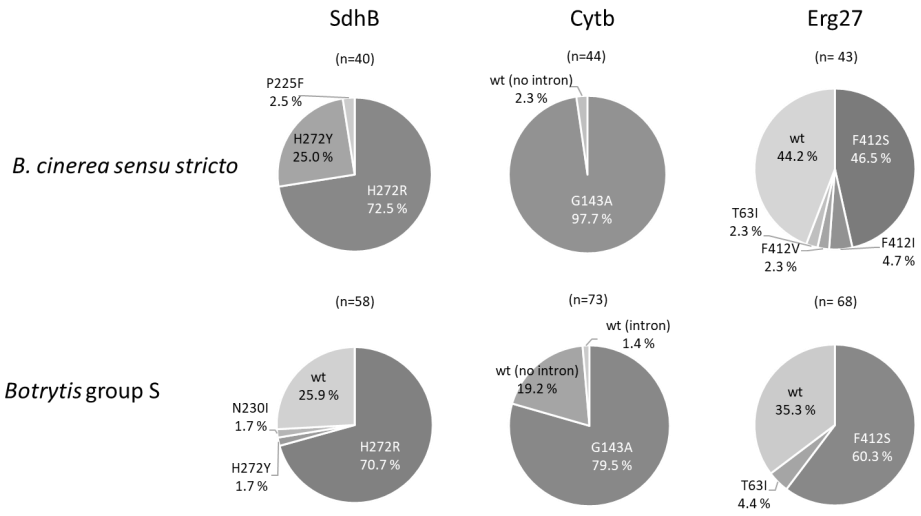


Figure 3. Proportions of substitutions that are known to confer resistance to boscalid, pyraclostrobin, and fenhexamid, in SdhB, Cytb, and Erg27, respectively, for isolates of Botrytis cinerea sensu stricto and Botrytis group S. Isolates lacking changes are designated as wild type (wt), and, in addition for Cytb, intron or no intron for detection of a specific 1205 bp intron known to preclude a mutation leading to G143A and resistance.

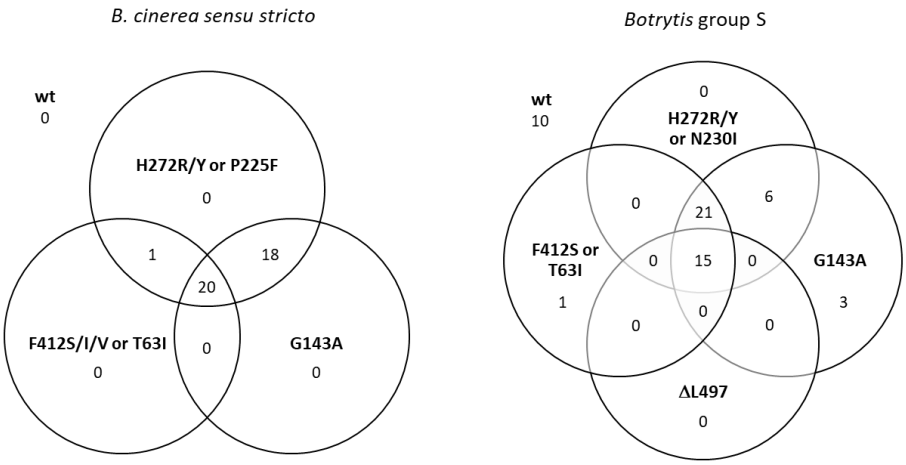


Figure 4. Accumulation of substitutions known to confer fungicide resistance in a selection of 39 B. cinerea sensu stricto and 56 Botrytis group S isolates. Substitutions in SdhB (H272R/Y, N230I, P225F), Cytb (G143A), Erg27 (F412S/I/V, T63I) and Mrr1 (L497 deletion) confer resistance to boscalid, pyraclostrobin, fenhexamid, and both fludioxonil and cyprodinil, respectively. Isolates for which these changes were not detected are designated as wt, wild type.

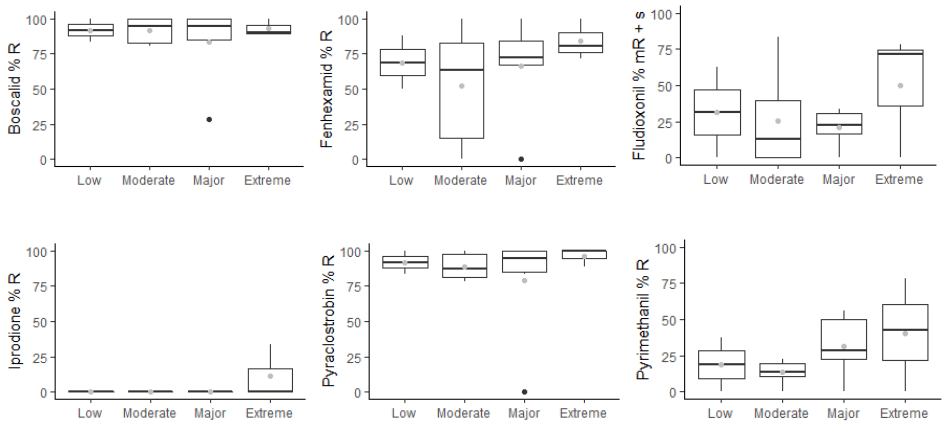


Figure 5. Fungicide resistance frequencies from Botrytis conidia samples from strawberry fields and the growers' perception of grey mould problem. Results from 17 fields (16 in the case of pyrimethanil) in Agder are grouped according to grower assessments of level of grey mould problem (low, moderate, 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 percent fully resistant conidia samples from the germination test except for fludioxonil, which is percent moderately resistant (mR) plus less sensitive (s).

Supporting Information Table 1. *Botrytis* reference sequences from GenBank® used in this study.

	Gene (accession number)					
Species	***nep2***	***g3pdh***	***mrr1***	***sdhB***	***cytb***	***erg27***
B. cinerea	DQ211825.1	KJ937075.1	JX266770.1	CP009805.1	KP795071.1	KP027846.1
B. cinerea		-	KF545941.1	-	KT318575.1	-
B. euroamericana	KX266752.1	KX266728.1	-	-	-	-
B. fabae	DQ211831.1	AJ705014.1	-	-	-	-
B. fragariae	KX429725.1	KX429699.1	-	-	-	-
B. paeoniae	AM087064.1	KY200511.1	-	-	-	-
B. prunorum	KR425424.1	KX196312.1	-	-	-	-
B. pseudocinerea	MK211256.1	JN692414.1	-	-	-	-
B. caroliniana	-	JF811584.1	-	-	-	-
B. mali	-	EF367128.1	-	-	-	-
B. californica	-	KJ937073.1	-	-	-	-
B. ricini	-	GQ860998.1	-	-	-	-

Supporting Information Table 2. Molecular results for *Botrytis* isolates included in this study, including accession numbers for sequences that have been submitted to GenBank®.

								Gene (accession numbers)		
Isolate[a]	Species/group[b]	Cytb	Intron[c]	Erg27	Mrr1	SdhB	Mating type	*nep2*	*g3pdh*	*mrr1*
1.1	S	G143A	N	F412S	ΔL497	H272R	MAT1-2	OK556246	OK637159	
1.2	Bc	G143A	N	F412S	NA	H272R	MAT1-2	OK556207		
1.3	S	G143A	N	F412S	wt	NA	NA	OK556247	OK637160	
1.4	NA	G143A	N	F412S	NA	H272R	MAT1-2	OK556317	OK637153	
1.5	NA	G143A	N	F412S	ΔL497	NA	NA		OK637154	
1.8	S	G143A	N	F412S	ΔL497	H272R	MAT1-2	OK556248	OK637161	
1.9	S	G143A	N	F412S	ΔL497	H272R	MAT1-1	OK556249	OK637162	
2.1	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556289	OK637198	
2.2	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556291	OK637200	
2.4	S	G143A	N	NA	wt	wt	NA	OK556292		
2.8	Bc	G143A	N	F412V	NA	H272Y	MAT1-1	OK556226	OK637132	
2.9	Bc	G143A	N	F412S	NA	H272R	MAT1-1	OK556227	OK637133	
2.10	S	wt	N	wt	wt	wt	MAT1-2	OK556290	OK637199	
4.2	S	wt	N	wt	wt	NA	MAT1-1/1-2[d]	OK556294		
4.8	Bc	G143A	N	wt	NA	H272R	MAT1-1	OK556233	OK637139	
5.1	S	G143A	N	NA	wt	NA	MAT1-1		OK637203	
5.2	Bp	wt	Y	wt	wt	wt	MAT1-1	OK556316	OK637224	OK626265
5.3	Bc	G143A	N	wt	NA	H272R	MAT1-1	OK556234	OK637140	
5.4	S	wt	N	wt	wt	wt	MAT1-1	OK556295	OK637204	
5.6	S	wt	N	wt	wt	wt	MAT1-1	OK556296	OK637205	
5.7	NA	G143A	N	F412S	ΔL497	NA	NA	OK556320	OK637158	
5.8	S	wt	N	wt	wt	wt	MAT1-1	OK556297	OK637206	
5.9	S	G143A	N	NA	ΔL497	NA	NA	OK556298		
6.3	S	wt	N	wt	wt	NA	MAT1-2	OK556300	OK637208	
6.6	S	wt	N	wt	wt	wt	MAT1-2	OK556301	OK637209	
6.8	S	wt	N	wt	wt	wt	MAT1-1	OK556302	OK637210	
6.9	S	wt	N	wt	wt	wt	MAT1-1	OK556303	OK637211	
6.10	S	wt	N	wt	wt	wt	MAT1-2	OK556299	OK637207	
7.1	S	G143A	N	wt	wt	H272R	MAT1-2	OK556304	OK637212	
7.3	S	G143A	N	wt	wt	H272R	MAT1-2	OK556306	OK637214	
7.4	NA	G143A	N	NA	ΔL497	NA	NA			
7.5	Bc	G143A	N	wt	NA	H272R	MAT1-1	OK556235	OK637141	
7.6	Bc	G143A	N	T63I	NA	H272R	MAT1-2		OK637142	
7.8	Bc	G143A	N	wt	NA	H272R	MAT1-2	OK556236	OK637143	
7.9	Bc	G143A	N	wt	NA	H272R	MAT1-1	OK556237	OK637144	
7.10	S	wt	Y	wt	wt	wt	MAT1-1	OK556305	OK637213	
8.2	Bc	G143A	N	wt	NA	H272R	MAT1-1	OK556238	OK637146	
8.3	Bc	G143A	N	F412S	NA	H272Y	MAT1-2	OK556239	OK637147	
8.5	Bc	G143A	N	F412S	NA	NA	MAT1-2	OK556240	OK637148	
8.6	Bc	G143A	N	wt	NA	H272R	MAT1-2	OK556241	OK637149	
8.7	Bc	G143A	N	F412S	NA	NA	MAT1-2	OK556242	OK637150	
8.9	S	wt	N	wt	wt	NA	MAT1-1	OK556307	OK637215	
8.10	Bc	G143A	N	F412S	NA	NA	MAT1-2		OK637145	
9.2	Bc	G143A	N	F412S	NA	H272Y	MAT1-2	OK556243	OK637151	
9.3	Bc	G143A	N	wt	NA	H272R	MAT1-1	OK556244	OK637152	
9.5	S	G143A	N	T63I	wt	NA	MAT1-2	OK556309	OK637217	
9.6	S	G143A	N	F412S	wt	NA	MAT1-1	OK556310	OK637218	
9.7	S	G143A	N	wt	wt	wt	MAT1-2	OK556311	OK637219	

9.9	S	wt	N	F412S	ΔL497	NA	MAT1-1	OK556312	OK637220	
9.10	S	G143A	N	F412S	ΔL497	H272R	MAT1-1	OK556308	OK637216	
10.3	NA	G143A	N	NA	NA	H272R	NA	OK556318	OK637155	
10.4	S	G143A	N	F412S	wt	NA	MAT1-2	OK556250		
10.5	NA	G143A	N	F412S	ΔL497	H272R	MAT1-2	OK556319		
10.8	A	G143A	N	wt	wt	H272R	MAT1-2	OK556245	OK637156	OK626263
10.9	Bc	G143A	N	wt	NA	H272R	MAT1-2	OK556208	OK637118	
10.10	S	G143A	N	F412S	ΔL497	H272R	MAT1-2		OK637163	
11.1	S	G143A	N	F412S	wt	H272R	MAT1-2	OK556251	OK637164	
11.3	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556252	OK637165	
11.4	S	G143A	N	F412S	ΔL497	H272R	MAT1-2	OK556253	OK637166	
11.5	S	G143A	N	F412S	ΔL497	H272R	MAT1-2	OK556254	OK637167	
11.6	S	G143A	N	F412S	wt	H272R	MAT1-2	OK556255	OK637168	
11.7	S	G143A	N	F412S	wt	N230I	MAT1-1	OK556256	OK637169	
11.8	S	G143A	N	F412S	ΔL497	H272R	MAT1-1	OK556257		
11.9	Bc	G143A	N	F412I	NA	H272R	MAT1-1	OK556209	OK637119	
12.1	S	G143A	N	F412S	ΔL497	H272R	MAT1-2	OK556258		
12.3	S	wt	N	F412S	wt	wt	MAT1-2	OK556259		
12.4	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556260		
12.6	X	wt	Y	wt	wt	wt	MAT1-1	OK556313	OK637221	OK626266
12.7	S	G143A	N	F412S	wt	H272R	MAT1-2	OK556261	OK637170	
13.1	S	wt	N	wt	wt	wt	MAT1-2	OK556262	OK637171	
13.2	Bc	G143A	N	wt	NA	H272R	MAT1-2	OK556210	OK637120	
13.3	S	G143A	N	wt	wt	H272R	MAT1-2	OK556263		
13.5	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556264	OK637172	
13.6	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556265	OK637173	
13.7	Bc	G143A	N	F412S	NA	H272R	MAT1-1	OK556211	OK637187	
13.8	Bp	wt	N	wt	wt	wt	MAT1-1	OK556314	OK637222	OK626262
13.10	S	G143A	N	F412S	wt	H272R	MAT1-1			
14.1	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556266	OK637174	
14.2	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556267	OK637175	
14.4	S	G143A	N	T63I	wt	H272R	MAT1-2	OK556268	OK637176	
14.5	Bc	G143A	N	F412S	NA	H272Y	MAT1-1	OK556212	OK637121	
14.6	S	G143A	N	wt	ΔL497	NA	MAT1-1	OK556269		
14.7	Bc	G143A	N	F412I	NA	H272R	MAT1-1	OK556213	OK637122	
14.8	Bc	G143A	N	F412S	NA	H272R	MAT1-1		OK637157	
14.9	S	G143A	N	F412S	wt	H272R	MAT1-2	OK556270	OK637177	
15.1	Bc	G143A	N	wt	NA	H272R	MAT1-2	OK556214		
15.3	Bc	G143A	N	wt	NA	H272R	MAT1-2	OK556216	OK637178	
15.4	S	G143A	N	wt	wt	wt	MAT1-1	OK556271	OK637179	
15.5	Bc	wt	N	F412S	NA	H272Y	MAT1-2	OK556217	OK637180	
15.6	S	G143A	N	wt	wt	wt	MAT1-1	OK556272	OK637181	
15.7	S	G143A	N	wt	wt	H272R	MAT1-1	OK556273	OK637182	
15.8	Bc	G143A	N	wt	NA	H272Y	MAT1-2	OK556218	OK637124	
15.9	S	G143A	N	NA	ΔL497	NA	MAT1-1	OK556274	OK637183	
15.10	Bc	G143A	N	F412S	NA	H272Y	MAT1-2	OK556215	OK637123	
16.1	Bc	G143A	N	NA	NA	H272R	MAT1-2	OK556219	OK637125	
16.3	S	G143A	-	F412S	ΔL497	NA	MAT1-1	OK556275	OK637184	
16.4	S	G143A	N	F412S	ΔL497	H272R	MAT1-1	OK556276	OK637185	
16.5	S	G143A	N	wt	wt	H272R	MAT1-2	OK556277	OK637186	
16.6	Bc	G143A	N	F412S	wt	P225F	MAT1-2	OK556220	OK637126	
16.8	S	G143A	N	F412S	ΔL497	H272R	MAT1-1	OK556278	OK637188	
16.9	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556279	OK637189	

17.1	S	G143A	N	F412S	wt	H272R	MAT1-2	OK556280	OK637190	
17.2	S	G143A	N	F412S	ΔL497	NA	MAT1-2	OK556281		
17.3	S	G143A	N	F412S	wt	H272R	MAT1-2	OK556282	OK637191	
18.1	Bc	G143A	N	F412S	NA	H272R	MAT1-1			
18.2	S	G143A	N	F412S	wt	H272Y	MAT1-1	OK556284	OK637193	
18.3	S	G143A	N	T63I	ΔL497	H272R	MAT1-2	OK556285	OK637194	
18.4	Bc	G143A	N	F412S	wt	H272Y	MAT1-2	OK556221	OK637127	
18.5	Bp	wt	Y	wt	wt	wt	MAT1-2	OK556315	OK637223	OK626264
18.6	S	G143A	N	F412S	wt	H272R	MAT1-1	OK556286		
18.7	S	G143A	N	F412S	ΔL497	H272R	MAT1-2		OK637195	
18.8	NA	G143A	N	NA	NA	NA	NA			
18.9	Bc	G143A	N	F412S	NA	H272R	MAT1-2		OK637128	
18.10	S	G143A	N	F412S	wt	H272R	MAT1-1/1-2	OK556283	OK637192	
19.1	S	G143A	N	F412S	ΔL497	H272R	MAT1-1	OK556287	OK637196	
19.2	Bc	G143A	N	wt	NA	H272R	MAT1-2		OK637129	
19.4	Bc	G143A	N	F412S	NA	H272Y	MAT1-2	OK556222		
19.5	S	G143A	N	NA	wt	NA	NA			
19.7	Bc	G143A	N	F412S	NA	H272Y	MAT1-2	OK556223	OK637130	
19.8	Bc	G143A	N	F412S	NA	H272R	MAT1-1	OK556224		
19.9	Bc	G143A	N	F412S	NA	H272R	MAT1-1	OK556225	OK637131	
19.10	S	G143A	N	F412S	ΔL497	H272R	MAT1-1	OK556288	OK637197	
20.1	Bc	G143A	N	wt	NA	H272R	MAT1-1	OK556228	OK637134	
20.4	Bc	G143A	N	wt	NA	H272R	MAT1-2	OK556229	OK637135	
20.5	NA	G143A	N	wt	wt	NA	NA			
20.6	Bc	G143A	N	wt	NA	H272R	MAT1-2	OK556230	OK637136	
20.7	Bc	G143A	N	wt	NA	NA	MAT1-2	OK556231	OK637137	
20.8	S	G143A	N	wt	wt	H272R	MAT1-1	OK556293	OK637202	
20.9	Bc	G143A	N	wt	wt	H272R	NA	OK556232	OK637138	
20.10	S	G143A	N	wt	NA	H272R	MAT1-1		OK637201	

[a] Single-spore *Botrytis* isolates with the prefix 96/16-

[b] A = Botrytis A, a unique isolate from this study; Bc = *B. cinerea sensu stricto*; Bp = *B. pseudocinerea*; S = *Botrytis* group S; X = *Botrytis* group X

[c] N = 1205 bp intron following codon 143 in *cytb* not detected; Y = yes, 1205 bp intron detected

[d] Both mating types detected for this isolate, but weak PCR product

Supporting Information Table 3. Results of statistical analysis in R version 4.1.0 to test for differences in expected fungicide resistance frequencies among problem level groups (low, moderate, major, extreme).

	Boscalid	Fenhexamid	Fludioxonil	Iprodione	Pyraclostrobin	Pyrimethanil
One-way ANOVA[a]						
P-value	0.848	0.662	0.608	0.200	0.771	0.360
F-value	0.268	0.542	0.632	1.784	0.377	1.175
Degrees of freedom	(3, 13)	(3, 13)	(3, 13)	(3, 13)	(3, 13)	(3, 12)
Levene's p-value[b]	0.661	0.591	0.467	0.200	0.657	0.182
Shapiro-Wilk normality p-value[c]	< 0.001	0.156	0.907	< 0.001	< 0.001	0.989
Durbin-Watson p-value[d]	0.126	0.198	0.184	0.128	0.468	0.074
Kruskal-Wallis H[a, e]						
P-value	0.990			0.198	0.729	

[a] Fungicide resistance frequency data used for the analysis was from the spore germination test of *Botrytis* conidia samples collected from strawberry fields in Agder, Norway in 2016. Data included was percent resistant (R) samples for all fungicides except fludioxonil, which was percent moderately resistant (mR) plus less sensitive (s).

[b] Check for homogeneity of variance of residuals

[c] Check for normality of residuals

[d] Check for independence of residuals

[e] Included in cases where p-values were statistically significant for the Shapiro-Wilk test, signifying that assumptions for ANOVA were not met.

Supporting Information Table 4. Fungicide resistance in *Botrytis* conidia samples from strawberry fields in Agder, Norway in 2016, based on a spore germination test[a].

		Boscalid	Fenhexamid	Fludioxonil		Iprodione	Pyraclostrobin	Pyrimethanil				
Field	n[b]	% R	% R	% mR	% s	% R	% R	% R	Problem[h]	Origin[i]	Planted	System
1	9	100	100	0.0	44.4	0.0	100	11.1	Moderate	N	2013	Matted-row
2	6	83.3	66.7	0.0	33.3	0.0	83.3	50.0	Major	N	2015	Plasticulture
4	6	83.3	50.0	0.0	0.0	0.0	83.3	0.0	Low	N	2016	Plasticulture
5	8[c]	75.0	28.6	0.0	87.5	33.3	50.0	0.0	NA[k]	N	NA	Matted-row
6	7[d]	28.6	0.0	0.0	0.0	0.0	0.0	0.0	Major	N	NA	Matted-row
7	10	80.0	0.0	0.0	0.0	0.0	90.0	10.0	Moderate	I	2016	Matted-row
8	8[e]	100	87.5	0.0	25.0	0.0	100	20.0	Moderate	N	2015	Matted-row
9	7[f]	100	71.4	0.0	71.4	0.0	100	42.9	Extreme	N	2014	Matted-row
10	6	100	66.7	33.3	50.0	0.0	83.3	16.7	Moderate	I	2016	Plasticulture
11	9	88.9	100	11.1	66.7	33.3	88.9	77.8	Extreme	N	NA	Matted-row
12	10	90.0	80.0	0.0	0.0	0.0	100	0.0	Extreme	N	2015	Matted-row
13	10[g]	80.0	60.0	0.0	0.0	0.0	80.0	22.2	Moderate	N	2015	Plasticulture
14	9	100	100	0.0	22.2	0.0	100	0.0	Major	N	2014	Plasticulture
15	9	100	66.7	0.0	22.2	0.0	100	22.2	Major	N	NA	Matted-row
16[j]	8	100	87.5	0.0	62.5	0.0	100	37.5	Low	I	2016	Plasticulture
17	7	100	85.7	0.0	14.3	0.0	100	28.6	Major	N	NA	Matted-row
18	10	100	100	0.0	20.0	0.0	90.0	50.0	NA	N	2014	Plasticulture
19	9	88.9	77.8	0.0	33.3	0.0	88.9	55.6	Major	I	2015	Plasticulture
20	9	88.9	0.0	0.0	0.0	0.0	77.8	0.0	Moderate	N	NA	Plasticulture

[a] Resistance categories for spore germination test: R (fully resistant), mR (moderately resistant), s (less sensitive), ss (highly sensitive), according to Weber and Hahn (2011)

[b] Number of conidia samples tested

[c] fenhexamid n=7, iprodione n=3

[d] pyrimethanil n=2

[e] pyrimethanil n=5

[f] boscalid n=6

[g] pyrimethanil n=9

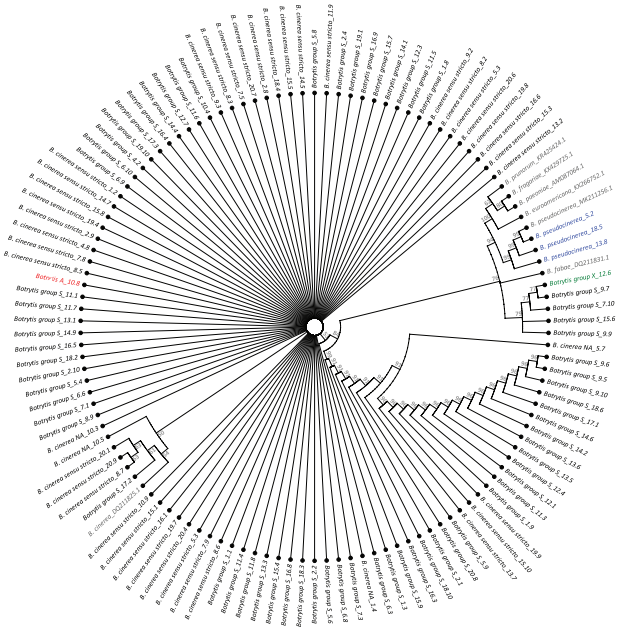
[h] Problem indicates grower assessment of grey mould problem level in the field for the 2016 season.

[i] Origin refers to where strawberry transplants used to establish the field were produced: “N” for Norway or “I” for imported.

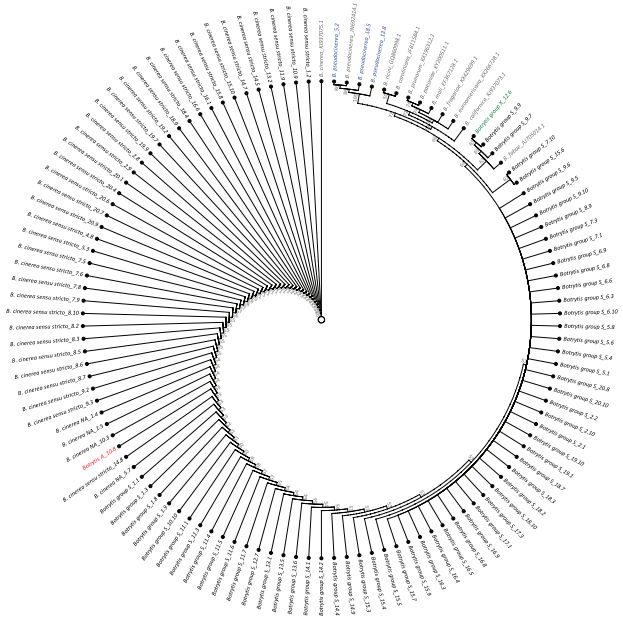
[j] Production in high tunnel

[k] NA for data not available

Necrosis and ethylene inducing peptide (*nep2*)



Glyceraldehyde 3-phosphate dehydrogenase *(g3pdh)*



Supporting Information Figure 1. Neighbour-joining phylogenetic trees of *nep2* and *g3pdh* sequences. The trees show the relationship between *Botrytis* species isolated from strawberry in Norway and other known species (see Supporting Information Table 1). Bootstrap percentages (n=1000) are indicated on the branches, and only branches with > 50 % bootstrap support are shown. Isolates identified as *Botrytis* group X, *Botrytis* A and *B. pseudocinerea* are green, red, and blue, respectively, whereas species used as references are grey.

Fungicide-resistant *Botrytis* in forest nurseries may impact disease control in Norway spruce

Katherine Ann Gredvig Nielsen, Magne Nordang Skårn, Venche Talgø, Martin Pettersson, Inger Sundheim Fløistad, Gunn Mari Strømeng, May Bente Brurberg, Arne Stensvand

Paper II

Fungicide-resistant *Botrytis* in Forest Nurseries May Impact Disease Control in Norway Spruce

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Abstract

Grey mould, caused by *Botrytis* spp., is a serious problem in Norway spruce seedling production in forest nurseries. From 2013 to 2019, 125 isolates of *Botrytis* were obtained from eight forest nurseries: 53 from Norway spruce seedlings, 16 from indoor air, 52 from indoor surfaces, and four from weeds growing close to seedlings. A mycelial growth assay was used to test for fungicide resistance, and resistance was detected to boscalid (8.8%), fenhexamid (33.6%), fludioxonil (17.6%), pyraclostrobin (36.0%), pyrimethanil (13.6%), and thiophanate-methyl (50.4%). Many isolates (38.4 %) were resistant to two to six different fungicides. A selection of isolates was analysed for the presence of known resistance-conferring mutations in the *cytb*, *erg27*, *mrr1*, *sdhB*, and *tubA* genes, and mutations leading to G143A, F412S, ΔL497, H272R, and E198A/F200Y were detected, respectively. *Botrytis pseudocinerea* was identified on Norway spruce seedlings for the first time, and *B. prunorum* was recovered from the air in one of the forest nurseries, which is its first documented presence in northern Europe. Detection of fungicide resistance in *Botrytis* from Norway spruce and forest nursery facilities reinforces the necessity of employing resistance management strategies to improve control and delay development of fungicide resistance in the grey mould pathogens.

Fungal disease management is a major challenge in forest nurseries in Norway, and grey mould caused by *Botrytis* spp. is considered an especially damaging disease in Norway spruce (*Picea abies*) seedling production. Seedlings that have been damaged or weakened by stress are considered more susceptible to infection (Lilja et al., 2010). The major high risk period for grey mould to develop is during cold storage, where *Botrytis* can enter storage as mycelium and conidia where it then thrives and spreads at low temperatures (Lilja et al., 2010; Petäistö, 2006; Unestam & Beyer-Ericson, 1980). Seedlings are also more prone to fungal disease during short-day treatment due to extra stress on the plants combined with humid conditions conducive for fungal infection (Lilja et al., 2010).

Grey mould management strategies in forest nurseries in Norway have traditionally included regular fungicide applications from June to September. Single-site fungicides currently approved for grey mould control in forest nurseries in Norway include fenhexamid (hydroxyanilide or HA, FRAC 17), fluopyram (succinate dehydrogenase inhibitor or SDHI, FRAC 7) and a product containing both cyprodinil (anilinopyrimidine or AP, FRAC 9) and fludioxonil (phenylpyrrole or PP, FRAC 12). Thiophanate-methyl (methyl benzimidazole carbamate or MBC, FRAC 1) and other benzimidazoles have been in use for at least 40 years, but approval for the last available MBC product expired in 2021. The multisite fungicide cuprous oxide (FRAC M 01) is also approved for grey mould control in forest nurseries in Norway. Resistance mechanisms for several fungicides have been studied in *Botrytis*, and common target-site mutations are therefore known for several of the fungicides used for grey mould control in forest nurseries. Mutations in the genes encoding cytochrome b (*cytb*), 3-ketoreductase (*erg27*), succinate dehydrogenase subunit B (*sdhB*), and beta-tubulin (*tubA*) can confer resistance to QoI fungicides, fenhexamid, fluopyram, and

thiophanate-methyl, respectively (Banno et al., 2008; Banno et al., 2009; Fernández-Ortuño et al., 2015; Fillinger et al., 2008; Grabke et al., 2013; Lalève et al., 2014; Veloukas et al., 2013). Mutations in the gene encoding multidrug resistance regulator 1 (*mrr1*) have been associated with multidrug resistance to AP and PP fungicides based on an efflux mechanism that gives partial resistance at a level that can be relevant for grey mould control (Fernández-Ortuño et al., 2014; Kretschmer et al., 2009; Leroch et al., 2013; Leroux and Walker, 2013; Li et al., 2014).

Botrytis cinerea, one of the causal agents of grey mould, is able to develop resistance to single-site fungicides with different modes of action in several hosts (Hahn, 2014; Fernández-Ortuño et al., 2015; Fillinger & Walker, 2016), and is therefore classified as a high-risk pathogen (FRAC, 2019). A polycyclic life cycle, production of large amounts of conidia, and a high degree of genetic variation are biological factors that contribute to the ability of *B. cinerea* to develop resistance to fungicides faster than many other fungal pathogens (Leroux et al., 2002; Veloukas et al., 2014).

DNA-based identification methods have contributed to the concept of a *Botrytis* species complex (Garfinkel, 2021; Taylor et al., 2000; Walker, 2016). Species composition of the *Botrytis* population is relevant for grey mould disease control because fungicide resistance varies between species (Hu et al., 2019; Notsu et al., 2021; Plesken et al., 2015; Walker et al., 2011). *Botrytis pseudocinerea*, for example, has natural fenhexamid resistance attributed to a detoxification mechanism (Debieu & Leroux, 2015). *Botrytis pseudocinerea*, originally identified in French vineyards by Walker et al. (2011), is a cryptic species that has since been detected in various hosts around the world (Azevedo et al., 2020; Fekete et al., 2012; Johnston et al., 2014; Li et al., 2015; Muñoz et al., 2016; Plesken et al., 2015; Saito et al., 2014; Wessels et al., 2013). Coexistence within the *Botrytis* species complex is possible; Hu et al. (2018) even found examples of two

Botrytis species in the same strawberry flower. *Botrytis prunorum*, one of several relatively new species, has been demonstrated to be polyphagous and capable of living in sympatry with *B. cinerea* (Acosta Morel et al., 2018; Elfar et al., 2017; Esterio et al., 2020; Ferrada et al., 2016; Ferrada et al., 2020). In a review about threats in reforestation, both *B. cinerea* and *B. allii* were mentioned, but *B. cinerea* was considered to be most problematic (Mittal et al., 1987). *Botrytis cinerea* has been referred to as the causal agent of grey mould in Norway spruce in several studies (Lilja et al., 2010; Petäistö et al., 2004; Unestam & Beyer-Ericson, 1980), but in light of recent understanding of the species complex, additional *Botrytis* species might be important in this host.

Resistance to benomyl (MBC, FRAC 1) was detected in forest nurseries as early as the 1970's in the western United States, and to the degree that alternatives were sought after (Cooley, 1980; Gillman & James, 1978; McCain and Smith, 1978). The challenge posed by fungicide resistance in the pathogen also featured in Mittal et al.'s (1987) review covering the threat of *Botrytis* to reforestation. Several of these publications clearly implicate extensive use of fungicides as contributing factors to resistance and grey mould control failure in forest nurseries.

In recent years, forest nurseries in Norway have experienced losses due to grey mould in Norway spruce despite using fungicides for control. In a preliminary study, resistance to some of the fungicides used in forest nurseries was found to be high in *Botrytis* from strawberry in Norway (Strømeng et al., 2018). Further investigation of the resistance situation in *Botrytis* in forest nurseries was deemed necessary to obtain knowledge about the *Botrytis* population. The aim of this study was to (i) characterize fungicide resistance in *Botrytis* from Norway spruce and forest nurseries using both *in vitro* and molecular methods, (ii) examine forest nursery facilities for the

presence of *Botrytis* inoculum, and (iii) identify the composition of the *Botrytis* species complex in Norway spruce.

Materials and Methods

Botrytis isolate collection

Asymptomatic or symptomatic Norway spruce seedlings were collected from production systems at eight forest nurseries (designated A through H) in Norway between 2013 and 2019. Most seedlings were two-year old, but some were one-year old. The seedlings were either symptomatic in the nursery or were asymptomatic and arbitrarily selected for incubation at high humidity until *Botrytis* developed. Conidia from *Botrytis* from infected spruce seedlings were transferred to water agar for germination. Single germinated conidia were excised using a dissecting microscope and scalpel and transferred to new agar plates to grow. A total of 53 single-spore *Botrytis* isolates were obtained from Norway spruce seedlings. Fungal tissue and/or conidia of single-spore isolates were stored in 20% glycerol at -20°C prior to testing for fungicide resistance and DNA extraction.

Sixty-eight *Botrytis* isolates were obtained from samples collected from the air and surfaces indoors in various areas at three nurseries (A, B, and C) in November 2018, January 2019, and June 2019, respectively. Samples were collected using pairs of agar plates. For the Nursery A, each pair of agar plates included one plate containing potato dextrose agar (PDA) (Difco^{TM} Potato dextrose agar) and one with acidified potato dextrose agar (APDA, PDA amended with 0.2% w/v tartaric acid to inhibit bacterial growth). For nurseries B and C, both agar plates in the pairs were APDA. For samples from the air, pairs of open 9 cm agar plates were placed on sheets of size A4

printer paper and allowed to stand open for three to four hours, except for one sample collected from a plate that stood open overnight. Open agar plates were placed in many locations throughout the nursery facilities, including greenhouses, cold storage, workshop, and on top of equipment in a packing room. For sampling from surfaces, a sterile cotton swab (Cotton Tipped Applicator, single tip, SELEFA®) was brushed over surfaces and transferred to agar plates by dilution streaking. Both abiotic and biotic surfaces were swabbed throughout the nurseries, e.g., watering and packing equipment, planting trays, washing machines, floors, and walls, in addition to seedlings in cold storage and greenhouses. Supplementary Table 1 describes sampling locations and surfaces for *Botrytis* isolates obtained from the air and surfaces.

After sample collection, the agar plates were placed in plastic bags in a cooler for transport back to the laboratory. The plates were incubated at ambient temperature, and potential *Botrytis* colonies were transferred to new APDA plates. Upon sporulation, conidia were transferred to water agar for germination and single-spore isolates made as described above and transferred to new APDA plates. Sporulating single-spore isolates were tested for fungicide resistance or stored until testing. Isolates were stored as conidia suspensions in 20% glycerol at -20°C.

Weeds in forest nurseries were collected from between rows of or right next to seedlings when Norway spruce seedlings were collected in 2016, and these yielded three single-spore *Botrytis* isolates: two from weeds of the genus *Epilobium* and one from the genus *Chenopodium*. A *Botrytis* isolate was also collected from a weed of genus *Epilobium* collected at nursery C in June 2019. Isolates were stored as described above.

Fungicides

Fungicides used for resistance testing in this study were product formulations of the following (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); pyraclostrobin (Comet® Pro, BASF; QoI); pyrimethanil (Scala®, BASF; AP); and thiophanate-methyl (Topsin ® WG, Nisso Chemical Europa GmbH; MBC).

Mycelial growth assay for testing fungicide resistance

A total of 125 single-spore isolates of *Botrytis* originating from Norway spruce and forest nursery facilities were tested for resistance to seven fungicides with a screening method adapted from Fernández-Ortuño et al. (2014). Briefly, stock solutions were made by dissolving fungicide products in liquid media. Molten agar growth media was amended with fungicide stock solutions to obtain discriminatory concentrations and pipetted into 24-well cell culture plates (Nunclon™ Delta Surface, Thermo Scientific), 1.5 ml amended medium per well (Table 1). Sterile toothpicks were used to transfer conidia from sporulating fungal cultures to the centre of the agar media in the wells. Following four days of incubation in darkness at 20°C, resistance category was assigned per isolate-fungicide combination by assessing the diameter of mycelial growth (%) in relation to well diameter (15 mm). Resistance categories for the mycelial growth assay included 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%). Isolates were tested once, except for a small selection that were re-tested when mycelial growth assay results did not correspond with detection of resistance-conferring mutations in the respective target genes. As we found MR to boscalid to be

associated with detection of the H272R substitution in SdhB, we include the MR category in results when presenting resistance for boscalid only.

DNA extraction and PCR

Mycelium, often sporulating, was scraped from agar plates containing single-spore *Botrytis* isolates and crushed using a mortar and pestle with liquid nitrogen. The DNeasy Plant Mini Kit (Qiagen) was used to isolate genomic DNA in accordance with the manufacturer's instructions. A total volume of 25 µl with one unit of Platinum Taq DNA polymerase (Invitrogen) was used for all PCR reactions. Supplementary Table 2 lists primers and PCR conditions. Sequencing of PCR products was conducted at Eurofins Genomics, using the same primers as for amplification. Sequence assembly and trimming was performed with CLC Main Workbench (Qiagen).

Molecular characterization of *Botrytis* isolates

For the identification of *Botrytis* group S isolates among those identified as *B. cinerea*, the primer pair Mrr1-spez-F/Mrr1-Pira (Leroch et al., 2013) was used to identify a characteristic 21 bp indel in the transcription factor encoding multidrug resistance regulator 1 (*mrr1*) gene. The PCR-product from Mrr1-spez-F/Mrr1-Pira was further digested with HpyH4V (Leroch et al., 2013) to detect a triplet deletion in *mrr1* that leads to ΔL497 and is associated with the MDR1h type of multidrug resistance. Mutations in the *sdhB* gene were detected by sequencing the PCR product of primers IpBcBeg and IpBcEnd2 (Leroux et al., 2010). Allele-specific PCR (AS-PCR) assays were used to identify the mutation in the cytochrome b gene (*cytb*) that leads to the G143A substitution and the 1205 bp intron that is found in some wild type (wt) alleles (K. A. G. Nielsen, unpublished data, Paper I, this thesis). Mutations in the *erg27* gene were characterized with multiplex AS-PCR

(Grabke et al., 2013) or by sequencing the PCR product generated with primers T63_F and F412_R. Mutations in the *tubA* were detected by sequencing the PCR product of primers TUB-F1 and TUB-R1 (Banno et al., 2008). Regions of the glyceraldehyde-3-phosphate dehydrogenase (*g3pdh*) and necrosis- and ethylene-inducing protein (*nep2*) genes were used for species identification and to generate phylogenetic trees. Alignments of consensus sequences with reference sequences from NCBI's nucleotide database (Supplementary Table 3) were used for characterization of mutations, species identification, and phylogenetic trees. For phylogenetic trees, sequences originating from the same gene were trimmed to equal length, and the neighbour-joining method and the Jukes Cantor substitution model were used with 1000 bootstrap replicates.

Pathogenicity test

Norway spruce seeds from the Norwegian seed orchard Hallen II (seed lot no. F19-019) were obtained from the Norwegian Forest Seed Center. Seeds were surface sterilized by submerging them in 70% ethanol for 10 seconds, followed by 0.5 % sodium hypochlorite for 90 seconds. Seeds were then left to dry on sterile filter paper in a laminar flow cabinet. A total of 245 seeds were placed in quintuplets on 9 cm APDA plates. The plates were incubated in transparent, sealed plastic bags in ambient conditions exposed to daylight. In some cases, fungal colonies appeared around seeds, presumably from internal or seed-borne infections, and these were removed. When the seeds had germinated and the seedlings were 1-3 cm tall (40 days after placing seeds on APDA), they were inoculated with four single-spore *Botrytis* isolates identified as *B. cinerea*, *Botrytis* group S, *B. prunorum*, and *B. pseudocinerea* (15/19-75B1, Pa7, 58/19-24A, and 15/19-66B1, respectively; Supplementary Table 4). Mycelial plugs (5 mm, 19-day-old plates) were transferred onto the surface of malt agar in glass test tubes (h-14 cm; d-2.5 cm) filled with malt

agar to approximately 2 cm h. A Norway spruce seedling was transferred to each tube, and the tubes were closed with a plastic cap (not airtight). Six tubes were used per isolate and there were 6 tubes as non-inoculated controls (sterile water agar plugs). The tubes were incubated in a growth room at 16°C and with a 16-hour light, 8-hour dark cycle. Results were assessed and the specimens were photographed 17 days post inoculation.

Results

Fungicide resistance

For all 125 isolates tested in the mycelial growth assay, 8.8%, 33.6%, 17.6%, 36.0%, 13.6%, and 50.4% were resistant to boscalid, fenhexamid, fludioxonil, pyraclostrobin, pyrimethanil, and thiophanate-methyl, respectively. For fluopyram, only LR to fluopyram was detected. For the 53 *Botrytis* isolates from Norway spruce seedlings, resistance to thiophanate-methyl was highest and pyraclostrobin second highest, to which isolates were R for approximately two thirds and one third, respectively (Table 2). Resistance frequencies for pyrimethanil, fludioxonil, and fenhexamid were approximately 5%, 10%, and 20%, respectively, while resistance to boscalid was not detected in these isolates from Norway spruce. Resistance frequencies for the 16 isolates originating from the air and 52 isolates from surfaces in forest nursery facilities varied among the three nurseries included in the investigation (Table 3). However, resistance to fenhexamid, pyraclostrobin, and thiophanate-methyl were most common, ranging from 30% to 40% resistant isolates. Of the four *Botrytis* isolates collected from weeds two were R to fenhexamid, fludioxonil, and thiophanate-methyl and MR to boscalid and pyrimethanil.

Analysis of multiple fungicide resistance frequencies revealed that isolates collected from the air and surfaces were resistant to up to six fungicides, and isolates from Norway spruce and weeds were resistant to up to five fungicides (Table 4). Detailed results from the mycelial growth assay are presented in Supplementary Table 4 combined with results from the molecular analysis of the isolates.

Mutations conferring resistance

The frequencies of substitutions in Erg27 and TubA conferring resistance to fenhexamid and thiophanate-methyl, respectively, were analysed for 121 *Botrytis* isolates (Table 5). All of the substitutions detected in Erg27 were F412S, and the majority in TubA were E198A. Discrepancies were found between genotype and phenotype for nearly 7 % of the isolates. Four isolates were of wild type (wt) in Erg27 but were characterized as MR or R to fenhexamid. One isolate had the F412S substitution in Erg27 but was only LR to fenhexamid. Two isolates were wt in TubA but were resistant to thiophanate-methyl, and one isolate had the E198A substitution but was sensitive to thiophanate-methyl.

A selection of 21 *B. cinerea* isolates from Norway spruce were tested for the mutation in the *cytb* gene that leads to the G143A substitution, and the intron known to preclude G143A. Six isolates had the G143A substitution, and all of these were R to pyraclostrobin in the mycelial growth assay. Of the 15 isolates that were wt in *cytb*, nine had the intron known to preclude G143A. Mycelial growth assay results for both isolates with and without the intron ranged from S to MR.

Nine isolates which were MR to boscalid were selected for analysis of the *sdhB* gene. These isolates originated from air, surface, or weeds, and the H272R substitution that confers resistance

to boscalid was detected in eight of the isolates. The ninth MR isolate, 58/19-64A, had wt *sdhB*, and was therefore tested again with the mycelial growth assay with the second result of R.

A total of 100 isolates, including 74 *Botrytis* group S isolates, were tested for the presence of ΔL497 in Mrr1 which is related to the MDR1h phenotype and associated resistance that includes resistance to fludioxonil, and it was detected in four *Botrytis* group S isolates. Of the four isolates in which ΔL497 was detected, two were from surfaces and two were from weeds of genus *Epilobium*. All four isolates with ΔL497 in Mrr1 were R to fludioxonil. Seventeen isolates which were R to fludioxonil did not have ΔL497.

Botrytis species in forest nurseries

A total of 121 *Botrytis* isolates were identified to species based on sequence data from *nep2* and *g3pdh*. Of those identified as *B. cinerea*, all but one was checked for the 21 bp indel in *mrr1* associated with *Botrytis* group S, and the majority of isolates were *Botrytis* group S (Table 6). Two isolates from Norway spruce were *B. pseudocinerea,* and two isolates from the air in a greenhouse were identified as *B. prunorum*. Two isolates from swabbed watering equipment in a greenhouse without seedlings under freezing conditions differed from the above species and are referred to as *Botrytis* sp. Based on alignments of gene sequences for *g3pdh* and *nep2*, these two isolates were found to be similar to isolates comprising the group AKBot3 described by Garfinkel et al. (2019). Isolate 15/19-49B1 had identical sequence alignments with AKBot3 isolates for both genes, and isolate 15/19-48B2 differed by only one bp for each of the genes. Phylogenetic trees constructed with sequences from *nep2* and *g3pdh* placed these isolates near the isolates identified as *B. prunorum* (Fig. 1). *Botrytis* group S isolates did not form distinct clades. Isolates identified as *B. pseudocinerea* from both Norway spruce and surfaces grouped together with the

corresponding reference isolate. There was no indication of isolates from Norway spruce, air, surfaces, or weeds forming distinct groups.

Pathogenicity of *Botrytis* spp. from forest nurseries

Necrotic plant tissue and sporulation was visible at 17 days post inoculation in six, six, four, and one Norway spruce seedling(s) of six inoculated with isolates of either *Botrytis* group S, *B. pseudocinerea, B. cinerea*, or *B. prunorum*, respectively. The *Botrytis* group S isolate caused profuse sporulation on all six seedlings and extended to the needles in three of them. Seedlings inoculated with *B. pseudocinerea* exhibited symptoms on all stems and on needles in four out of six seedlings. Four seedlings inoculated with the *B. cinerea* isolate developed necrotic tissue on the stems, and necrotic tissue was also visible on needles of one of these four seedlings. For the *B. prunorum* isolate, only one of the six seedlings was completely necrotic and covered with conidia (Fig. 2, D). The other five seedlings inoculated with *B. prunorum* were mostly green (Fig. 2, E), with the exception of one which had some mycelial growth at the base of the needles, and another where the dead seed coat stuck to the seedling's needles was covered in sporulating *Botrytis*. Control seedlings remained healthy and green.

Discussion

Fungicide resistance in *Botrytis* obtained from Norway spruce and forest nursery facilities was characterized using both *in vitro* and molecular methods. Although multiple studies have addressed fungicide resistance in conifers in North America (Cooley, 1980; Gillman & James, 1978; Gillman & James, 1980; James & Gilligan, 1985), this is the first characterization of fungicide resistance in the grey mould pathogen in Norway spruce. *Botrytis pseudocinerea* was identified

for the first time from Norway spruce, and *B. prunorum* was identified for the first time in northern Europe. We also recovered fungicide-resistant *Botrytis* from the air, surfaces, and weeds in the forest nurseries.

In most cases, detection of known mutations in the *erg27* and *tubA* genes corresponded to the results obtained from the mycelial growth assay for fenhexamid and thiophanate-methyl, respectively. The F412S substitution was detected in Erg27 in isolates resistant to fenhexamid, and resistance to thiophanate-methyl was associated with the E198A, or in one case, the F200Y substitution in TubA. These are some of the most common substitutions that have been detected in Erg27 (Amiri & Peres, 2014; Grabke et al., 2013), and TubA (Banno et al., 2008; Fernández-Ortuño et al., 2015). Resistance to thiophanate-methyl was high in this study's *Botrytis* isolates. This fungicide lost approval for use in Norway in autumn 2021. Nonetheless, we expect this resistance to remain in the *Botrytis* population, as tolerance to thiophanate-methyl does not seem to have a large fitness cost (Walker et al., 2013). Furthermore, resistance was recently detected in *Botrytis* from raspberry and strawberry in Norway (K. A. G. Nielsen et al., unpublished data), where use has been discontinued for more than three decades. There was one *B. cinerea* isolate for which E198A was detected but which was S to thiophanate-methyl. Although examination of the *tubA* sequence fragment for this isolate revealed no additional mutations, pathogen populations continue to evolve and it is possible that mutations outside of the fragment we amplified could have had an impact on resistance phenotype (Allen et al., 2017).

Resistance to fludioxonil detected in 17 of the *Botrytis* isolates in this study could not be explained by ΔL497 in Mrr1 that is associated with the MDR1h type of multidrug resistance. The presence of this deletion was detected by enzymatic digestion of a PCR product and not sequencing, so

there could have been other mutations in the *mrr1* gene that could not be detected by the test used in this study. Some studies indicate that fludioxonil resistance in *Botrytis* may be conferred through target-site resistance, although specific targets have not yet been identified (Leroch et al.; 2013, Kilani & Fillinger; 2016). Recently, isolates with high resistance to fludioxonil have been recovered from greenhouses, and some of these h Fludioxonil together with cyprodinil comprise one of the fungicide products approved for grey mould control in forest nurseries. Cyprodinil is an AP fungicide that has cross-resistance with the AP fungicide pyrimethanil (Hilber and Schüepp, 1996), so the pyrimethanil resistance detected in this study is relevant for the product containing cyprodinil with fludioxonil. In all, 8.8% of *Botrytis* isolates in this study were R to both fludioxonil and pyrimethanil.

Resistance screening with the mycelial growth assay revealed isolates that were R to the QoI pyraclostrobin, and the associated G143A substitution in CytB was detected when a selection of isolates was analysed. No QoI fungicides are approved for grey mould control in forest nurseries, but a product containing the QoI trifloxystrobin combined with prothioconazole (DMI, FRAC 3) has been used for control of the fungal pathogens *Gremmeniella abietina* and *Lophodermium* spp. since 2014. In addition, a different product containing the QoI trifloxystrobin combined with propiconazole (DMI, FRAC 3) has been registered for more than a decade but was phased out in 2020. There is cross-resistance among FRAC 11 QoI fungicides (FRAC, 2020), so use of a product containing trifloxystrobin for control of other pathogens has likely selected for resistance to QoIs in *Botrytis*.

Results from the mycelial growth assay also revealed several isolates that were MR to boscalid, an SDHI fungicide which has not been approved in forest nurseries. Nine of these isolates were

selected for analysis of *sdhB*, and eight of these had the mutation causing the H272R substitution that is associated with resistance to boscalid. The one wt isolate was a *B. pseudocinerea* isolate with multiple fungicide resistance (58/19-64A). There is incomplete cross-resistance among SDHI fungicides, but in some cases, substitutions in SdhB can confer resistance to both boscalid and fluopyram (Lalève et al., 2014). Fluopyram, the only SDHI fungicide that has been approved in forest nurseries in Norway, has been used since 2017. As the H272R substitution is known to confer resistance to boscalid while making isolates sensitive to fluopyram (Veloukas et al., 2013; Weber et al., 2015), we assume fluopyram has not selected for boscalid resistance detected in this study. The origin of the isolates with boscalid resistance is therefore a matter of speculation. *Botrytis* conidia are easily dispersed in the air (Jarvis, 1977), so it is possible that inoculum from other production systems in which boscalid is used, such as berry or fruit, could have travelled by wind and entered forest nursery greenhouses through vents (Sutherland et al., 1990). Alternatively, other organisms (James et al., 1995) or perhaps equipment and workers could have transported the pathogen into forest nurseries. Although *Botrytis* is not generally considered a seed-borne pathogen in conifers, it has been detected in seeds of *Abies* spp. (Talgø et al., 2010).

For five of eight *B. pseudocinerea* isolates in this study, resistance in the mycelial growth assay did not match the expected substitution in Erg27. Four of the isolates identified as *B. pseudocinerea* were R or MR to fenhexamid but were wt in Erg27. This may be explained by resistance to fenhexamid in *B. pseudocinerea* conferred through a detoxification mechanism (Debieu & Leroux, 2015). The fifth *B. pseudocinerea* isolate, however, had the F412S substitution associated with fenhexamid resistance but was LR to fenhexamid. Two of the above four *B. pseudocinerea* isolates were also wt in TubA but R to thiophanate-methyl. The *B. pseudocinerea*

isolate 58/19-64A was resistant to multiple fungicides despite being of wt in the commonly associated genes. Resistance in several of the *B. pseudocinerea* isolates in this study would not have been detected if we had relied solely on our molecular characterization of resistance. This highlights the importance of screening resistance phenotypes when monitoring fungicide resistance.

Prior to this study, no records of *B. pseudocinerea* in Norway spruce have been found. Two of the *B. pseudocinerea* isolates in this study, FG49 and FG50, were isolated from Norway spruce in 2017. One of the *B. pseudocinerea* isolates that was collected from the surface of a sowing machine in one of the nurseries was used in the inoculation test with Norway spruce seedlings *in vitro*. All six seedlings inoculated with this *B. pseudocinerea* isolate developed necrotic tissue from which conidia developed, indicating that this species is able to infect Norway spruce seedlings at this early stage of development. This confirms that grey mould in Norway spruce can be caused by a *Botrytis* species complex, and not only *B. cinerea*. Several *B. cinerea* isolates from Norway spruce in this study were identified as *Botrytis* group S. As in other studies (Leroch et al., 2013, Yin et al., 2016), *Botrytis* group S isolates in trees generated from *nep2* and *g3pdh* gene sequences did not form a separate phylogenetic group.

Two isolates collected from the air in a forest nursery greenhouse in mid-June where seeds had recently been sown were identified as *B. prunorum*. There is no previous record of *B. prunorum* being identified in northern Europe. *Botrytis prunorum* was first identified in Chile as a causal agent of blossom blight in Japanese plums (Ferrada et al., 2016), and it has also been identified in several other fruit crops in the country (Elfar et al., 2017; Esterio et al., 2020; Ferrada et al., 2016; Ferrada et al., 2020; Riquelme et al., 2021). Outside of Chile, *B. prunorum* has been identified in

vineyards in Spain (Acosta Morel et al., 2018) and alfalfa in Canada (Reich et al., 2017). This species is therefore characterized as polyphagous but so far found to be less virulent than *B. cinerea* (Garfinkel, 2021; Ferrada et al., 2016; Wang et al., 2018). As the *B. prunorum* isolates were from the air in a forest nursery, the role of this species as a pathogen in Norway spruce is uncertain. One of the isolates of *B. prunorum* was included in the inoculation test to determine whether it could cause grey mould symptoms, and one of the inoculated seedlings was completely necrotic and covered with conidia. The other five seedlings, however, were mostly green despite some mycelial growth on one seedling and *Botrytis* sporulation on a seed coat of another seedling. The seedlings inoculated with *B. pseudocinerea* and *B. cinerea* developed much more necrotic tissue in comparison, so if *B. prunorum* is part of the species complex in Norway spruce, it may play a minor role, as was found to be the case in plum in Chile (Ferrada et al., 2016). More research is needed to establish the role of *B. prunorum* as a pathogen, or possibly a saprotroph, in this host.

In this study, two isolates (15/19-48B2 and 15/19-49B1) identified as *Botrytis* sp. were obtained from samples collected from watering equipment in an empty greenhouse in January where the inside temperature was below freezing. Based on sequence analysis of the *g3pdh* and *nep2* genes, the two isolates in this study were nearly identical to three isolates from peony (*Paeonia lactiflora*) in Alaska that comprised a monophyletic group dubbed AKBot3 by Garfinkel et al. (2019). The authors observed that the three AKBot3 isolates had distinct stellate mycelial growth when grown on malt extract agar and incubated in the dark at 20°C for 7 days. We transferred the two *Botrytis* sp. isolates from this study to malt extract agar and subjected them to the same incubation conditions, but there was no stellate growth pattern (*data not shown*). Garfinkel et al. (2019) noted that more work remains to determine whether the isolates comprising AKBot3

and other unique clades they identified are in fact new species or potentially other recently described species, such as *B. prunorum*.

In this study, we detected resistance to fungicides constituting products available for grey mould control in *Botrytis* isolates obtained from the air, surfaces, and weeds in forest nursery facilities. *Botrytis* can be found everywhere (Bardin et al., 2018; Pady, 1951), and this study shows that fungicide-resistant *Botrytis* can also be found all over forest nurseries. *Botrytis cinerea* can infect many different plant species (Elad et al., 2016), so infected weeds in planting trays or in the vicinity of the seedlings in forest nurseries could function as reservoirs of *Botrytis* between production seasons. *Botrytis* can survive as mycelium and sclerotia in dead plant material and then produce conidia over several weeks (Carisse, 2016; Strømeng et al., 2009). Infected weeds or other dead plant material can also contribute to spread of disease in cold storage, where *Botrytis* can continue to grow even at 0°C (Lilja et al., 2010; Petäistö, 2006; Unestam and Beyer-Ericson, 1980). Limiting primary inoculum helps to prevent and suppress *Botrytis* epidemics (Carisse, 2016), and is also an important principle of integrated pest management (Barzman et al., 2015). Sanitation measures, including removing weeds, and disease monitoring, including removing infected plants and screening pathogens for resistance, can be incorporated in an integrated approach to achieve better disease control and reduce the need for chemical input (Shrimpton, 1992). Fungicide treatments that are deemed necessary should be timed to protect Norway spruce seedlings when they are most susceptible to *Botrytis* infection in the early part of the season (Petäistö et al., 2004). Timing and duration of short-day treatment before cold storage in the autumn can be manipulated to avoid a late summer bud flush that can make seedlings more susceptible to grey mould (Fløistad & Granhus, 2013). Rapid thawing of seedlings coming out of

cold storage can slow spread of grey mould between tightly packed seedlings (Petäistö, 2006) and improve seedling quality by conferring better frost tolerance and later bud break (Fløistad & Kohmann, 2001).

Our results indicate that *Botrytis* in Norway spruce has developed resistance to the products containing fenhexamid and cyprodinil with fludioxonil, which are currently permitted for grey mould control. In a screening program for resistance in *Botrytis* in strawberry the U. S., resistance frequencies over 20 % were considered as high risk for resistance development and those between 10 and 20 % were considered medium risk (Schnabel et al., 2015). This study clearly revealed risk-level resistance frequencies for all fungicides permitted for use against grey mould in forest nurseries, with the exception of fluopyram. Only isolates LR or S to fluopyram were detected in this study, but *Botrytis* isolates from strawberry that were R to fluopyram have been detected in Norway (K. A. G. Nielsen, unpublished data), so resistance in *Botrytis* in Norway spruce should be expected to develop with continued use of fluopyram. The prevalence of multiple fungicide resistance further complicates the situation. It behoves forest nurseries to employ fungicide resistance management strategies in conjunction with cultural control methods to delay loss of efficacy for the few remaining products for as long as possible.

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Table 1. Discriminatory fungicide concentrations and growth media for the mycelial growth assay[a] used to test *Botrytis* isolates for fungicide resistance.

Active ingredient	Concentration (mg/L)	Growth medium
Boscalid	75	Yeast Bacto acetate agar
Fenhexamid	50	Malt extract agar
Fludioxonil	0.5	Malt extract agar
Fluopyram	10	Yeast Bacto acetate agar
Pyraclostrobin + SHAM[b]	10 + 100	Malt extract agar
Pyrimethanil	4	Czapek-Dox agar
Thiophanate-methyl	100	Malt extract agar
Control	-	Czapek-Dox agar

[a] As described by Fernández-Ortuño et al. (2014)

[b] Salicylhydroxamic acid (SHAM) was dissolved in methanol and added for alternative oxidase inhibition.

Table 2. Frequencies of fungicide resistance in isolates of *Botrytis* from Norway spruce (*Picea abies*).

Resistance category[a]	Bos	Fen	Flud	Fluo	Pyra	Pyri	Thio
% R	0.0	18.9	9.4	0.0	32.1	3.8	67.9
% MR	0.0	1.9	1.9[b]	0.0	15.1	9.4[e]	0.0
% LR	9.4	0.0	1.9	3.8	43.4[c]	20.8	0.0
% S	90.6	79.2	86.8	96.2	9.4[d]	66.0	32.1[d]

[a] Fungicide resistance category determined by mycelial growth assay for boscalid (Bos), fenhexamid (Fen), fludioxonil (Flud), fluopyram (Fluo), pyraclostrobin (Pyra), pyrimethanil (Pyri), and thiophanate-methyl (Thio). Categories include resistant (R), moderately resistant (MR), low resistant (LR), and sensitive (S) as in Fernández-Ortuño et al. (2014).

[b] Includes one MR/R isolate which was re-tested and had a different result than the initial test.

[c] Includes one LR/MR isolate which was re-tested and had a different result than the initial test.

[d] Includes one S/LR isolate which was re-tested and had a different result than the initial test.

[e] Includes one LR/MR/R isolate which was re-tested and had a different result than the initial test.

Table 3. Frequencies of fungicide resistance in *Botrytis* isolates from the air and surfaces in three forest nurseries in Norway[a].

Nursery[b]	Total isolates	Bos MR+R	Fen R	Flud R	Fluo MR+R	Pyra R	Pyri R	Thio R
Air								
A	4	1	3	3	0	3	2	3
B	7	1	2	1	0	2	2	2
C	5	1	1	1	0	1	1	0
Total %	16	18.8 %	37.5 %	31.3 %	0.0 %	37.5 %	31.3 %	31.3 %
Surface								
A	2	1	1	1	0	1	1	1
B	15	0	1	1	0	2	0	3
C	35	5[c]	21	8	0	16	9[d]	15
Total %	52	11.5[c] %	44.2 %	19.2 %	0.0 %	36.5 %	21.2[d] %	36.5 %

[a] Fungicide resistance category determined by mycelial growth assay for boscalid (Bos), fenhexamid (Fen), fludioxonil (Flud), fluopyram (Fluo), pyraclostrobin (Pyra), pyrimethanil (Pyri), and thiophanate-methyl (Thio). Categories include resistant (R), moderately resistant (MR), low resistant (LR), and sensitive (S) as in Fernández-Ortuño et al. (2014).

[b] Visits to nurseries A, B, and C took place in November 2018, January 2019, and June 2019, respectively.

[c] Includes one MR/R isolate which was re-tested and had a different result than the initial test.

[d] Does not include one MR/R isolate which was re-tested and had a different result than the initial test.

Table 4. Frequencies of multiple fungicide resistance in *Botrytis* isolates from Norway spruce (*Picea abies*), air, surfaces, and weeds of forest nurseries.

		Multiple fungicide resistance [b]						
Origin	n[a]	% 0R	% 1R	% 2R	% 3R	% 4R	% 5R	% 6R
Norway spruce	53	28.3	35.8	20.8	9.4	1.9	3.8	0.0
Air	16	62.5	0.0	0.0	0.0	12.5	12.5	12.5
Surface	52	48.1	13.5	1.9	11.5	17.3	0.0	7.7
Weeds	4	25.0	0.0	0.0	25.0	0.0	50.0	0.0

[a] Number of isolates

[b] Fungicide resistance determined by mycelial growth assay for boscalid, fenhexamid, fludioxonil, fluopyram, pyraclostrobin, pyrimethanil, and thiophanate-methyl (Fernández-Ortuño et al., 2014). Multiple resistant phenotypes were determined by number of resistant (R) results per isolate (0R-6R), as no resistance was detected for fluopyram), except for boscalid where moderately resistant (MR) was also included.

Table 5. Frequencies of substitutions in Erg27 and TubA conferring resistance to fenhexamid and thiophanate-methyl, respectively, in *Botrytis* isolates from Norway spruce (*Picea abies*), air, surfaces, and weeds from forest nurseries in Norway.

	Erg27		TubA		
Origin	n[a]	% F412S	n[a]	% E198A	% F200Y
Norway spruce	53	17.0	52	67.3	0.0
Air	16	37.5	16	25.0	6.3
Surface	48	41.7	49	34.7	0.0
Weeds	4	75.0	4	75.0	0.0
Total	121	31.4	121	48.8	0.8

[a] Number of single-spore *Botrytis* isolates analyzed

Table 6. Species/group/clade of single-spore *Botrytis* isolates and their source in forest nurseries in Norway.

Species[a]	*P. abies*	Air	Surface	*Chenopodium*	*Epilobium*	Total
B. cinerea[b]	19	4	9	1	0	33
Botrytis group S[c]	30	10	32	0	3	75
B. pseudocinerea	2	0	6	0	0	8
B. prunorum	0	2	0	0	0	2
Botrytis sp.[d]	0	0	2	0	0	2

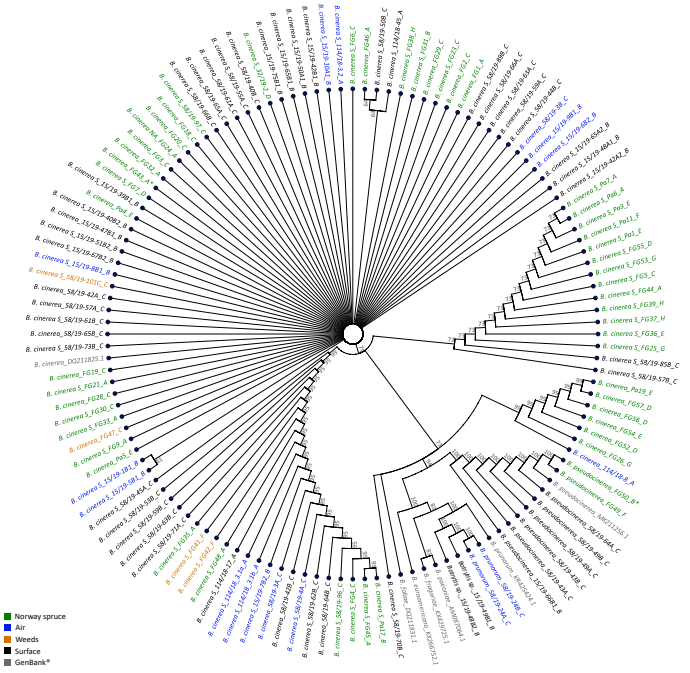
[a] Species identified using sequence data from glyceraldehyde-3-phosphate dehydrogenase (*g3pdh*) and necrosis- and ethylene-inducing protein (*nep2*) genes.

[b] Lacked 21 bp indel in *mrr1* gene characteristic of *Botrytis* group S (Leroch et al., 2013).

[c] Identified by detection of a characteristic 21 bp indel in the *mrr1* gene as in Leroch et al. (2013).

[d] Similar to a unique phylogenetic clade called AKBot3 described by Garfinkel et al. (2019).

Necrosis and ethylene inducing peptide (*nep2*)



Glyceraldehyde 3-phosphate dehydrogenase (*g3pdh*)

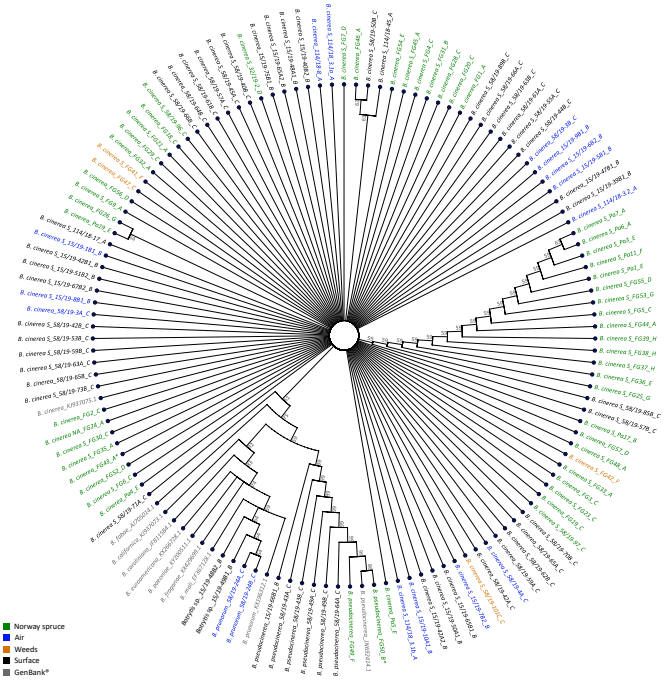


Figure 1. Phylogenetic trees of nep2 and g3pdh genes from *Botrytis* isolates obtained from Norway spruce forest nurseries. The trees were constructed using the neighbor-joining method. Branches with > 50% bootstrap are shown and labelled with bootstrap percentages (n = 1000), and reference sequences are listed in Supplementary Table 3. Letter codes at the end of the isolate name indicate forest nursery of origin. Two isolates marked with * were collected from Norway spruce that had been planted out but originated from the nursery indicated by the code.

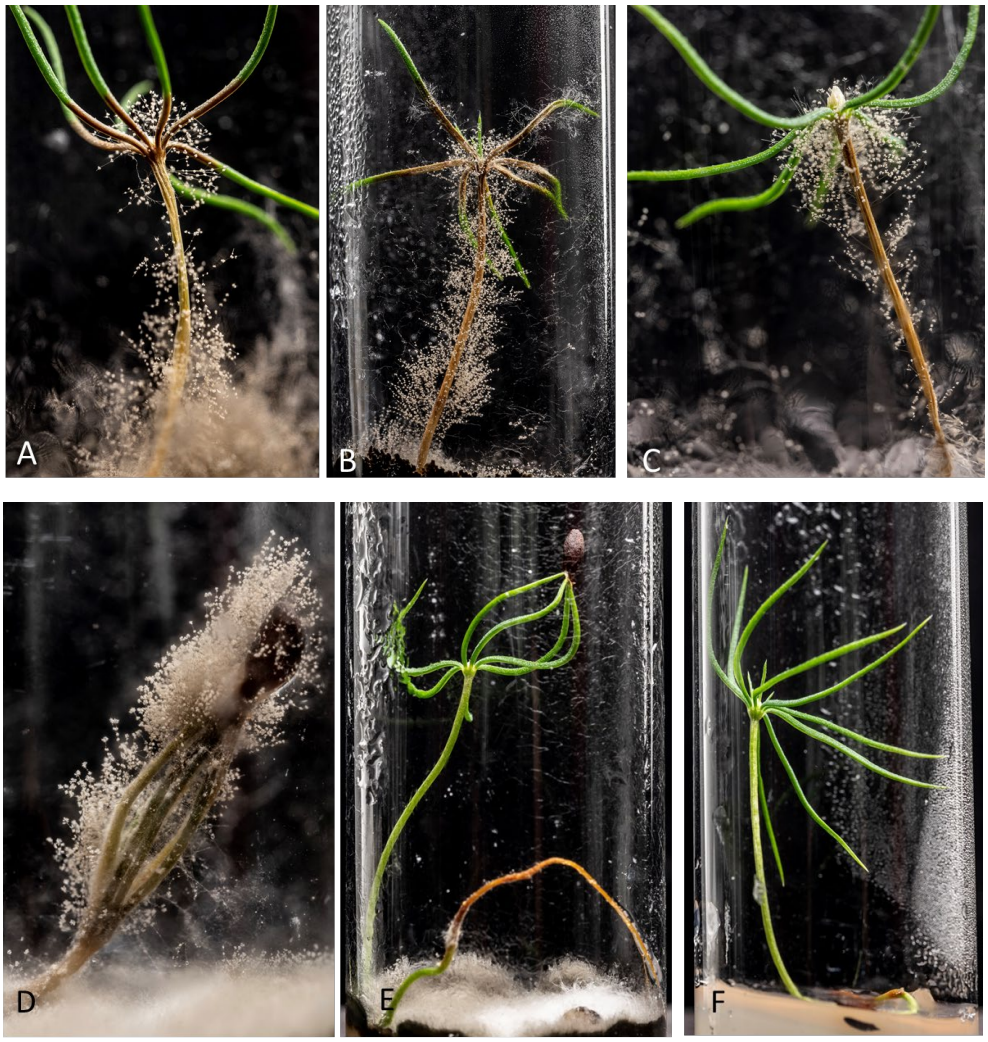


Figure 2. Norway spruce (*Picea abies*) seedlings inoculated with different *Botrytis* isolates: *B. cinerea* (A); *Botrytis* group S (B); *B. pseudocinerea* (C); *B. prunorum* (D, E), and control with no *Botrytis* (F). Photographs were taken 17 days post inoculation.

Supplementary Table 1. *Botrytis* isolates obtained from air and surface samples in three different Norway spruce forest nurseries.

Isolate[a]	Nursery[b]	Origin[c]	Sampling location of agar plates or surfaces swabbed	
114/18-8	A	Air	Greenhouse	
114/18-17	A	Surface	Packing room	Countertop
114/18-3.1a	A	Air	Greenhouse	
114/18-3.1b	A	Air	Greenhouse	
114/18-3.2	A	Air	Greenhouse	
114/18-45	A	Surface	Greenhouse	Tub with weeds in it
15/19-8B1	B	Air	Greenhouse	
15/19-9B1	B	Air	Greenhouse	
15/19-10A1	B	Air	Greenhouse	
15/19-42B1	B	Surface	Greenhouse	Wall
15/19-47B1	B	Surface	Greenhouse	Wall, metal racks for plant trays stacked on the ground
15/19-48A1	B	Surface	Greenhouse	Watering hoses
15/19-48B2	B	Surface	Greenhouse	Watering hoses
15/19-65B1	B	Surface	Sowing room	Conveyer belt, stereo, jacket, end of sowing machine, lots of dust
15/19-75B1	B	Surface	Packing room	Top of caged-in part, metal along conveyer belts
15/19-1B1	B	Air	Greenhouse	
15/19-5B1	B	Air	Greenhouse	
15/19-6B2	B	Air	Greenhouse	On trays
15/19-7B2	B	Air	Greenhouse	
15/19-49B1	B	Surface	Greenhouse	Watering boom
15/19-51B2	B	Surface	Cold storage	Garage, wall, things stored on windowsill
15/19-65A2	B	Surface	Sowing room	Conveyer belt, stereo, jacket, end of sowing machine (lots of dust)
15/19-67B2	B	Surface	Sowing room	Conveyer rollers on a trailer parked in the middle of the room
15/19-39B1	B	Surface	Greenhouse	Watering boom
15/19-40B2	B	Surface	Greenhouse	Moss, wall, old chairs, shelf, plant
15/19-42A2	B	Surface	Greenhouse	Wall
15/19-50A1	B	Surface	Greenhouse	Tarp over something, wall moss, watering boom
15/19-66B1	B	Surface	Sowing room	Sowing machine
58/19-3A	C	Air	Cold storage	Pallet storage
58/19-3B	C	Air	Cold storage	Pallet storage
58/19-4A	C	Air	Cold storage	Pallet storage
58/19-24A	C	Air	Greenhouse	5 m from watering boom
58/19-24B	C	Air	Greenhouse	5 m from watering boom
58/19-40B	C	Surface	Cold storage	Plants in cold storage
58/19-41A	C	Surface	Cold storage	Empty plant trays
58/19-42A	C	Surface	Cold storage	Plants in cold storage
58/19-42B	C	Surface	Cold storage	Plants in cold storage
58/19-43A	C	Surface	Cold storage	Floor
58/19-43B	C	Surface	Cold storage	Floor
58/19-44B	C	Surface	Pallet storage	Pallets and trays
58/19-45A	C	Surface	Pallet storage	Walls and plastic trays
58/19-50B	C	Surface	Cold storage	Plants with gray mold
58/19-52A	C	Surface	Cold storage	Healthy-looking plants
58/19-53B	C	Surface	Cold storage	Plants with apparent gray mold

58/19-55A	C	Surface	Cold storage	Plants on the floor, white mycelium
58/19-57A	C	Surface	Sowing room	Peat machine, conveyer belt, and washing machine
58/19-57B	C	Surface	Sowing room	Peat machine, conveyer belt, and washing machine
58/19-59A	C	Surface	Sowing room	Sick plants, sorted out
58/19-59B	C	Surface	Sowing room	Sick plants, sorted out
58/19-61A	C	Surface	Sowing room	On top of spraying machine
58/19-61B	C	Surface	Sowing room	On top of spraying machine
58/19-62B	C	Surface	Sowing room	Pallets
58/19-63A	C	Surface	Sowing room	Garbage container
58/19-63B	C	Surface	Sowing room	Garbage container
58/19-64A	C	Surface	Sowing room	Sorting machine
58/19-64B	C	Surface	Sowing room	Sorting machine
58/19-65A	C	Surface	Sowing room	Windowsill
58/19-65B	C	Surface	Sowing room	Windowsill
58/19-66A	C	Surface	Sowing room	Washing machine and peat sack
58/19-66B	C	Surface	Sowing room	Washing machine and peat sack
58/19-68B	C	Surface	Fan room	Wall and fan
58/19-70B	C	Surface	Greenhouse	Hoses and boom
58/19-85B	C	Surface	Greenhouse	Weeds between rows and Norway spruce plants
58/19-49A	C	Surface	Cold storage	Plants with white mycelium
58/19-49B	C	Surface	Cold storage	Plants with white mycelium
58/19-71A	C	Surface	Greenhouse	Random plant tray with germinating plants (1 week)
58/19-73B	C	Surface	Greenhouse	Wall and door
58/19-89B	C	Surface	Greenhouse	Plastic along the wall, lots of condensation on glass walls

[a] Single-spore *Botrytis* isolates

[b] Samples were collected from forest nurseries A, B, and C in November 2018, January 2019, and June 2019, respectively.

[c] Air indicates collection from open agar plates, and surface indicates sterile cotton swabs swiped over surfaces and then onto agar plates.

Supplementary Table 2. Primers and polymerase chain reaction conditions[a]

Target	Primer	Sequence (5´- 3´)	Annealing temperature (˚C)	Extension time (s)	Reference
cytb	Qo_G143A_F	CGGGCAAATGTCACTGTGAGC	64	90	K. A. G. Nielsen, *unpublished data*[d]
	Qo_wt_F	CGGGCAAATGTCACTGTGAGG	64	90	K. A. G. Nielsen, *unpublished data*[d]
	Qo-universal_R	TCCGTAGGTTTCCTGCTGAT	64	90	K. A. G. Nielsen, *unpublished data*[d]
	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[b]	45[c]	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[b]	45[c]	Grabke et al., 2013
	T63_int	CACCTCTGAAGACACGATTCACA	63	45	Grabke et al., 2013
	T63_R	CGCCTTCAGACCCTTCCTTC	63	45	Grabke et al., 2013
g3pdh	G3PDHfor+	ATTGACATCGTCGCTGTCAACGA	55	90	Staats et al. 2005
	g3pdh_R2	GAGTGGTTGTCACCGTTCATGTCAG	55	90	K. A. G. Nielsen, *unpublished data*[d]
mrr1	Mrr1-spez-F	TATCGGTCTTGCAGTCCGC	56	45	Leroch et al., 2013
	Mrr1-Pira	CCACCACAATCTTGGATCATTGGGATCAGAACCTGC	56	45	Leroch et al., 2013
nep2	NEP2forD	TTGCCTTCTCAAAATCATTACAGC	55	90	Staats et al., 2007
	NEP2revD	TCTAGAAAGTAGCCTTCGCAAGAT	55	90	Staats et al., 2007
sdhB	IpBcBeg	CCACTCCTCCATAATGGCTGCTCTCCGC	60	60	Leroux et al., 2010
	IpBcEnd2	CTCATCAAGCCCCCTCATTGATATC	60	60	Leroux et al., 2010
tubA	TUB-F1	GCTTTTGATCTCCAAGATCCG	56	60	Banno et al., 2008
	TUB-R1	CTGGTCAAAGGAGCAAATCC	56	60	Banno et al., 2008

[a] Initial denaturation at 95˚C for 5 minutes; 35 cycles of amplification (denaturation at 95˚C for 30 seconds, annealing at the indicated temperature for 30 seconds, extension at 72˚C for time indicated); and final elongation at 72˚C for 7 minutes.

[b] When used for sequencing, 60˚C

[c] When used for sequencing, 90s

[d] Paper I, this thesis

Supplementary Table 3. GenBank® reference sequences for *Botrytis* species used in this study.

	Gene (accession numbers)						
Species	*cytb*	*erg27*	*g3pdh*	*mrr1*	*nep2*	*sdhB*	*tubA*
B. californica			KJ937073.1				
B. caroliniana			JF811584.1				
B. cinerea	KP795071.1 KT318575.1	KP027846.1	KJ937075.1	JX266770.1 KF545941.1	DQ211825.1	CP009805.1	MH680908.1
B. euroamericana			KX266728.1		KX266752.1		
B. fabae			AJ705014.1		DQ211831.1		
B. fragariae			KX429699.1		KX429725.1		
B. mali			EF367128.1				
B. paeoniae			KY200511.1		AM087064.1		
B. prunorum			KX196312.1		KR425424.1		
B. pseudocinerea			JN692414.1		MK211256.1		
B. ricini			GQ860998.1				
Botrytis sp.			KY200400.1		KY230672.1		
Botrytis sp.			KY200444.1		KY230684.1		
Botrytis sp.			KY200460.1		KY230686_1		

Supplementary Table 4. Fungicide resistance and corresponding genotype of *Botrytis* isolates from forest nurseries (A through H) in Norway.

				Resistance[b]															Gene (accession numbers[h])	
Isolate	Year	Nursery	Origin[a]	Bos	Fen	Flud	Fluo	Pyra	Pyri	Thio	Cytb	Intron[c]	Erg27	Mrr1	SdhB	TubA	Species[d]	21bp[e]	*g3pdh*	*nep2*
32/19-2[i]	2018	D	P	S	S	S	S	MR	S	S	wt	Y	wt	wt		wt	Bc	S	OL743310	OL743431
58/19-3A	2019	C	A	MR	R	R	S	R	R	S			F412S	wt	H272R	wt	Bc	N	OL743314	OL743435
58/19-3B	2019	C	A	S	S	S	S	MR	S	S			wt	wt		wt	Bc	N	OL743315	OL743436
58/19-4A	2019	C	A	S	S	S	S	MR	S	S			wt	wt		wt	Bc	S	OL743325	OL743446
58/19-24A	2019	C	A	S	S	S	S	LR	S	S			wt	wt		wt	Bpru	N	OL743312	OL743433
58/19-24B	2019	C	A	S	S	S	S	LR	S	S			wt	wt		wt	Bpru	N	OL743313	OL743434
58/19-40B	2019	C	S	S	S	S	S	MR	S	S			wt	wt		wt	Bc	S	OL743316	OL743437
58/19-41A	2019	C	S	S	S	S	S	MR	S	S										
58/19-42A	2019	C	S	LR	R	S	S	R	R	R			F412S	wt		E198A	Bc	N	OL743317	OL743438
58/19-42B	2019	C	S	S	R	S	S	R	R	S			F412S	wt		wt	Bc	S	OL743318	OL743439
58/19-43A	2019	C	S	S	R	S	S	MR	S	S			F412S	wt		wt	Bpse	N	OL743319	OL743440
58/19-43B	2019	C	S	S	R	S	S	MR	S	S			F412S	wt		wt	Bpse	N	OL743320	OL743441
58/19-44B	2019	C	S	S	S	S	S	MR	S	S			wt	wt		wt	Bc	S	OL743321	OL743442
58/19-45A	2019	C	S	S	R	R	LR	R	R	S			F412S	wt		wt	Bc	S	OL743322	OL743443
58/19-50B	2019	C	S	S	S	S	S	MR	S	S			wt	wt		wt	Bc	S	OL743326	OL743447
58/19-52A	2019	C	S	S	R	S	S	MR	S	S										
58/19-53B	2019	C	S	MR	R	R	S	R	R	R			F412S	wt	H272R	E198A	Bc	S	OL743327	OL743448
58/19-55A	2019	C	S	MR	R	R	S	R	R	R			F412S	ΔL497	H272R	E198A	Bc	S	OL743328	OL743449
58/19-57A[i]	2019	C	S	S	R	S	LR	S	S	S			F412S	wt		E198A	Bc	N	OL743329	OL743450
58/19-57B	2019	C	S	S	S	S	S	LR	R	R			wt	wt		E198A	Bc	S	OL743330	OL743451
58/19-59A	2019	C	S	S	S	S	S	MR	S	S			wt	wt		wt	Bc	N	OL743331	OL743452
58/19-59B	2019	C	S	LR	R	R	LR	R	MR	R			F412S	wt		E198A	Bc	S	OL743332	OL743453
58/19-61A	2019	C	S	MR	R	S	S	R	MR	R			F412S	wt	H272R	E198A	Bc	N	OL743333	OL743454
58/19-61B	2019	C	S	LR	R	R	S	R	MR	S			F412S	wt		wt	Bc	S	OL743334	OL743455
58/19-62B	2019	C	S	S	S	S	S	MR	S	S				wt		wt	Bc	S	OL743335	OL743456
58/19-63A	2019	C	S	S	S	S	S	MR	S	S			wt	wt		wt	Bc	S	OL743336	OL743457
58/19-63B	2019	C	S	MR	R	R	S	R	R	R			F412S		H272R	E198A	Bc	S	OL743337	OL743458
58/19-64A[i]	2019	C	S	MR/R	R	S	S	R	MR/R	R			wt	wt	wt	wt	Bpse	N	OL743338	OL743459
58/19-64B	2019	C	S	LR	R	S	S	R	R	R			F412S	wt		E198A	Bc	N	OL743339	OL743460
58/19-65A	2019	C	S	LR	R	S	S	R	R	R			F412S	wt		E198A	Bc	N	OL743340	OL743461
58/19-65B	2019	C	S	LR	R	S	S	R	MR	R			F412S	wt		E198A	Bc	N	OL743341	OL743462
58/19-66A	2019	C	S	LR	R	R	S	R	MR	R			F412S	wt		E198A	Bc	S	OL743342	OL743463
58/19-66B	2019	C	S	LR	R	R	S	R	MR	R			F412S	wt		E198A	Bc	S	OL743343	OL743464

58/19-68B	2019	C	S	LR	R	MR	S	R	MR	R										
58/19-70B	2019	C	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743344	OL743465
58/19-85B	2019	C	S	S	S	S	S	MR	S	R			wt	wt		E198A	Bc	S	OL743347	OL743468
58/19-49A	2019	C	S	S	LR	S	S	LR	S	S			wt	wt		wt	Bpse	N	OL743323	OL743444
58/19-49B[i]	2019	C	S	S	R	S	S	S	S	LR			wt	wt		wt	Bpse	N	OL743324	OL743445
58/19-71A	2019	C	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743345	OL743466
58/19-73B	2019	C	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743346	OL743467
58/19-89B	2019	C	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743348	OL743469
58/19-96	2019	C	P	S	S	S	S	S	S	S	wt	N	wt	wt		wt	Bc	S	OL743349	OL743470
58/19-97	2019	C	P	S	S	S	S	LR	S	S	wt	N	wt	wt		wt	Bc	S	OL743350	OL743471
58/19-101C	2019	C	E	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743311	OL743432
114/18-8	2018	A	A	S	S	S	S	MR	S	S			wt			wt	Bc	N	OL743287	OL743408
114/18-17	2018	A	S	MR	R	R	S	R	R	R			F412S	ΔL497		E198A	Bc	S	OL743284	OL743405
114/18-3.1a	2018	A	A	S	R	R	S	R	MR	R			F412S	wt		E198A	Bc	S	OL743282	OL743403
114/18-3.1b	2018	A	A	S	R	R	S	R	R	R			F412S	wt		E198A	Bc	S	OL743283	OL743404
114/18-3.2	2018	A	A	MR	R	R	S	R	R	R			F412S	wt		E198A	Bc	S	OL743285	OL743406
114/18-45	2018	A	S	S	S	S	S	MR	S	S			wt	wt		wt	Bc	S	OL743286	OL743407
15/19-8B1	2019	B	A	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743308	OL743429
15/19-9B1	2019	B	A	LR	R	S	S	R	R	R			F412S	wt		F200Y	Bc	N	OL743309	OL743430
15/19-10A1[i]	2019	B	A	S	S/LR	S	S	LR	LR	LR			wt	wt		wt	Bc	S	OL743288	OL743409
15/19-42B1	2019	B	S	S	S	S	S	MR	S	S			wt	wt		wt	Bc	S	OL743293	OL743414
15/19-47B1[i]	2019	B	S	S	S	R	S	R	LR	R			wt	wt		E198A	Bc	N	OL743294	OL743415
15/19-48A1	2019	B	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743295	OL743416
15/19-48B2	2019	B	S	S	S	S	S	S	S	S			wt	wt		wt	B. sp.	N	OL743296	OL743417
15/19-65B1	2019	B	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743302	OL743423
15/19-75B1	2019	B	S	LR	R	S	S	R	LR	R			F412S	wt		E198A	Bc	N	OL743306	OL743427
15/19-1B1	2019	B	A	S	S	S	S	S	S	S			wt	wt		wt	Bc	S	OL743289	OL743410
15/19-5B1	2019	B	A	S	S	S	S	S	S	S			wt	wt		wt	Bc	S	OL743300	OL743421
15/19-6B2	2019	B	A	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743305	OL743426
15/19-7B2	2019	B	A	MR	R	R	S	R	R	R			F412S	wt	H272R	E198A	Bc	S	OL743307	OL743428
15/19-49B1	2019	B	S	S	S	S	S	S	S	S			wt	wt		wt	B. sp.	N	OL743297	OL743418
15/19-51B2	2019	B	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743299	OL743420
15/19-65A2	2019	B	S	S	S	S	S	S	S	S			wt	wt		wt	Bc	S	OL743301	OL743422
15/19-67B2	2019	B	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743304	OL743425
15/19-39B1	2019	B	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743290	OL743411
15/19-40B2	2019	B	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743291	OL743412
15/19-42A2	2019	B	S	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743292	OL743413

15/19-50A1	2019	B	S	S	S	S	S	LR	S	S			wt	wt	wt	Bc	S	OL743298	OL743419
15/19-66B1[i]	2019	B	S	S	LR	S	S	S	LR	R			F412S		wt	Bpse		OL743303	OL743424
Pa1	2013	E	P	S	S	S	S	LR	S	R			wt	wt	E198A	Bc	S	OL743394	OL743515
Pa3	2013	E	P	S	S	S	S	LR	S	R	wt	Y	wt	wt	E198A	Bc	S	OL743398	OL743519
Pa4	2013	E	P	S	S	R	S	R	LR	R	G143A	N	wt	wt	E198A	Bc	N	OL743399	OL743520
Pa5	2013	E	P	S	S	R	S	R	LR	R	G143A	N	wt	wt	E198A	Bc	N	OL743400	OL743521
Pa6	2013	A	P	S	S	S	S	LR	S	R	wt	Y	wt	wt	E198A	Bc	S	OL743401	OL743522
Pa7	2013	A	P	S	S	S	S	LR	S	R			wt	wt	E198A	Bc	S	OL743402	OL743523
Pa11	2013	F	P	S	S	S	S	LR	S	R			wt	wt	E198A	Bc	S	OL743395	OL743516
Pa17	2013	B	P	S	R	R	S	R	R	R	G143A	N	F412S	wt	E198A	Bc	S	OL743396	OL743517
Pa19	2013	E	P	S	S	S	S	LR	S	R			wt	wt	E198A	Bc	N	OL743397	OL743518
FG1	2014	A	P	S	S	S	S	R	LR	R			wt		E198A	Bc	N	OL743351	OL743472
FG2	2014	C	P	S	S	S	S	R	LR	R			wt	wt	E198A	Bc	N	OL743354	OL743475
FG3	2014	C	P	S	S	LR	S	R	LR	R			wt		E198A	Bc	N	OL743363	OL743484
FG4	2014	C	P	S	S	S	S	LR	MR	R			wt	wt	E198A	Bc	S	OL743373	OL743494
FG5[i]	2014	C	P	S	R	S	LR	MR	S	R	wt	Y	F412S	wt	E198A	Bc	S	OL743383	OL743504
FG6	2014	C	P	S	S	S	S	MR	S	S			wt	wt	wt	Bc	S	OL743391	OL743512
FG7	2014	D	P	S	S	S	S	MR	S	S			wt	wt	wt	Bc	S	OL743392	OL743513
FG9[i]	2014	A	P	S	S	S	S	MR/LR	S	S/LR	wt	N	wt	wt	wt	Bc	S	OL743393	OL743514
F18	2014	C	P	S	S	S	S	R	LR	R			wt		E198A	Bc	N	OL743352	OL743473
FG19	2014	C	P	LR	S	S	S	R	S	R			wt		E198A	Bc	N	OL743353	OL743474
FG20	2014	C	P	S	S	S	S	R	MR	R			wt		E198A	Bc	N	OL743355	OL743476
FG21	2014	A	P	S	S	S	S	S	S	S	wt	N	wt	wt	wt	Bc	S	OL743356	OL743477
FG23	2014	C	P	S	S	S	S	LR	S	S	wt	Y	wt	wt	wt	Bc	S	OL743357	OL743478
FG24	2015	A	P	S	R	S	S	R	LR	R			F412S		E198A	Bc		OL743358	OL743479
FG25[i]	2015	G	P	S	S	S	S	MR	S	R	wt	Y	wt	wt	E198A	Bc	S	OL743359	OL743480
FG26	2015	G	P	S	S	S	S	LR	S	R			wt	wt	E198A	Bc	N	OL743360	OL743481
FG27	2014	C	P	LR	S	S	S	R	LR	R			wt						
FG28	2014	C	P	LR	S	S	S	R	S	R	G143A	N	wt		E198A	Bc	N	OL743361	OL743482
FG29	2014	C	P	S	S	S	S	R	S	R			wt		E198A	Bc	N	OL743362	OL743483
FG30	2014	C	P	S	S	S	S	LR	S	S			wt	wt	wt	Bc	S	OL743364	OL743485
FG31	2015	B	P	S	S	S	S	LR	S	S			wt	wt	wt	Bc	S	OL743365	OL743486
FG32	2015	A	P	S	R	S	S	LR	LR	R	wt	Y	F412S		E198A	Bc	N	OL743366	OL743487
FG33	2015	A	P	S	S	S	S	LR	S	S			wt	wt	wt	Bc	S	OL743367	OL743488
FG35[i]	2015	A	P	S	R	R/MR	S	R	LR	R			F412S	wt	E198A	Bc	S	OL743368	OL743489
FG36	2016	E	P	S	S	S	S	S	S	R	wt	Y	wt	wt	E198A	Bc	S	OL743369	OL743490
FG37	2015	H	P	S	S	S	S	LR	S	R			wt	wt	E198A	Bc	S	OL743370	OL743491

FG38	2015	H	P	S	S	S	S	LR	S	S	wt	N	wt	wt		wt	Bc	S	OL743371	OL743492
FG39	2015	H	P	S	S	S	S	LR	S	S			wt	wt		wt	Bc	S	OL743372	OL743493
FG41	2016	F	E	MR	R	R	S	R	MR	R			F412S	ΔL497	H272R	E198A	Bc	S	OL743374	OL743495
FG42	2016	F	E	MR	R	R	S	R	MR	R			F412S	ΔL497	H272R	E198A	Bc	S	OL743375	OL743496
FG43	2016	A[f]	P	LR	R	S	S	R	MR	R			F412S			E198A	Bc	N	OL743376	OL743497
FG44	2015	A	P	S	S	S	S	MR	S	R			wt	wt		E198A	Bc	S	OL743377	OL743498
FG45	2015	A	P	S	R	R	S	R	MR	R	G143A	N	F412S	wt		E198A	Bc	S	OL743378	OL743499
FG46[i]	2016	A	P	LR	R	S	S	S	LR/MR/R	S			F412S			wt	Bc	N	OL743379	OL743500
FG47	2016	C	C	LR	R	S	S	R	LR	R			F412S			E198A	Bc	N	OL743380	OL743501
FG48	2017	A	P	S	R	R	S	R	R	R	G143A	N	F412S	wt		E198A	Bc	S	OL743381	OL743502
FG49[i]	2017	F	P	S	R	S	LR	S/LR	LR	S			wt			wt	Bpse	N	OL743382	OL743503
FG50	2017	B[g]	P	S	MR	S	S	LR	S	S			wt			wt	Bpse	N	OL743384	OL743505
FG52	2017	D	P	S	S	S	S	LR	S	R	wt	Y	wt			E198A	Bc	N	OL743385	OL743506
FG53	2017	G	P	S	S	S	S	LR	S	R	wt	N	wt	wt		E198A	Bc	S	OL743386	OL743507
FG54	2017	E	P	S	S	S	S	MR	S	R			wt			E198A	Bc	N	OL743387	OL743508
FG55	2017	D	P	S	S	S	S	MR	S	S			wt	wt		wt	Bc	S	OL743388	OL743509
FG56	2017	D	P	S	S	S	S	LR	S	R			wt			E198A	Bc	N	OL743389	OL743510
FG57	2017	D	P	S	S	S	S	LR	S	R			wt			E198A	Bc	N	OL743390	OL743511

[a] Origin of isolates indicated as: "P" for Norway spruce (*Picea abies*), "A" for air, "S" for surfaces, and "C" or "E" for weeds of genus *Chenopodium* or *Epilobium*, respectively.

[b] Fungicide resistance category determined by mycelial growth assay for boscalid (Bos), fenhexamid (Fen), fludioxonil (Flud), fluopyram (Fluo), pyraclostrobin (Pyra), pyrimethanil (Pyri), and thiophanate-methyl (Thio). Categories include resistant (R), moderately resistant (MR), low resistant (LR), and sensitive (s) as in Schnabel et al. (2015).

[c] Intron refers to the 1205 bp intron known to preclude G143A. "Y" indicates intron detected and "N" indicates intron not detected.

[d] *Botrytis* species are indicated as: *B. cinerea* (Bc), *B. prunorum* (Bpru), *B. pseudocinerea* (Bpse), and *Botrytis* sp. (B. sp.) that are similar to isolates in a unique phylogenetic clade called AKBot3 described by Garfinkel et al. (2019).

[e] Indicates analysis of the *mrr1* gene for the 21 bp indel associated with *Botrytis* group S. "S" indicated indel detected and characterization of isolate as group S, and "N" indicates not detected.

[f] Indicates isolate from Norway spruce tree planted out autumn 2015.

[g] Indicates isolate from Norway spruce tree planted out

[h] GenBank®

[i] Re-tested with mycelial growth assay

Fenhexamid resistance and fitness dynamics in *Botrytis* in strawberry and raspberry fields in Norway

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Paper III

Fenhexamid resistance and fitness dynamics in *Botrytis* in strawberry and raspberry fields in Norway

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Abstract

Grey mould, caused by *Botrytis* spp., is a problematic fungal disease in open field strawberry and raspberry production in Norway. Growers face challenges of fungicide resistance in the pathogen, few remaining approved fungicides, and limited prospects of fungicides with new modes of action reaching the Norwegian market. Thus, there is interest in reversing resistance to the *Botrytis*-specific fungicide fenhexamid. An experimental strawberry field and six commercial raspberry fields were sampled over periods of two and three years, respectively. The crops were perennial, with no replacement of plants during the experimental period. High resistance to fenhexamid (90%) was intentionally selected for in the strawberry field, fungicide use was subsequently discontinued, and fenhexamid resistance steadily decreased to about 7% at the final sampling. In the six raspberry fields, growers had discontinued use of fenhexamid but continued to use other available fungicides. In general, minor changes in fenhexamid resistance were observed after three years. Prevalence of multiple fungicide resistance in *Botrytis* combined with continued fungicide use, particularly of a product containing boscalid and pyraclostrobin, presumably contributed to the lack of decrease in fenhexamid resistance in the raspberry fields. A selection of raspberry field isolates resistant and sensitive to fenhexamid did not show differences in conidia production and growth rate. However, there appears to be a fitness cost associated with fenhexamid resistance, and multiple fungicide resistance, in the grey mould pathogen in the field in Norway, but multiple fungicide resistance may make fitness cost difficult to exploit in resistance management efforts.

Introduction

Grey mould, caused by *Botrytis* spp., causes devastating losses in open field strawberry (*Fragaria* × *ananassa*) and raspberry (*Rubus idaeus*) production in Norway. Cool and wet summers create conditions conducive to grey mould infection and spread, and chemical control is a cornerstone of growers' control regimes. The efficacy of the fungicides available to growers in Norway and elsewhere in the world are at risk due to fungicide resistance developing in the grey mould pathogen (Hahn, 2014; Fernández-Ortuño et al., 2015; Grabke et al., 2013; Nielsen et al., 2020; Nielsen et al., 2021). The fungicides approved for grey mould control in strawberry and raspberry in Norway are single-site fungicides. Such fungicides affect a specific enzyme in the target organism and exert strong selective pressure, which translates into high risk of resistance development, particularly in pathogens with a polycyclic life cycle such as *Botrytis* spp. (Lucas et al., 2015).

Fenhexamid is a single-site fungicide which has been available for grey mould control in strawberry and raspberry in Norway for two decades. It is a hydroxyanilide derivative that has a preventative effect on the related genera *Botrytis*, *Monilinia*, and *Sclerotinia* (Debieu et al., 2001). Fenhexamid's mode of action is inhibition of sterol biosynthesis, and while it does not affect spore germination, both germ tube elongation and mycelial growth are inhibited in *B. cinerea* (Debieu et al., 2001). High resistance to fenhexamid in *Botrytis* is associated with mutations in the gene encoding 3-ketoreductase (*erg27*), and most often at codon F412 (Amiri & Peres, 2014; Fillinger et al., 2008; Grabke et al., 2013; Saito et al., 2014). In *B. pseudocinerea*, a low-level of resistance to fenhexamid is likely conferred through detoxification by a cytochrome P450 monooxygenase, but this level of resistance is not considered to have consequences for practical resistance (Debieu & Leroux, 2015). A rearrangement of the

promoter for the gene encoding the MfsM2 drug transporter can also confer fenhexamid resistance in *Botrytis* as part of the multidrug resistance phenotypes known as MDR2 and MDR3, although these have only been identified in vineyards in France and Germany so far (Kretschmer et al., 2009; Mernke et al., 2011).

Mutations in the genes that confer target-site fungicide resistance may result in less efficient enzymes that can affect pathogen metabolism, reproduction, and survival, thus leading to a competitive disadvantage once the selective pressure exerted by the fungicide is removed (De Miccolis Angelini et al., 2015). Fitness cost has been measured in several studies of fenhexamid resistance in *Botrytis,* but with varying results. Saito et al. (2014) reported reduced mycelial growth and spore germination in several fenhexamid-resistant isolates from vineyards in the U. S. Esterio et al. (2021) found no difference in mycelial growth, reduced conidia production and sclerotia development only for isolates with the F412V substitution, and more aggressiveness in isolates with the F412S and -I substitutions compared to sensitive isolate. Lab-generated resistant isolates had reduced sporulation, germination, and sclerotia production (Ziogas et al., 2003). Billard et al. (2012) generated isogenic strains in order to compare fitness parameters correlated with the F412S, -I, and -V substitutions without the potential effects of genetic variability found in field isolates. Certain mutants had lower production of conidia, lower growth rates at specific temperatures, and slower growth from sclerotia after storage. In some studies, authors noted that the detected fitness costs could have practical relevance for winter or outside survival of *Botrytis* (Billard et al., 2012; Chen et al., 2016; Ziogas et al., 2003). If this is the case, then Norway's harsh winters could provide negative selection of the fenhexamid-resistant proportion of *Botrytis* populations overwintering in strawberry and raspberry fields.

In more recent studies, fitness has been examined in isolates with multiple fungicide resistance that increasingly dominate in production systems where fungicides with several modes of action have been used. Both Chen et al. (2016) and Hu et al. (2016) found that sensitive *Botrytis* isolates outcompeted isolates with multiple fungicide resistance when co-inoculated on apple and without fungicide exposure. However, Rupp et al. (2017) found isolates with multiple fungicide resistance had no disadvantage in co-inoculation experiments with sensitive isolates. When Cosseboom et al. (2020) inoculated blackberry flowers in research fields, isolates with multiple fungicide resistance survived and spread to plots that had not been inoculated.

Controlling *Botrytis* by chemical means is becoming increasingly complicated as the number of available fungicides dwindles and resistance to remaining fungicides continues to develop. Fenhexamid controls sensitive populations of *Botrytis* effectively and is less harmful to non-target organisms and the environment relative to some other fungicides (Debieu et al., 2001), so it would be advantageous if resistance could be reversed. If the mutations in *erg27* that confer high resistance to fenhexamid in *Botrytis* also impose a fitness cost under field conditions in Norway, then growers may be able to implement resistant management strategies to use fenhexamid again. The objectives of this study were therefore to: (i) monitor fenhexamid resistance over two years in an experimental perennial strawberry field where resistance was first selected for and then subsequent fungicide use discontinued; (ii) monitor fenhexamid resistance in six commercial perennial raspberry fields where growers had stopped using fenhexamid in their fungicide rotations; (iii) compare fitness, of fenhexamid-resistant and -sensitive *Botrytis* isolates from the commercial raspberry fields by comparing mycelial growth rates and conidia production.

Materials and Methods

Experimental strawberry field and sample collection

An experimental strawberry field was established at the Norwegian University of Life Sciences (NMBU) in Ås, Norway. Plug transplants of strawberry cv. Korona were planted out in six rows at the end of May 2018. To achieve high fenhexamid resistance in the field as quickly as possible, three sporulating fenhexamid-resistant *Botrytis* (Table 1) isolates on potato dextrose agar (PDA, Difco™ Potato dextrose agar) were placed along the plant rows, and the field was treated five times with the fenhexamid product Teldor® WG 50 (Bayer) at the label rate and sprayed until run-off. All five fenhexamid treatments were within the first month after planting. The *Botrytis* isolates used to generate inoculum for the experimental strawberry field were originally collected from strawberry fields in Norway and characterized for resistance in an earlier study (K.A.G. Nielsen, unpublished, Paper I, this thesis) (Table 1). Isolates stored as conidia in 20% glycerol at -80°C, were recovered by plating on potato dextrose agar (PDA, Difco™ Potato dextrose agar). Mycelial plugs from the peripheral edge were transferred to 16 new PDA plates for each of the three isolates each time inoculum was produced for the field. Agar containing sporulating mycelium in each plate was cut into 6 pieces, and 96 agar triangles were evenly distributed in the field. The field was watered after placing out inoculum if conditions were hot and dry. Inoculum was placed in the field in this manner three times in June 2018, once at the beginning of July 2018, and once in the middle of September 2018. The field was otherwise managed as a standard conventional perennial strawberry field, with insecticides and herbicides applied as needed. Plants were covered with fleece during the winters. Drip lines were installed for watering as required through the experiment, and there was also some overhead watering in the summer of 2018 that was unusually hot and dry.

The first sample was collected from the field two days after planting (t0), thereafter plant material was collected from the field seven times (t1 – t7) from summer 2018 to summer 2020 (Table 2). Forty samples of leaves were collected from 40 arbitrary locations evenly distributed over the four middle rows except the ends of each row (approximately one meter). Each sample included approximately 5-10 leaves (old and new if possible). Fruit samples were also taken from different locations within the four centre rows. Plant material from each sample was placed together in a plastic bag and all material was frozen until further analysis. Thawed samples were placed in separate containers with moistened paper towels to maintain high humidity for incubation at ambient temperatures. *Botrytis* conidia were harvested from sporulating lesions and spread over water agar. Single germinating conidia were excised from water agar and transferred to acidified potato dextrose agar (APDA, PDA amended with 0.2% w/v tartaric acid) to inhibit bacterial growth. After incubation for approximately 5 days, the plates were placed under UV lamps to stimulate conidia production. Conidia were then used directly for resistance testing or stored until testing. Single-spore isolates of *Botrytis* were stored as conidia in 20% glycerol at -20°C. In 2019, *Botrytis* was also obtained by harvesting conidia from infected fruit in the field with dry cotton swabs. Conidia were then transferred directly from the swabs to water agar, and single-spore isolates were made as described above.

Sample collection from commercial raspberry fields

Advisors from the Norwegian Agricultural Extension Service helped select commercial raspberry fields for sampling to monitor fenhexamid resistance in *Botrytis* from spring 2018 to spring 2021. Six fields were selected where growers had removed fenhexamid from their fungicide rotation. Five fields were located in the districts Sogn and Nordfjord in Vestland county, and one field was in Bærum municipality in Viken county (field D). One of the growers

in Vestland had converted to tunnel production in 2017 (field F), and the rest were open field production. Advisors collected samples in the form of overwintered canes in the spring and raspberry fruits at the end of the growing season. In 2018 and 2019, plant samples were individually wrapped and sent to NIBIO, where it was frozen until analysis, as described above for strawberry. In 2020 and 2021, the advisors incubated the plant material, whereafter they wiped cotton swabs across conidia and sent the swabs by post. The cotton swabs were treated as described above for strawberry. Single-spore isolates were made and stored as described above. The advisors also collected fungicide treatment data for the raspberry fields and submitted this with the samples (Supplementary Table 1).

Fungicides

In this study, fungicides used for resistance testing were the following product formulations (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); pyraclostrobin (Comet® Pro, BASF; QoI); pyrimethanil (Scala®, BASF; AP); and thiophanate-methyl (Topsin ® WG, Nisso Chemical Europa GmbH; MBC) (Table 3).

Mycelial growth assay

Single-spore field isolates of *Botrytis* from both strawberry (208 isolates) and raspberry (624 isolates) were tested for resistance to fenhexamid using a mycelial growth assay adapted from Fernández-Ortuño et al. (2014). In addition, 98 and 230 of the isolates from strawberry and raspberry, respectively, were tested for resistance to boscalid, fludioxonil, fluopyram, pyraclostrobin, pyrimethanil, and thiophanate-methyl. Briefly, fungicide products were dissolved in liquid media to make stock solutions, which were used to amend agar growth

media to obtain discriminatory concentrations (Table 3). The amended medium was added in aliquots of 1.5 ml to 24-well cell culture plates (Nunclon™ Delta Surface, Thermo Scientific). Conidia from sporulating *Botrytis* cultures were transferred with sterile wooden toothpicks to the centre of the agar medium surface of each well, and plates were incubated for four days at 20°C in the dark. Each isolate-fungicide combination was assigned a resistance category of sensitive (S, no growth), low resistant (LR, less than 20%), moderately resistant (MR, more than 20% and less than 50%), or resistant (R, more than 50%), based on mycelial growth diameter in the well in relation to the 1.5 cm diameter of the well. In a previous study (K.A. G. Nielsen, unpublished data, Paper II, this thesis), isolates MR to boscalid often had the H272R substitution in SdhB, so when referring to resistance in the results, we include the MR category for boscalid only.

Quantification of conidia production

A total of 92 isolates collected in the spring 2020 and 2021 from all six commercial raspberry fields were selected to test their conidia production ability. In total, there were 46 each of isolates that were S and R to fenhexamid in the mycelial growth assay. Isolates were transferred from stocks in the freezer to PDA plates that were incubated in the dark at 20°C for four days. Mycelial plugs were harvested from the periphery of the colony and transferred with the mycelium side down to the centre of new 5.5 cm PDA plates, two plates per isolate. The plates were sealed with parafilm and placed in the dark at 20°C for four days before being randomly placed in a growth chamber (Versatile Environmental Test Chamber MLR-352H, PHC Corporation, Japan) at 20°C, 70% RH, with a 12 h light/dark cycle at light setting 4 LS with fluorescent lamps (Panasonic, FL40SS-ENW/37). After 10 days, plates were flooded with 1.8 ml 20% glycerol solution, and conidia were dislodged using a sterile glass rod stave, after which 1.5 ml of the resulting spore suspension was filtered through autoclaved, hospital quality

absorbent cotton into microtubes (one tube per plate). Tubes were refrigerated overnight, and conidia were disposed in disposable Glasstic® slides with grids (Kova International; California, USA) and counted using a microscope. For each sample, the number of conidia in nine squares on the diagonal across the grid were averaged, and two counts were conducted and averaged to obtain the concentration of conidia per ml. Conidia suspensions were stored at -20°C for use with the growth rate experiment. The experiment was repeated; however, in the second experiment, plates were only taped at opposite sides of the lids instead of being sealed with parafilm, to reduce humidity, which seemed to prevent sporulation in the first experiment.

Measuring mycelial growth rate

A photometric microplate absorbance reader (Bioscreen C° Pro™, Oy Growth Curves, Finland) was used to incubate and automatically measure three-dimensional fungal growth, by optical density (OD), in semi-solid medium (Medina et al., 2012). Conidia suspensions from 73 isolates were adjusted to 2 x 10^4 conidia per ml in 20 % glycerol solution, and four microtubes per isolate were stored at -20°C until use. For each isolate, 250 µl conidia suspension was mixed with 250 µl semi-solid PDA (4.8 g Difco™ Potato Dextrose Broth and 0.25 g Bacto™ Agar per 100 ml distilled water) using a vortex mixer, and 200 µl of the mixture was pipetted into one well in each of two microplates (100-well Honeycomb 2 Microplates). For each experiment, OD measurements were recorded automatically and simultaneously with a 405 nm filter. Two experiments, each with two microplates, were conducted at both 18°C and 28°C for seven days. In all experiments, growth rate was calculated by subtracting the OD value at 0 hours from that at 96 hours for each isolate in each microplate well.

Statistical analysis

Statistical analysis and generation of box and whisker plots was done using R version 4.1.2 (2021-11-01). Conidia production and optical density data were analysed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons of means. Conidia production data was transformed before conducting statistical analysis.

Results

Fungicide resistance in experimental strawberry field

The frequency of fenhexamid resistance in *Botrytis* isolates obtained from the experimental perennial strawberry field increased quickly from 3.4% at the end of May (t0) to 90.0% by mid-July 2018 (t1) (Fig. 1). Subsequent sampling and testing revealed a steady decline in fenhexamid resistance to 5.3 % and 6.9% for the two final samples collected in April (t6) and June 2020 (t7), respectively. Isolates from the first two sampling dates were also tested for resistance to boscalid, fludioxonil, fluopyram, pyraclostrobin, pyrimethanil, and thiophanate-methyl, and resistance frequencies to all these fungicides, except fluopyram which remained at 0%, increased from May (t0) to mid-July 2018 (t1) (Table 4). Boscalid and pyraclostrobin resistance reached 90% and 95% of the isolates, respectively. Resistance to fludioxonil was undetected in May 2018 (t0) but had increased to 12.5% by July 2018 (t1). In June 2020 (t7), isolates from samples were also tested for resistance to six fungicides in addition to fenhexamid. Apart from fluopyram, which remained at 0%, resistance frequencies for all fungicides were lower in the final sampling in June 2020 (t7) than they were in the initial sampling in May 2018 (t0). Frequencies of multiple fungicide resistance show a dramatic shift from May 2018 (t0), when there were no isolates resistant to five or six fungicides (5R or 6R), to July 2018 (t1) when 47.5% of isolates collected were 5R or 6R. By the final sampling in June

2020 (t7), however no isolates were detected that were resistant to more than three fungicides and the frequency of 0R isolates had increased to 75.0% (Fig. 2).

Fungicide resistance in commercial raspberry fields

In total, 624 single-spore isolates of *Botrytis* were obtained from pre- and post-season samples collected from six commercial perennial raspberry fields and tested for resistance to fenhexamid. Changes in fenhexamid resistance were generally minor within each field (Table 5). Five of the six fields had fenhexamid resistance frequencies of 50% or higher for the final sample collected pre-season in 2021. Isolates from samples collected pre-season in both 2018 and 2021 were also tested with the mycelial growth assay for resistance to boscalid, fludioxonil, fluopyram, pyraclostrobin, pyrimethanil, and thiophanate-methyl (Table 6). Resistance to boscalid increased in all but one field (field F) from pre-season 2018 to pre-season 2021. Pyraclostrobin resistance increased in fields A-C and decreased in fields D-F. Resistance frequencies for thiophanate-methyl generally decreased. There was no clear trend observed for resistance to pyrimethanil and fludioxonil from pre-season 2018 to pre-season 2021. Multiple fungicide resistance was detected in many of the 230 raspberry field isolates from pre-season 2018 and pre-season 2021 (Table 7). Resistance to fenhexamid alone was rare and only detected in four isolates in pre-season 2018 and one in pre-season 2021. There was an observable trend in change in fenhexamid resistance plotted against change in frequency of isolates resistant to four or more fungicides (Fig. 3). Change in fenhexamid resistance was also compared to fungicide application data, with some fields showing a trend relative to total Signum® applications (Fig. 3). All but one of the raspberry field isolates from pre-season 2018 and pre-season 2021 that were resistant to both boscalid and pyraclostrobin were also resistant to fenhexamid. The one isolate resistant to both boscalid and pyraclostrobin and not to fenhexamid was from field F in pre-season 2021.

Fitness parameter testing of *Botrytis* field isolates from raspberry

Due to poor sporulation, the method of closing the agar plates was changed for the second experiment to attempt to reduce humidity levels inside the plates during incubation. Data from the two experiments was analysed separately.

For each of the conidia production experiments, one-way analysis of variance resulted in a significant ($p < 0.05$) effect of field of isolate origin on conidia production, however, Tukey multiple comparisons of means revealed significant difference ($p < 0.05$) only between fields A and E in the first experiment. There were no significant differences in conidia production between isolates that were sensitive or resistant to fenhexamid, in either experiment (Fig. 4).

Growth rate experiments were performed with a selection of 73 of the *Botrytis* isolates that were used to test conidia production. There were no statistically significant differences between isolates sensitive or resistant to fenhexamid based on the OD measurements of mycelial growth. There were, however, significant effects ($p < 0.05$) of experiment, field origin, and plate for tests conducted when replicate experiments were analysed together. The most extreme outliers in the data were observed for the sensitive isolates (Fig. 5).

Discussion

Results from the experimental strawberry field give a strong indication that multiple fungicide resistance, including fenhexamid resistance, in *Botrytis* entails a fitness cost under field conditions in Norway. General lack of comparable reduction in fenhexamid resistance in commercial raspberry fields, however, may illustrate how the prevalence of multiple fungicide resistance can interfere with exploiting fitness cost in an effort to reverse resistance.

In the experimental strawberry field, we successfully selected for very high fenhexamid resistance over the course of one and a half months by applying five treatments with the product containing fenhexamid and distributing resistant inoculum. Resistance testing in July 2018 also revealed an increase in resistance frequencies for other fungicides. As multiple fungicide resistance was already present in the field at the time of first sampling, treatment with fenhexamid may have contributed to the increase. Resistance to fludioxonil, however, was detected in the sampling in July 2018, but not the initial sampling. The sporulating *Botrytis* mycelium placed in the field as inoculum included an isolate for which the triplet deletion leading to ΔL497 had been detected in the multidrug resistance regulator 1 (*mrr1*) gene. This triplet deletion is associated with the MDR1h type of multidrug resistance which is known to confer resistance to fludioxonil (Leroch et al., 2013). Therefore, the inoculum placed in the strawberry field could have infected the strawberry plants, been a source of the fludioxonil resistance we detected in July 2018, and contributed to increasing fenhexamid resistance at the beginning of the experiment. Isolates used for inoculum had mutations known to confer resistance to boscalid and pyraclostrobin and may also have contributed to the high multiple fungicide resistance detected in July 2018.

Fenhexamid resistance in *Botrytis* in the experimental strawberry field declined steadily when fungicide use stopped, signifying a fitness cost for resistant isolates in the absence of selective pressure exerted by fungicides. It took approximately two years without fungicide input for the fenhexamid resistance frequency to return to its original level, and gradual reduction, is typical for reversion of resistance when there is fitness cost (Mikaberidze & McDonald, 2015). In studies with lab-generated strains of *Botrytis* resistant to fenhexamid, fitness costs were identified which could have implications for overwintering and outdoor survival (Billard et al., 2012; Ziogas et al., 2003). *Botrytis* overwinters in strawberry plant material (Strømeng et al.,

2009), and strawberry plants in the experimental field were protected from Norwegian winter conditions by only a thin fleece layer, so *Botrytis* was exposed to overwintering and outdoor survival conditions. Fenhexamid resistance was detected in isolates which were also resistant to other fungicides, so fitness costs associated with multiple fungicide resistant phenotypes must also be considered. We observed a shift in multiple resistant phenotypes towards fewer accumulated resistances at the end of the experiment. This is consistent with a study in which sensitive isolates outcompeted multiple resistant isolates of *Botrytis* from strawberry in competition experiments in the absence of fungicide pressure (Chen et al., 2016). In the same study, authors reported slower growth of multiple fungicide resistant MDR1h isolates compared to sensitive isolates at the lowest temperature they tested, 4°C. Hu et al. (2016) found that isolates resistant to boscalid, fenhexamid, pyraclostrobin, and thiophanate-methyl were unable to compete with sensitive isolates when co-inoculated in a detached fruit assay without fungicide application. Even though we observed a clear reversion of resistance in the experimental strawberry field which we attribute to fitness cost, we cannot rule out the potential effect of other factors, such as genetic drift or indirect costs associated with linked genes, that could have also contributed to the reduction in resistance frequencies (Hawkins and Fraaije, 2018).

Six commercial raspberry growers discontinued fenhexamid use in their fields to see if reversal of resistance was possible, but there was minimal change in fenhexamid resistance frequencies in most fields. There could be several explanations for this. There was a high degree of multiple fungicide resistance in the raspberry field isolates, and few isolates were resistant to fenhexamid only. Results from studies of fitness associated with multiple fungicide resistance have varied; some have found multiple fungicide resistant *Botrytis* isolates to have comparable fitness to sensitive isolates (Fernández-Ortuño et al., 2015; Rupp et al., 2017).

Cosseboom et al. (2020) registered survival and spread of multiple fungicide resistant *Botrytis* over the course of three years in untreated blackberry fields. Apparent lack of fitness cost in these cases and in our study could be attributed to compensatory mutations arising in the resistant proportion of the population (Ishii, 2015; Jeger et al., 2008). Such mutations can restore, or even improve, fitness in isolates that remain resistant, as demonstrated in a study with fludioxonil-resistant *Aspergillus nidulans* (Schoustra et al., 2006).

Another explanation is that continued fungicide application in the raspberry fields selected for the proportion of the populations with multiple fungicide resistance that included resistance to fenhexamid. Raspberry growers applied fungicide treatments, mainly the two products containing boscalid with pyraclostrobin and cyprodinil with fludioxonil, during the growing seasons from 2018 to 2020. In our data, increases in fenhexamid resistance over the course of the three-year sampling period were associated with increases in frequencies of isolates resistant to four or more fungicides. Hu et al. (2016) referred to inadvertently selecting for resistance to certain fungicides through the use of others in different groups as "selection by association" and demonstrated the effect with a detached-fruit assay in which application of pyraclostrobin selected for isolates resistant to boscalid, pyraclostrobin, fenhexamid, and thiophanate-methyl. Selection for multiple fungicide resistance in the detached fruit assay went at a faster pace than the increase of multiple fungicide resistance they witnessed in the field, so the authors assumed the multiple resistant strains had reduced fitness under field conditions (Hu et al., 2016). Selection by association can explain why fenhexamid resistance frequencies did not change dramatically in this study as all of the isolates from pre-season 2018 that were resistant to both boscalid and pyraclostrobin were also resistant to fenhexamid. This also explains decreases in fenhexamid resistance being associated with decreased number of applications of the product containing boscalid and pyraclostrobin.

Perhaps fewer treatments with boscalid and pyraclostrobin reduced fungicide selection pressure in the field, allowing for selection against isolates with fitness costs associated with fungicide resistance. Due to the patterns of multiple fungicide resistance and fungicide treatment data, selection for fenhexamid through use of the product containing boscalid and pyraclostrobin seems the most likely explanation for the persistence of fenhexamid resistance in the commercial raspberry fields.

The most reduction in fenhexamid resistance was observed in field F, with resistance starting at 83.3%, dropping to 20.8%, and increasing to 50.0% by the final sampling. Multiple fungicide resistance was also reduced in this field over the course of the sampling period. Fitness cost could have played a role in reduction in fenhexamid resistance as this field received the fewest number of total fungicide treatments. Only the product containing cyprodinil and fludioxonil was used in both 2018 and 2019 when fenhexamid resistance decreased. Fenhexamid resistance started to increase again after the product containing boscalid and pyraclostrobin was used in 2020. This grower may have used fewer total fungicide treatments because this field was under a high plastic tunnel, which can help prevent conditions conducive to grey mould (Nes et al., 2017; Xiao et al., 2001). Using cultural methods to reduce the need for fungicide input may be an approach to exploit potential fitness costs in the resistant proportion of the *Botrytis* population.

We conducted laboratory tests of fitness parameters to determine whether the raspberry field isolates showed reduced fitness in the absence of selective pressure from fungicides. *Botrytis* isolates from samples collected in the spring were selected for testing due to relevance for production of primary inoculum for the upcoming season. *Botrytis* is known for both its genetic variability and morphological plasticity (Atwell et al., 2015; Jarvis, 1977, Garfinkel,

2021; Walker, 2016), so we chose to test a large number of isolates, 92 for conidia production and 73 for growth rate. There were no significant differences between isolates resistant and sensitive to fenhexamid for conidia production. We chose to analyse growth rates using OD measurements to increase efficiency of testing a large number of isolates. No significant differences between isolates resistant and sensitive to fenhexamid were detected at either 18°C or 28°C. However, the outlier with the highest growth rate in one of the experiments conducted at 28°C was an isolate sensitive to fenhexamid, so this isolate may have an advantage at warmer temperatures.

The absence of any difference between raspberry field isolates resistant and sensitive to fenhexamid in conidia production and growth rate tests may indicate a lack of reduced fitness for resistant isolates for these parameters, however, our laboratory tests of fitness were not comprehensive. Other parameters, such as pathogenicity and stress survival, could be more relevant for fitness in the field (De Miccolis Angelini et al., 2015). In addition, we performed the tests on PDA, and ample nutrient access for the fungus can mask negative effects of fitness (Hawkins and Fraaije, 2018). In a study of the effect of genetic variation and plasticity on the manifestation of *B. cinerea* phenotypes, environment was shown to influence sporulation on growth media more than genotype (Corwin et al., 2016). We observed this when sporulation in the second conidia production experiment increased due to changing the way the agar plate lids were closed, which likely affected humidity levels within the plates. *In planta*, the interaction between environment and genotype contributes significantly to phenotype (Corwin et al., 2016), but interaction is, by nature of the tests, not included in *in vitro* analyses of conidia production and growth rate. Fitness as measured *in vitro* in a laboratory can therefore be difficult to translate into fitness in the field. Saito et al. (2014) also questioned the usefulness of laboratory studies for assessing practical fitness cost. Despite lack of

evidence of fitness cost for resistant isolates in conidia production and growth rate, fitness cost that is relevant for field survival still could exist and may have contributed to the reduction in fenhexamid resistance in raspberry field F.

Multiple fungicide resistance in *Botrytis* is a major problem in strawberry and raspberry production in Norway. Growers have access to few single-site fungicides, and continuing to use strategies of mixtures and alternations when multiple fungicide resistance already exists may only exacerbate the situation (Hu et al., 2016; Rupp et al., 2017). Results from the experimental strawberry field revealed a potential fitness cost for *Botrytis* resistant to fenhexamid and other fungicides, however, this reversal of resistance was obtained through halting fungicide use entirely. Fungicide use continued in the commercial raspberry fields, and this likely contributed to selection for fenhexamid due to the prevalence of multiple fungicide resistance in these fields. A trend of reduction of resistance associated with reduced number of fungicide treatments should be explored further. Using cultural methods to reduce grey mould disease pressure, and concurrently the need for fungicide applications, may therefore be the most important strategy to adopt in order to exploit potential fitness cost and reverse resistance.

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Table 1. Genetic information about single-spore isolates of *Botrytis cinerea* used for inoculum production for an experimental strawberry field.

						Gene (accession numbers[b])	
Isolate[a]	Cytb	Erg27	Mrr1	SdhB	Mating type	*nep2*	*g3pdh*
96/16-2.8	G143A	F412V	NA	H272Y	MAT1-1	OK556226	OK637132
96/16-9.2	G143A	F412S	NA	H272Y	MAT1-2	OK556243	OK637151
96/16-19.10	G143A	F412S	ΔL497	H272R	MAT1-1	OK556288	OK637197

[a] All isolates are *B. cinerea*, and 96/16-19.10 is in addition characterized as *Botrytis* group S.
[b]GenBank®

Table 2. Sampling data and number of *Botrytis* isolates obtained from an experimental perennial strawberry field over three years.

Sample number and time		Material	Number of isolates obtained
t0	May 2018	leaves	29
t1	July 2018	leaves	40
t2	October 2018	leaves	20
t3	April 2019	leaves	20
t4	July 2019	asymptomatic ripe fruit	6
		conidia swabs of infected fruit	25
t5	October 2019	leaves	20
t6	April 2020	leaves	19
t7	June 2020	leaves	20
		asymptomatic unripe fruit	9

Table 3. Fungicide groups, fungicides, discriminatory fungicide concentrations and growth media used in a mycelial growth assay[a] to test isolates of *Botrytis* for fungicide resistance.

Active ingredient	Group (abbreviation)	Concentration (mg/L)	Growth medium
Boscalid	Succinate dehydrogenase inhibitor (SDHI)	75	Yeast Bacto acetate agar
Fenhexamid	Ketoreductase inhibitor (KRI)	50	Malt extract agar
Fludioxonil	Phenylpyrrole (PP)	0.5	Malt extract agar
Fluopyram	Succinate dehydrogenase inhibitor (SDHI)	10	Yeast Bacto acetate agar
Pyraclostrobin + SHAM[b]	Quinone outside inhibitor (QoI)	10 + 100	Malt extract agar
Pyrimethanil	Anilinopyrimidine (AP)	4	Czapek-Dox agar
Thiophanate-methyl	Methyl benzimidazole carbamate (MBC)	100	Malt extract agar
Control	-	-	Czapek-Dox agar

[a] Adapted from Fernández-Ortuño et al. (2014).

[b] Amended with salicylhydroxamic acid (SHAM) dissolved in methanol for alternative oxidase inhibition.

Table 4. Fungicide resistance frequencies for *Botrytis* isolates from an experimental perennial strawberry field in Norway.

Sampling time[b]	Number of isolates	Fungicide resistance[a]					
		Boscalid %MR+R	Fludioxonil %R	Fluopyram %R	Pyraclostrobin %R	Pyrimethanil %R	Thiophanate-methyl %R
t0	29	34.5	0	0	62.1	10.3	34.5
t1	40	90.0	12.5	0	95.0	62.5	72.5
t7	29[c]	6.9	0	0	14.3	0	13.8

[a] Resistance categories are based on a mycelial growth assay, and include resistant (R), moderately resistant (MR), low resistant (LR), and sensitive (S) as in Fernández-Ortuño et al. (2014).
[b] Samples t0, t1, and t7 were collected in May 2018, July 2018, and June 2020, respectively.
[c] For t7, n = 28 for pyraclostrobin only.

Table 5. Fenhexamid resistance frequencies for *Botrytis* isolates obtained pre-season and post-season from six commercial perennial raspberry fields (A to F), from 2018 to 2021.

	% *Botrytis* isolates resistant to fenhexamid[a] (Number of isolates tested)						
Field	Pre-18	Post-18	Pre-19	Post-19	Pre-20	Post-20	Pre-21
A	87.0 (23)	100.0 (3)	100.0 (15)	84.6 (13)	36.8 (19)	NA[b]	95.0 (20)
B	72.0 (25)	100.0 (4)	93.8 (16)	92.3 (13)	75.0 (24)	NA[b]	76.2 (21)
C	92.0 (25)	NA	93.3 (15)	100.0 (12)	89.3 (28)	NA[b]	100.0 (20)
D	26.7 (15)	100.0 (5)	11.1 (18)	17.6 (17)	16.7 (18)	26.9 (26)	20.0 (5)
E	66.7 (24)	78.6 (14)	50.0 (4)	72.7 (11)	88.9 (9)	76.5 (34)	50.0 (14)
F	83.3 (18)	50.0 (16)	52.6 (19)	27.3 (11)	20.8 (24)	25.0 (16)	50.0 (10)

[a] Resistance determined by a mycelial growth assay adapted from Fernández-Ortuño et al. (2014).

[b] Not available

Table 6. Fungicide resistance frequencies for *Botrytis* isolates obtained from six commercial perennial raspberry fields in Norway, at the start of the experiment in 2018 and at the end of the experiment in 2021

			Fungicide resistance[a]					
			Boscalid	Fludioxonil	Fluopyram	Pyraclostrobin	Pyrimethanil	Thiophanate-methyl
Field	Sampling time[b]	Isolates	%MR+R	%R	%R	%R	%R	%R
A	Pre-18	23	8.7	34.8	0	78.3	82.6	26.1
	Pre-21	20	85.0	25.0	0	80.0	85.0	10.0
B	Pre-18	25	56.0	8.0	0	20.0	64.0	24.0
	Pre-21	21	71.4	52.4	0	33.3	47.6	19.0
C	Pre-18	25	84.0	20.0	0	64.0	92.0	8.0
	Pre-21	20	100	25.0	0	70.0	90.0	0
D	Pre-18	15	13.3	20.0	0	40.0	40.0	26.7
	Pre-21	5	20.0	20.0	0	0	20.0	20.0
E	Pre-18	24	50.0	20.8	0	37.5	33.3	16.7
	Pre-21	14	78.6	71.4	0	7.1	57.1	7.1
F	Pre-18	28	64.3	35.7	0	50.0	67.9	35.7
	Pre-21	10	50.0	20.0	0	30.0	60.0	20.0

[a] Determined by a mycelial growth assay adapted from Fernández-Ortuño et al. (2014). Categories moderately resistant (MR) and resistant (R) are included in the calculations for the table as indicated.
[b] Pre-season 2018 (Pre-18) and pre-season 2021 (Pre-21).

Table 7. Frequencies of multiple fungicide resistance in *Botrytis* isolates from six commercial raspberry fields in Norway.

			Multiple fungicide resistance[a]							
Field	Sample[b]	Isolates	%0R	%1R	%2R	%3R	%4R	%5R	%6R	%BP[c]
A	Pre-2018	23	13.0	0	8.7	43.5	13.0	13.0	8.7	8.7
	Pre-2021	20	5.0	0	5.0	20.0	50.0	10.0	10.0	70.0
B	Pre-2018	25	16.0	12.0	12.0	36.0	20.0	4.0	0	12.0
	Pre-2021	21	9.5	19.0	0.0	33.3	14.3	19.0	4.8	33.3
C	Pre-2018	25	4.0	0	4.0	28.0	56.0	4.0	4.0	52.0
	Pre-2021	20	0	0	0	30.0	55.0	15.0	0	70.0
D	Pre-2018	15	46.7	20.0	6.7	0.0	6.7	13.3	6.7	6.7
	Pre-2021	5	80.0	0	0	0	0	20.0	0	0
E	Pre-2018	24	25.0	16.7	8.3	16.7	25.0	8.3	0	25.0
	Pre-2021	14	14.3	7.1	0	57.1	14.3	7.1	0	7.1
F	Pre-2018	28	14.3	3.6	17.9	7.1	17.9	28.6	10.7	42.9
	Pre-2021	10	40.0	0	10.0	20.0	10.0	10.0	10.0	30.0

[a] Resistance tested with a mycelial growth assay for boscalid, fenhexamid, fludioxonil, fluopyram, pyraclostrobin, pyrimethanil, and thiophanate-methyl adapted from Fernández-Ortuño et al. (2014). Multiple fungicide resistant phenotypes are represented by the number of accumulated resistant (R) results per isolate (0R-6R), except for boscalid where moderately resistant (MR) was also included.

[b] Pre-season 2018 (Pre-2018) and pre-season 2021 (Pre-2021).

[c] MR or R to boscalid and R to pyraclostrobin.

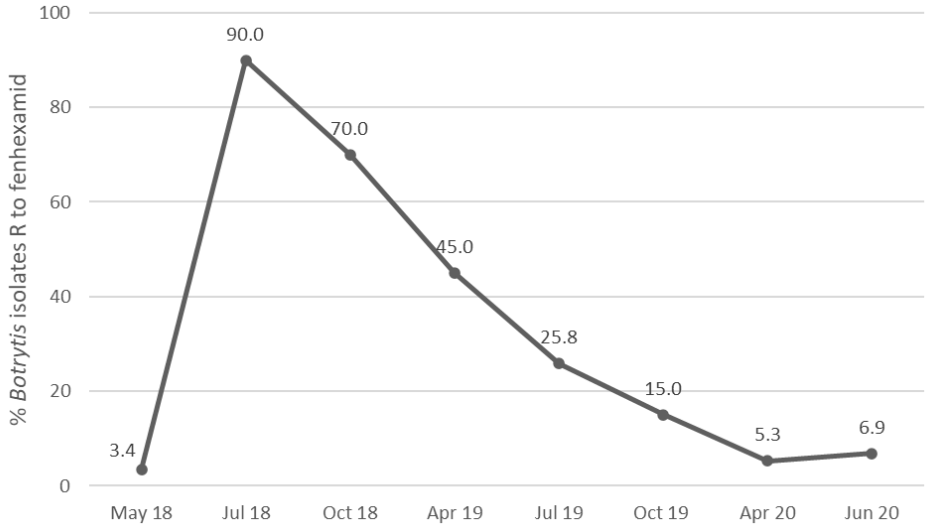

Figure 1. Fenhexamid resistance frequencies in *Botrytis* isolates from an experimental perennial strawberry field as determined with a mycelial growth assay. Resistant inoculum (sporulating mycelium on agar) was placed in the field four times between sampling in May 2018 and July 2018 and once in September 2018. The field received five fenhexamid treatments in June 2018 and no further fungicide treatments.

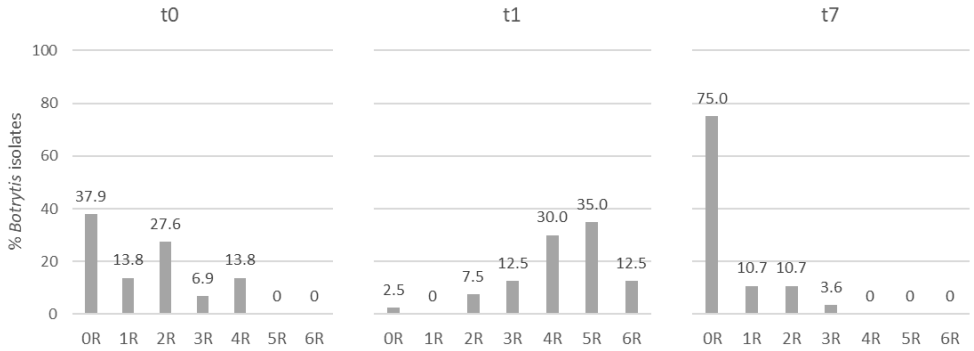

Figure 2. Frequencies of multiple fungicide resistance in *Botrytis* isolates collected at three times from an experimental strawberry field. Times of collection (t0, t1, and t7) were in May 2018, July 2018, and June 2020, respectively. Resistance was determined by a mycelial growth assay adapted from Fernández-Ortuño et al. (2014). Multiple fungicide resistant phenotypes are represented by the number of accumulated resistant (R) results per isolate (0R-6R), except for boscalid where moderately resistant (MR) was also included. Number of isolates assessed for multiple fungicide resistance for sampling times t0, t1, and t7 were 29, 40, and 28, respectively.

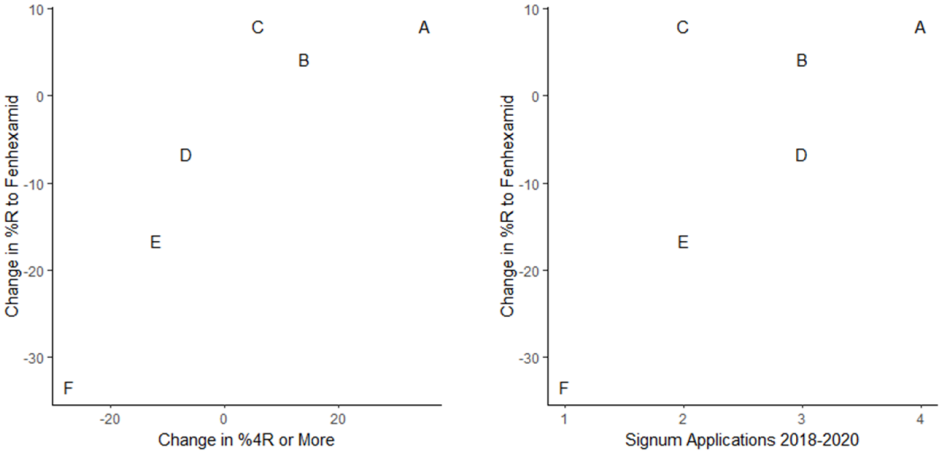

Figure 3. Changes in fenhexamid resistance frequencies for Botrytis isolates from six commercial perennial raspberry fields (A to F) compared in frequency of isolates resistant to four (4R) or more fungicides and to total number of fungicide applications (Signum® - boscalid and pyraclostrobin). Change in resistance frequencies and multiple resistant phenotypes is based on isolates from samples collected pre-season 2018 and pre-season 2021. Resistance results were determined with a mycelial growth assay adapted from Fernández-Ortuño et al. (2014), and multiple resistance phenotypes were determined based on number of resistant (R) results for all fungicides except boscalid, which also included moderately resistant (MR) results. Number of Signum® applications from 2018 to 2020 includes all applications between the sampling times for resistance testing.

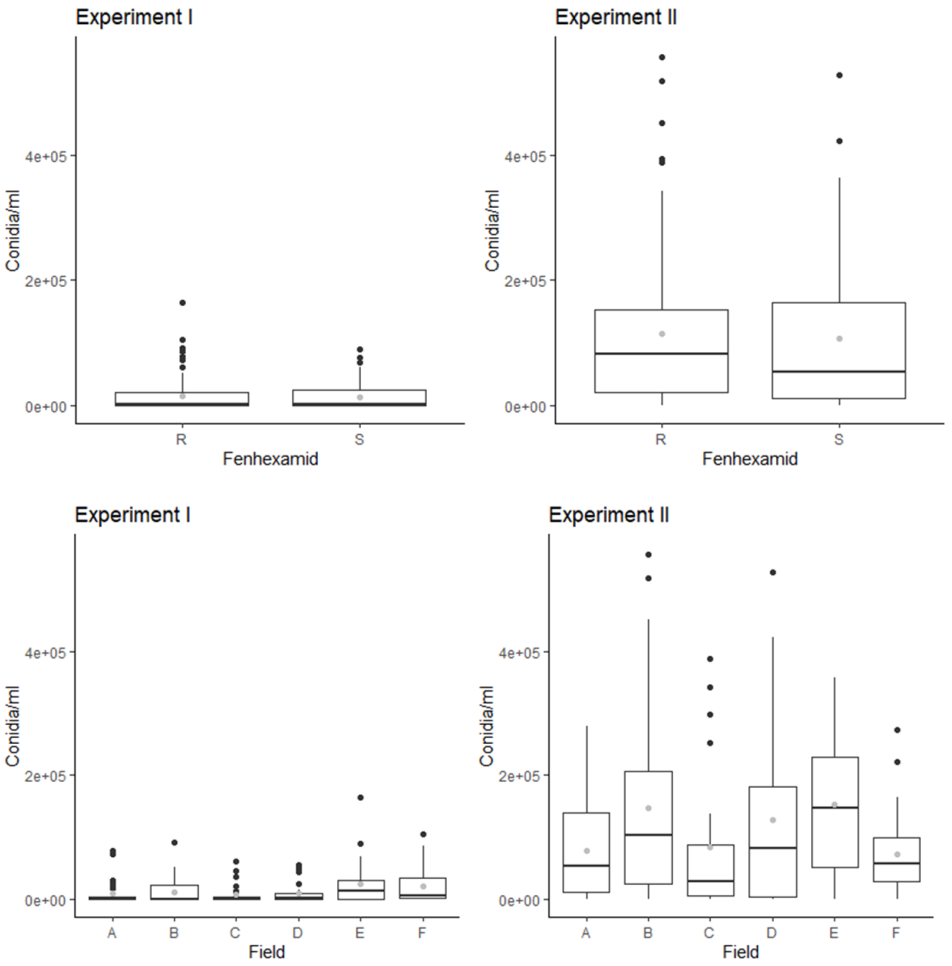

Figure 4. Conidia production by *Botrytis* isolates obtained from commercial perennial raspberry fields. Conidia production is given as concentrations of conidia suspensions harvested from isolates (conidia/ml) that were resistant (R) or sensitive (S) to fenhexamid. Conidia production is shown by commercial perennial raspberry fields (A to F) from which *Botrytis* isolates were obtained pre-season 2020 and pre-season 2021. Agar plates with isolates were completely sealed with parafilm during incubation in experiment I, but only taped on two spots on the plates for experiment II. Median values are indicated by bold, horizontal bars, means by grey dots, and outliers by black dots. Hinges are at the 1st and 3rd quantiles.

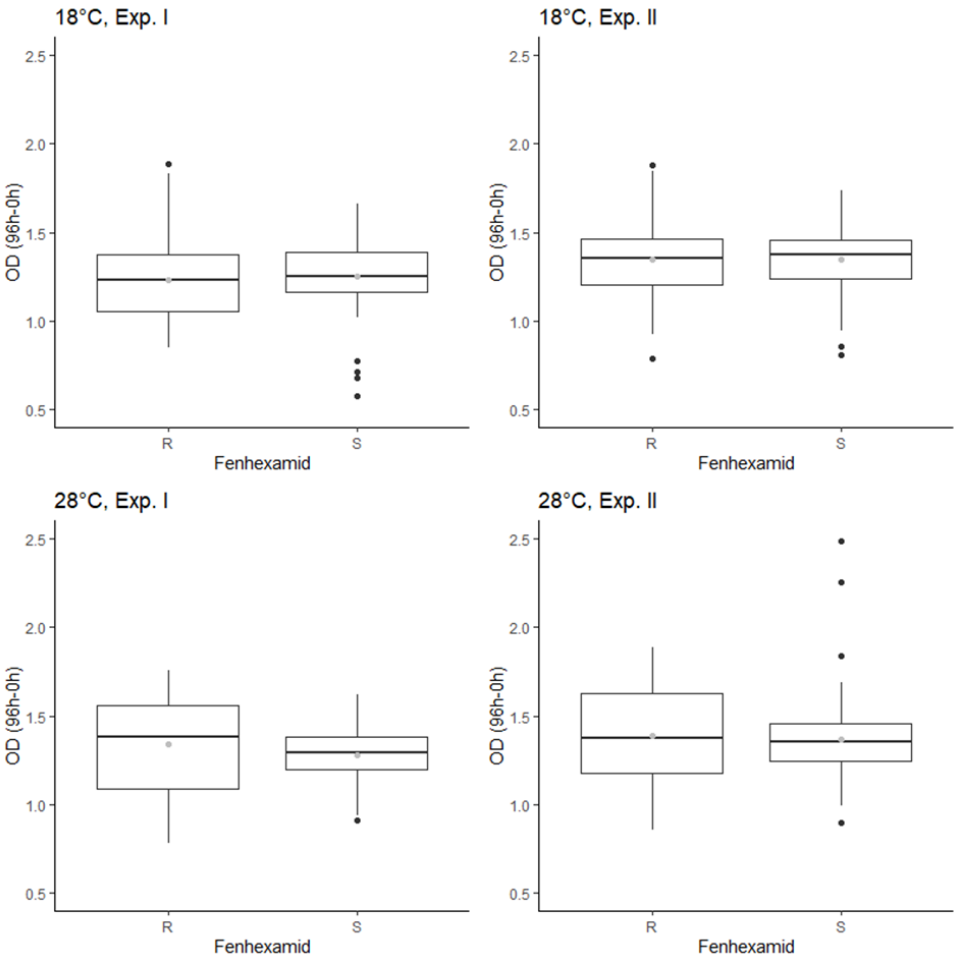

Figure 5. Growth rate as measured by increase in optical density (OD) from 0 to 96 hours for 73 *Botrytis* isolates resistant (R) or sensitive (S) to fenhexamid. Conidia were suspended in semi-solid medium and incubated at 18°C and 28°C, with two experiments at each temperature. There was no statistically significant difference between isolates R and S to fenhexamid in any of the experiments. Median values are indicated by bold, horizontal bars, means by grey dots, and outliers by black dots. Hinges are at the 1st and 3rd quantiles.

Supplementary Table 1. Fungicide treatments for six commercial perennial raspberry fields where samples were collected for *Botrytis* isolation and fungicide resistance testing.

Field	Fungicide products[a] (number of treatments)		
	2018	2019	2020
A	Signum® (1) Switch® 62.5 WG (2)	Signum® (2) Switch® 62.5 WG (2)	Signum® (1) Switch® 62.5 WG (1)
B	Signum® (1) Switch® 62.5 WG (2)	Signum® (1) Switch® 62.5 WG (2)	Signum® (1) Switch® 62.5 WG (1)
C	Switch® 62.5 WG (2)	Signum® (1) Switch® 62.5 WG (2)	Signum® (1) Switch® 62.5 WG (2)
D	Signum® (1) Switch® 62.5 WG (2)	Signum® (1) Switch® 62.5 WG (2)	Signum® (1) Switch® 62.5 WG (2)
E	Rovral® (1) Switch® 62.5 WG (2)	Signum® (1) Teldor® WG 50 (2)	Signum® (1) Switch® 62.5 WG (2)
F	Switch® 62.5 WG (2)	Switch® 62.5 WG (2)	Signum® (1) Switch® 62.5 WG (1)

[a] Fungicide products included the following (active ingredients and group in parentheses): Rovral® (iprodione, dicarboximide), Signum® (boscalid, SDHI; pyraclostrobin, QoI), Switch® 62.5 WG (cyprodinil, AP; fludioxonil, PP), and Teldor® WG 50 (fenhexamid, KRI).

Steam thermotherapy strongly reduces *Botrytis* in strawberry transplants without negative effects on plant growth and yield

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Paper IV

Steam thermotherapy strongly reduces *Botrytis* in strawberry transplants without negative effects on plant growth and yield

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Abstract

The effect of steam treatments in reducing *Botrytis* spp. populations in strawberry transplants was studied. Tray plants (rooted in 0.2 L peat plugs) of seasonal flowering cvs. Falco, Sonsation, and Soprano, and everbearing cvs. Favori and Murano were exposed to a pre-treatment with steam at 37°C for 1 h, followed by 1 h at ambient temperature and air humidity, and then 2 h or 4 h steam treatment at 44°C. For all cultivars, there was no or a negligible negative effect of the steam treatments on plant growth and yield. In mean of all five cultivars for plants exposed to 2 and 4 h at 44°C compared to the untreated, the incidence of *Botrytis* was significantly reduced by 43 and 86%, respectively. If comparing treatments within cultivars, except for the two experiments with cv. Soprano, the 2 h treatment was not significantly different from the untreated. For the 4 h treatment, incidence was reduced with 71 to 100% compared to the untreated, and it was significant in 3 of 6 experiments (one with cv. Murano and both with cv. Soprano). In separate experiments, 90 to 100% (mean 97%) of sclerotia originating from four different isolates of *Botrytis* spp. were killed when exposed to the pre-treatment and 4 h at 44°C. Screening for fungicide resistance of recovered isolates

from the strawberry transplants, revealed that 27.4, 38.7, 11.3, 12.9 and 3.2% of the isolates were resistant to 1, 2, 3, 4, or 5 fungicides, respectively. Overall, our findings clearly indicate that aerated steam treatment strongly reduces populations of *Botrytis* spp. and therefore the quantity of fungicide-resistant strains in strawberry transplants, with no or insignificant negative impacts on the transplants.

Introduction

Grey mould, caused by *Botrytis* spp., can lead to severe losses in strawberry. The infection often remains asymptomatic in the early stage, and invasion and sporulation gradually increase with fruit maturity and leaf senescence (Bristow et al., 1986; Carisse, 2016; Petrasch et al., 2019). Latent infection of *Botrytis* spp. can remain undetected in planting material, and healthy-appearing transplants produced under certified guidelines can become a primary source of inoculum (Oliveira et al., 2017).

Current management of grey mould is mostly based on use of fungicides (Petrasch et al., 2019; Wedge et al., 2007). Since *Botrytis* spp. are polycyclic pathogens and able to cause latent infections, timing and frequency of fungicide applications are vital for effective disease control (Mertely et al., 2002; Powelson, 1960). However, the genetic variability within the *Botrytis* population, a polycyclic lifecycle and abundant sporulation contributes to making it a high-risk pathogen for developing fungicide resistance (Hahn, 2014). An increasing number of cases with fungicide resistance in *Botrytis* populations challenge the available chemical control measures (Fernández-Ortuño et al., 2014; Hahn, 2014; Leroch et al., 2013).

In commercial strawberry production, transplants are shipped from nurseries to fruit production sites. Previous studies have shown the presence of fungicide resistant strains of *Botrytis* spp. in strawberry transplants (Nielsen et al., 2020; Oliveira et al., 2017; Weber & Entrop, 2017). The introduction of resistant strains with planting material poses a great risk of failure in controlling grey mould in the strawberry fruit production. With the increasing threat of fungicide resistance, the need for non-chemical approaches to reduce the pathogen population is therefore crucial.

Thermotherapy, primarily warm water dipping of strawberry transplants has been practised successfully against numerous phytopathogenic microbes and pests (Goheen & McGrew, 1954; Klingler, 1969; Miller & Stoddard, 1956; Smith & Goldsmith, 1936; Staniland, 1953). Strawberry cultivars vary in their ability to survive heat treatments, but plants tolerated better heat treatments if they had well developed roots and soil moisture was low (Mellor & Fitzpatrick, 1961). Furthermore, the more dormant the plants were, the better they tolerated warm water treatments, and storing plants for two weeks at 0°C increased the tolerance of the hot-water treatments (Goheen et al., 1956). However, in experiments with four strawberry cultivars, Turechek and Peres (2009) had the opposite effect; cold-stored plants of cv. Ventana were quite intolerant to heat treatments, but when fresh-dug plants were used, it was among the cultivars which performed best. Another cultivar, Strawberry Festival, was very tolerant to warm water treatments independent whether it was cold stored or not (Turechek & Peres, 2009). Non-dormant runner plants of seven cultivars all tolerated a treatment killing endoparasitic nematodes (Staniland, 1953).

A technique for warm water dipping of strawberry transplants was developed in Florida (Brown et al., 2016). This included a pre-conditioning treatment at 37°C inducing heat shock proteins that increase heat tolerance in the strawberry plants, followed by one hour at ambient temperature and then a pathogen-eradicative treatment for four hours at 44°C. This protocol has been used with warm water steaming of transplants against several strawberry pathogens, including *B. cinerea*, *Colletotrichum acutatum*, *Podosphaera aphanis* and *Xanthomonas fragariae* (Da Silva Jr et al., 2019; Wang et al., 2017; Turechek et al., 2021; Zuniga, 2018; Zuniga & Peres, 2017). Bare root plants of six everbearing strawberry cultivars

exhibited no negative effects of this treatment (Wang et al., 2019), and in nursery propagation fields where plants had received the above steam treatment, there was no reduction in plant yield (Turechek et al., 2021). The main objective of this study was to investigate the effect of warm steam treatments of rooted plug plants (tray plants) of strawberry transplants on subsequent quality and yield after planting and the presence of latent infections of *Botrytis* spp. Furthermore, a study on the effect of steam on viability of *Botrytis* sclerotia was included. Finally, the presence of fungicide resistant strains in the planting material was investigated.

Materials and methods

The steam chamber

The unit used for aerated steam treatments in Norway was constructed by the company Myhrene AS, currently named Plantsauna AS (Lier, Norway). Similar large- and small-scale research units for steaming of strawberry transplants were constructed by the same manufacturer in California and Florida (Turechek et al., 2021). The unit used in the present experiments contained two rooms (chambers) on top of each other, separated by a horizontal wall, i.e., ceiling of the lower antechamber and floor of the upper treatment chamber. The whole unit had insulated walls with a thickness of 12.5 cm, and the inside floor/ceiling area measured 94 × 150 cm. The height of the treatment chamber was 112 cm, while the height of the antechamber was 4.7 cm. The antechamber was an air-filled space that contained a 15 mm wide copper pipe attached to a 1.2 KW steam generator (10KOHM, Tylö AB, Halmstad, Sweden). The pipe had ten circular openings with diameters ranging from 2.5 to 6 mm, and the diameter of the opening increased with the increase in distance from the steam generator, to ensure uniform dispersion of steam. The steam left the pipe and went into the

antechamber and passed further up into the main treatment chamber through 2 cm wide slits along each of the long sides of the floor of the main chamber.

The temperature of the steam leaving the generator was 100°C, and when released into the air of the antechamber, which had a lower temperature, the air became water saturated. The temperature of the water saturated air in the treatment chamber was regulated by a thermostat. An electronic sensor regulated the steam production in the generator, and subsequently the temperature in the antechamber. The sensor was placed in a position so that none of the slits along the main chamber floor had higher air temperature than the sensor itself. At start of the steaming process, the main chamber had ambient temperature. Water saturated warm air from the antechamber raised along the walls of the main chamber, and when it met the cooler air in the main chamber, the air condensed and heated the air in the chamber. The steam production stopped when the main chamber reached the desired temperature, and the temperature distribution was balanced (± 0.5°C). When the temperature decreased due to loss of energy to the room outside the chamber, the generator re-started to produce warm steam, and a vertical balance in temperature and steam was maintained in the main chamber. Since the plants placed in the main chamber were colder than the water saturated air, water condensed on their surfaces. When plants and the chamber had the same temperature, plants would remain at a constant temperature and wet throughout the treatment period.

Effect of aerated steam treatment on strawberry transplants with *Botrytis*

In the current experiments with cold stored tray plants (rooted in 0.2 L peat plugs) plants of five strawberry cultivars were exposed to a pre-treatment at 37°C in aerated steam, followed

by 1 h in ambient air (20-25°C and around 30-50% RH), and then 2 or 4 h at 44°C in aerated steam. These treatments were originally developed in Florida (Brown et al., 2016; Turechek et al., 2013 & 2021).

For the seasonal flowering cv. Soprano, two replicated experiments were conducted in April 2020, and 120 strawberry transplants were randomly and equally divided into three treatment groups. Transplants to be steam treated were placed into four 20 L IFCO trays (IFCO Systems GmbH, Pullach, Germany). Each tray had 20 transplants and one Tinytag TGP-4500 temperature and RH logger (Gemini data loggers, Chichester, UK) placed centrally in close vicinity to the plants. After steam treatment, for half of the transplants in each treatment group (20 plants) the plug and root parts were removed, and the aerial parts were stored at -20°C before further analysis. The other half of the plants were potted in 3.5 L pots filled with fertilized and limed peat soil for further growth and yield assessments. The pots were placed on wooden pallets, approximately 15 cm above ground, in open air between two greenhouses. For each of the two runs, plants were placed in a randomized block design. The plants were overhead irrigated by a shower-hose when needed. From the sixth week of potting, fertilizing was done once weekly. When fruiting started, plants were covered with a nylon net to prevent the loss of fruits to birds. There was no use of pesticides throughout the growth period. There were five plants in each of four replicates in each treatment, and there were two replicates over time of the experiment.

For the remaining four cultivars, there were two steam experiments carried out in late April and early May 2020, respectively. The first experiment was with two everbearing cultivars (Favori and Murano) and the second with two seasonal flowering cultivars (Falco and

Sonsation). The plants of each cultivar were randomly divided into three treatment groups as mentioned above. After the heat treatments, plant parts including whole leaves and leaflets were collected from the transplants and stored at -20°C for laboratory analysis. All plants were then brought to a high plastic tunnel research facility for further growth and yield assessments. Plants were potted in 50 cm rectangular plastic trays, either with three (everbearing cultivars) or four (seasonal flowering cultivars) plants in each tray, with either three or two trays per treatment plot, respectively. The experiments were randomized block designs with three replicates. The growth medium was fertilized and limed peat mixed with perlite (10% v/v), and the plants were drip irrigated with fertilized water.

Various growth and yield parameters were assessed for the five cultivars, including first day of flowering, number of runners, crowns, shoots, leaves, shoots, plant height, number of fruits, fruit weight, above ground fresh and dry weight, and root dry weight. It took 14, 16, and 23 weeks from potting to final assessments for cv. Soprano (dry weight recorded two weeks later), cvs. Falco and Sonsation, and cvs. Favori and Murano, respectively.

To analyse the efficiency of aerated steam treatment to eradicate *Botrytis* spp., incubation of freezer stored plant material was done. Frozen samples were allowed to thaw, rinsed in running tap water, dried and placed in 14 cm diameter Petri dishes with 12.5 cm diameter Whatman™ filter papers moistened with 2 ml autoclaved water to maintain high humidity. The Petri dishes were sealed in plastic bags and incubated at ambient room temperature. On the seventh day following start of incubation, plant parts were observed under a stereo microscope (14 ×) for the presence of conidiophores and conidia of *Botrytis* spp.

In each of the two experimental runs with cv. Soprano, there were five transplants in each of four replicates. There were three to four leaves per plant, and since number and size of the leaves varied, the leaf tissue of each plant was incubated in two or three Petri dishes. A mean incidence (%) per plant was calculated by dividing number of leaves with *Botrytis* with the total number of leaves incubated. For the remaining cultivars, both leaflets and whole leaves (leaf units) were collected and arbitrarily split in four replicates for each cultivar before incubation as described above. The number of Petri dishes per replicate were either one or two. Incidence (%) was calculated as described above, and in addition, the area of each leaf unit covered with *Botrytis* was used to calculate a mean severity (%) per replicate.

Effect of aerated steam on sclerotia of *Botrytis* spp.

Four single spore isolates from strawberry in Norway that had been part of an investigation on fungicide resistance were exposed to steam treatments. Two of the isolates were of *Botrytis* group S (hereafter named BS1 and BS2) and the other two were *B. cinerea sensu stricto* and *B. pseudocinerea* (hereafter named Bc and Bp), and the four isolates used were 96/16-16.4, 96/16-19.10, 96/16-15.3 and 96/16-18.5 in Nielsen et al. (Paper I in this thesis, unpublished), respectively, where more details regarding their resistance profiles may be found. The isolates had been stored as single spore isolates in glycerol solution at -20˚C. The isolates were allowed to thaw at room temperature and vortexed for 20 seconds to make the solution homogenous. Then 5 µl of solution from each isolate was transferred to 4-cm Petri dishes with acidified (0.2% w/v tartaric acid) PDA (Difco™ Potato dextrose agar) and incubated at 20°C. Once the mycelium grew to the edge of the dishes, isolates were transferred to 9-cm PDA Petri dishes and incubated at 10°C in continuous darkness in an incubation chamber (Versatile Environmental Test Chamber, MLR-352H-PE, PHC Corporation,

Japan). Sclerotia were harvested with sterile forceps after 3 months of incubation. Excess agar was removed by rubbing them between filter papers and then transferred to a Retsch stainless steel sieve (CISA, Spain) and rinsed under running tap water. Rinsed sclerotia were placed on top of the stainless-steel sieve with filter paper and allowed to dry at room temperature. The size of the sclerotia varied greatly among the isolates. When weighing 100 dry sclerotia arbitrarily collected within each of the isolates BS1, BS2, BC, and BP, the mean weights of one sclerotium were 1.6, 12.6, 15.3, and 10.4 mg, respectively. Seven sclerotia of each isolate were packed in 5 × 5 cm^2 muslin cloth and closed with wire twist ties.

Aerated steam treatment of sclerotia took place in the same unit as described above. There were two replicated experiments over time, each with two treatment groups (untreated control and heat-treated), and in each of four replicates there were four muslin cloth bags included. The heat treatment was as described above but included only the 4 h treatment at 44°C. Bags belonging to different replicates were hung in four different IFCO trays, and each one of them were equipped with a Tinytag TGP-4500 logger. After completion of heat treatment, both treatment and control groups of sclerotia were brought to the laboratory and surface sterilized in a 1% NaOCl solution. Then they were transferred individually to 24-well plates (Nunclon^{TM} Delta Surface, Thermo Fisher Scientific, Denmark) containing 1 ml PDA in each well. The plates were incubated in the growth chamber as described earlier at 20°C, with 12h light/dark cycle and 85% RH. Assessment of germination was based on observation under a stereo microscope (14×) on the 14th day following incubation.

Fungicide sensitivity test

Cold-stored bulk isolates of *Botrytis* spp. (species not identified) obtained from cvs. Falco, Favori and Murano, including 25, 23 and 14 isolates from untreated, 2 h or 4 h treated transplants, respectively, were subjected to a fungicide sensitivity test. The isolates were tested for sensitivity to seven fungicides (product and manufacturer in parentheses): boscalid (Cantus®, BASF); fenhexamid (Teldor® WG 50, Bayer); fludioxonil (Geoxe® 50 WG, Syngenta); fluopyram (Luna® Privilege, Bayer); pyraclostrobin (Comet® Pro, BASF); pyrimethanil (Scala®, BASF); and Thiophanate-methyl (Topsin® WG, Nisso Chemical Europe). Previously determined discriminatory doses of the fungicides on suitable growth media were used (Fernández-Ortuño et al., 2014, Schnabel et al., 2015; Weber & Hahn 2011; Weber et al., 2015). The prepared amended growth media with fungicides were poured into a designated row in two 24-well plates (Nunclon™ Delta Surface, Thermo Scientific, Denmark), as described by Fernández-Ortuño et al. (2014). In the control row, growth medium (Czapek's agar, CZA) was used without added fungicide.

Once all the *Botrytis* isolates sporulated on PDA plates, conidia were transferred with the help of sterile toothpicks to 24-well plates, to designated columns of plates. After conidial transfer, plates were incubated in the same incubation chamber and under the same conditions as mentioned above for sclerotia. Assessment of fungicide resistance was done on the fourth day of incubation. Resistance categories used for assessment were based on diametric growth of mycelium on 15 mm diameter agar wells; sensitive (no mycelium growth), low resistant (< 20 %), moderately resistant (20-50%) and resistant (> 50 %) (Schnabel et al. 2015). Visual assessment was done with a stereo microscope (14×).

Statistical analysis

Data obtained from the experiments were analysed with either one- or two-way analysis of variance (ANOVA) in R version 4.0.4 (2020-06-22). Fisher's protected least significant difference (LSD) test at $P \leq 0.05$ was used to obtain mean and grouping information after one-way analysis of variance and Tukey's HSD test was used to obtain mean and grouping information after two-way analysis of variance. A Chi-square test ($\chi 2$ test, $P \leq 0.05$) of independence was carried out to see if there was a link between isolates and the sensitivity of sclerotia to aerated steam treatment using the same R version as above. Post-hoc analysis of the Chi-square test was done with Fisher's Exact Test.

Results

Temperature profiles

The temperature profile for one experiment with cv. Soprano is shown in Fig. 1; profiles for the other experiments did not deviate from this, and data are therefore not presented. The temperature was reached around 20 minutes after it was set to either 37 or 44°C and was stable (± 0.4°C) thereafter. During the experiments with transplants, removal of two boxes from the chamber after 2 hours treatment was followed by a slight but very brief drop in temperature.

Growth and yield of strawberry transplants

Growth and yield parameters, including days to first flowering, number of runners, leaves and crowns, plant height and weight were examined. There were only marginal differences among the cultivars for the different treatments, and steaming did not seem to have any overall positive or negative effects on plant performance (Table 1).

Effect of steam on *Botrytis* in five cultivars

In comparison with the untreated in mean of six experiments (two experiments with cv. Soprano and one each for the other four cultivars), *Botrytis* incidence was reduced with 43 and 86%, respectively, for transplants exposed to 2 or 4 h at 44°C (Fig. 2, $P < 0.001$). However, if comparing within cultivars, the 2 h treatment was not significantly lower than the untreated, except for cv. Soprano. In the 4 h treatment, incidence was reduced with 71 to 100% compared to the untreated, but it was significant only for cv. Murano ($P = 0.018$) and for the two experiments with cv. Soprano ($P < 0.001$). The mean severity of *Botrytis* for cvs. Falco, Favori, Murano and Sensation (not recorded for cv. Soprano) was reduced with 32 and 84% for the 2 h and 4 h treatments, respectively, but it was significant only for the latter (Table 2).

Effect of steam on sclerotia of *Botrytis* spp.

All untreated sclerotia of the four *Botrytis* spp. isolates germinated in the 24-well agar plates. Of totally 192 sclerotia of each isolate in both experiments, number of sclerotia surviving the 1 h pre-treatment at 37°C + the 4 h treatment at 44°C was 0, 2, 3 and 18 for the isolates BS1, BS2, Bp and Bc, respectively. This means that 90 to 100% (mean 97%) of the conidia were killed by the steam treatment. The Chi square test showed that sclerotia from the four isolates were different in their sensitivity to heat treatment, χ^2 (3, N = 768) = 36.7, $P < 0.001$. The post hoc test indicated that sclerotia of isolate BC were significantly less sensitive to heat treatment than the other three isolates ($P < 0.001$).

Fungicide sensitivity test

Of the 62 isolates, 93.5% were resistant (R) to one or more fungicides. In mean of all isolates, R varied from 1.6 % against fluopyram to around 90% against pyraclostrobin (Table 3). Moreover, 27.4, 38.7, 11.3, 12.9 and 3.2% of the isolates were resistant to 1, 2, 3, 4, or 5 fungicides, respectively.

Discussion

The strawberry transplants used in these experiments were imported material from commercial nurseries, they carried quiescent infections of *Botrytis* spp., and isolates obtained from the plants were resistant to up to five fungicides. There was no or insignificant negative effect of the steam treatments on plant growth and yield, and the 4 h treatment at 44°C effectively suppressed *Botrytis*. While all untreated sclerotia were viable, a mean of only 3% of them germinated following steam treatment. The present experiments therefore clearly showed the potential in reducing *Botrytis* in strawberry transplants, and consequently also a reduction in the content of fungicide resistant strains, without negative impact on the planting material. The present results confirm previous findings in Florida and Norway showing very good effect of steam treatments of strawberry transplants against several pathogens (Da Silva Jr. et al., 2019; Weber et al., 2017; Turechek et al., 2021; Zuniga, 2018; Zuniga & Peres, 2017), including *Botrytis* (Zuniga, 2018; Zuniga & Peres, 2017), without negative growth and yield effects (Turechek et al., 2021; Wang et al., 2019; Zuniga, 2018).

Strawberry transplants may have different abilities to tolerate heat treatments (Goheen et al., 1956; Mellor & Fitzpatrick 1961; Turechek & Peres, 2009). Fresh dug plants performed better than cold-stored plants of the same cultivar when plants were dipped in warm water (Turechek & Peres, 2009), while in another report, cold-stored, dormant plants tolerated heat

treatments the best (Goheen et al., 1956). Both cold-stored steam treated bare root transplants of six cultivars (Wang et al., 2019), and the cold-stored plug plants (tray plants) of the five cultivars used in the present experiments, tolerated the steam treatments well. For neither of the two plant qualities, there were any or minimal negative growth and yield effects; however, further studies are needed to reveal if other plant qualities and cultivars may be as tolerant.

Although the effect varied when considering each cultivar separately, the overall effect of the warm steam treatments gave a considerable and significant reduction in *Botrytis*, both for the 2 and 4 h treatments, but there was also a significant reduction from 2 to 4 h. Dormant structures of *Botrytis* spp. were probably either killed or deactivated upon exposure to the high temperature. Similar results were obtained in a study in Florida, where warm steam treatment of strawberry transplants significantly reduced the incidence of *B. cinerea*, and conidia were killed after 30 min if exposed to 44°C in a water bath (Zuniga, 2018; Zuniga & Peres, 2017). *Botrytis* spp. are known to survive freezing temperature, but high temperatures on the other hand may hamper disease development and survivability of overwintering structures (Droby & Lichter, 2007; Jarvis, 1977; Oliveira et al., 2017). This heat susceptibility may thus make *Botrytis* spp. a suitable candidate for heat treatment.

In the present trials, steam treatments strongly reduced viability of sclerotia of four isolates when exposed to the 37°C pre-treatment followed by 4 h at 44°C. In an investigation in Florida, from zero to 44% of sclerotia survived when immersed in test tubes in a water bath at 44°C for 4 h, and even at 48 and 52°C for 4 h sclerotia could survive the treatment (Zuniga, 2018). In the latter experiments with sclerotia, there was a great variability among isolates in

susceptibility to heat treatments, but there was no indication provided for this discrepancy. For sclerotia of the four isolates used in the present experiments, there also seemed to be a difference in heat susceptibility, possibly due to differences in size. None of the sclerotia of BS1 germinated, and these were by far the smallest, while the largest sclerotia of isolate BC had the highest germination rate; sclerotia of BC weighed around 10 times as much as those of BS1.

Oliveira et al. (2017) in Florida found that grey mould on plant debris in strawberry fields did not survive the warm summer temperatures occurring there. It is not unlikely that the planting material in those experiments contained both mycelia and sclerotia as survival structures, as was found in numerous strawberry fields in Norway (Strømeng et al., 2009), thus indicating that neither form of the fungus survived the heat. Heat treatment may inactivate the much larger sclerotia of *Sclerotinia sclerotiorum*; however, the temperature and time required for total inactivation was 120 ºC for 20 minutes, and at 100°C for 80 minutes around 10% of the conidia survived (Dueck et al., 1981).

Although sclerotia are hardy overwintering structures of *Botrytis* spp., aerated steam treatment in the 'Plantsauna' were at least as effective against sclerotia as against dormant mycelium. Symptomless endophytic growth in the apoplast during latent infection is typical of *Botrytis* (Barnes & Shaw, 2003, Sowley et al., 2010). Although water on the plant surface during steam treatment should rapidly lead heat into the plant, to some extent and for a period after start of the treatment, the inner plant tissue containing *Botrytis* structures may have been protected against the high temperature during steaming. Although protected by muslin tissue, the sclerotia in these experiments may have been more exposed to the heat

than if they had been partly embedded in plant tissue. In future experiments, it may thus be advisable to include planting material and soil that contain sclerotia.

In the present work, the frequency of resistance against pyraclostrobin was very high, followed in descending order of resistance to thiophanate-methyl, pyrimethanil, boscalid, fenhexamid, fludioxonil and fluopyram. Many isolates had resistance to two to five fungicides, and this is in accordance with other studies, where transplants were found to carry isolates resistant to multiple fungicides (Nielsen et al., 2020; Oliveira et al., 2017; Weber & Entrop 2017). Nurseries are known to use multiple sprays of pyraclostrobin, boscalid, cyprodinil and fludioxonil in their strawberry transplant production (Weber & Entrop, 2017), and the high frequency of fungicide applications favour selection of resistant strains of *Botrytis* spp. (Fernández-Ortuño et al., 2012; Hu et al., 2016; Weber & Entrop 2017). This problem needs to be addressed, as strains of *Botrytis* spp. with multiple fungicide resistance can enter the production system along with transplants and thus decrease the efficacy of periodic fungicide applications (Amiri et al., 2014; Fernández-Ortuño et al., 2012; Weber & Entrop, 2017). In this study, thermotherapy seemed detrimental to the *Botrytis* population regardless of resistance. This reduction in the initial pathogen population may reduce the burden of disease management during the production season.

Warm water treatments have been used in commercial plant nurseries to control pathogens, mites, and insects; however, it has been speculated that this treatment practice could increase cross-contamination and in some cases injure the transplants (Buchner, 1991; Turechek & Peres, 2009). To overcome this problem, aerated steam treatment as an enhanced means of thermotherapy came forward (Turechek et al., 2021); steam treatments

will not, or to a very small extent, lead to plant-to-plant dissemination of pathogens. For minimum damage to the plants, precision and uniformity of the chamber temperature, especially during the four hours at 44°C, are critical for successful heat treatment, with minimum damage to the plants (Turechek et al., 2021). In our case, the temperature varied within 0.5°C. The present work was carried out with a small-scale research unit. In California, a larger steam chamber was used in a commercial nursery (Turechek et al., 2021), still with some limitations in efficacy and volume of plants. However, large-scale units of an improved model are now being marketed internationally. In the future, this technology may also be used to produce healthy planting material of nursery stock of other dormant deciduous fruit, berry and ornamental crops. This may strongly reduce the need for fungicide control after planting and increase the longevity of important chemical compounds in the control of *Botrytis* and other pathogens.

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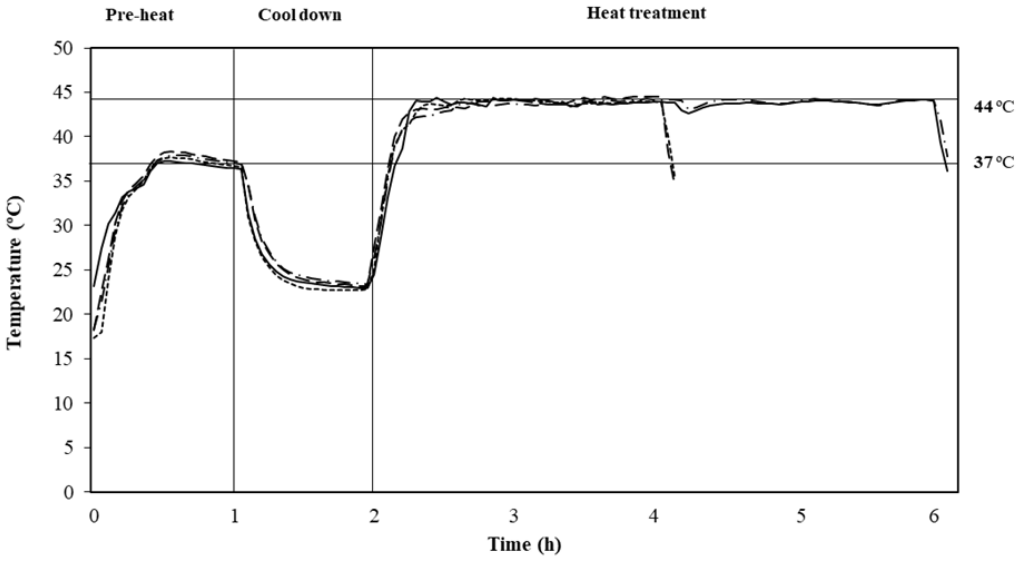

Figure 1. Temperature profile inside the steam chamber as recorded by four loggers placed at plant height in the middle of each of four plant boxes; two sensors removed after 2 h and two after 4 h at 44°C.

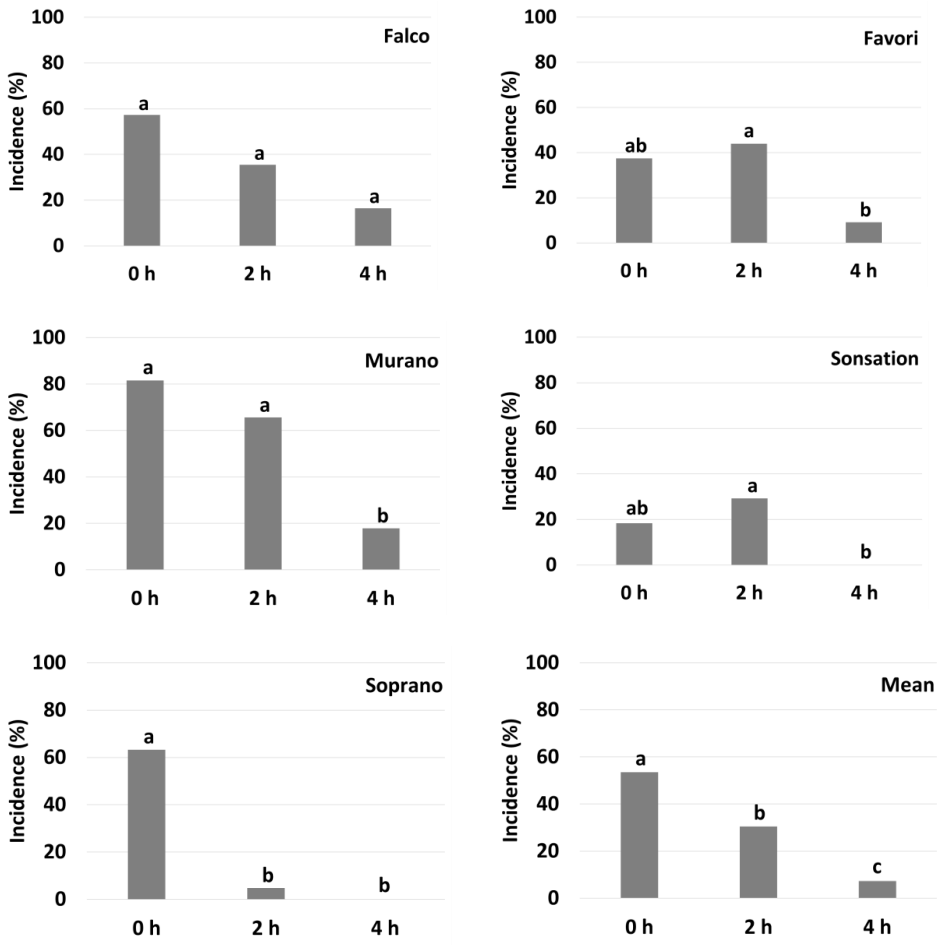

Fig. 2. Incidence (%) of *Botrytis* spp. in plant tissue of five strawberry cultivars; mean of six experiments, two with cv. Soprano and one each with the other cultivars. Plants were either not treated (0 h) or pre-treated at 37 °C in aerated steam, 1 h in ambient air, then either 2 (2 h) or 4 (4 h) at 44°C in aerated steam.

Table 1. Days to anthesis and growth and yield performance per plant of strawberry transplants after aerated steam treatments.

Cultivar	Treatment[a]	Days to anthesis	No. of runners	No. of leaves	No. of crowns	Yield (g)	Plant height (cm)	Fresh shoot weight (g)	Dry shoot weight (g)	Dry root weigt (g)
'Falco'	0 h	33.5 a[b]	17.7 b	65.2 a	5.6 a	447.1 a	34.2 a	246.6 ab	-[c]	-
	2 h	33.6 a	17.6 b	58.3 a	5.6 a	438.1 a	34.8 a	237.2 b	-	-
	4 h	34.5 a	28.7 a	57. 9 a	5.6 a	349.6 b	37.2 a	319.8 a	-	-
	P-value	0.24	<0.01	0.51	1	0.03	0.27	0.06	-	-
'Favori'	0 h	19.7 a	6.5 a	32.5 a	3.9 a	980 a	30.8 a	272.2 a	-	-
	2 h	15.4 b	6.0 a	41.7 a	4.9 a	968.9 a	29.6 a	330 a	-	-
	4 h	18.7 a	6.6 a	40.5 a	5.2 a	934.1 a	30.9 a	295.2 a	-	-
	P-value	0.03	0.5	0.2	0.36	0.4	0.8	0.37	-	-
'Murano'	0 h	21.4 a	12.2 b	77.4 a	9.6 a	748.9 a	28.4 a	290.2 a	-	-
	2 h	20.4 a	13.3 b	73 a	11.4 a	790.7 a	26.9 a	266 a	-	-
	4 h	23.3 a	18.2 a	62.7 a	9.43 a	861.2 a	29.8 a	263.7 a	-	-
	P-value	0.13	<0.01	0.1	0.6	0.2	0.69	0.8	-	-
'Sonsation'	0 h	36.6 a	16.2 a	51.5 a	5.2 a	462.2 a	36 a	331.3 ab	-	-
	2 h	36.5 a	13.5 a	52.6 a	5.5 a	459.7 a	37.4 a	321 b	-	-
	4 h	37.3 a	17.3 a	57.3 a	5.7 a	451.7 a	37.7 a	357.4 a	-	-
	P-value	0.4	0.12	0.6	0.8	0.9	0.6	0.07	-	-
'Soprano'	0 h	37.2 a	3.7 a	18.8 a	5.9 a	129.9 a	39.2 a	133.5 a	31.7 a	8.9 a
	2 h	37.8 a	3.6 a	18.8 a	6.2 a	141.9 a	37.3 b	127.7 a	29.9 ab	9.01 a
	4 h	37.6 a	3.2 a	19.5 a	6.3 a	142.0 a	39.6 a	122.7 a	27.7 b	8.3 a
	P-value	0.7	0.2	0.7	0.68	0.3	< 0.001	0.08	< 0.001	0.3

[a]No treatment (0 h) or pre-treatment at 37°C in aerated steam, 1 h in ambient air, then either 2 (2 h) or 4 (4 h) at 44°C in aerated steam.

[b]The data presented are means per plant of 5 (cv. Soprano), 8 (cvs. Falco and Sonsation) or 9 (cvs. Favori and Murano) potted plants per treatment in each of three replicates; columns for each cultivar with mean values followed by different letters are significantly different at $P \leq 0.05$ by Fisher's LSD test; experiments were run two times for cv. Soprano and one for the other cultivars.

[c]- is not examined.

Table 2. Severity (%) of *Botrytis* spp. on leaves detached from steam-treated groups of four strawberry cultivars, either non-treated or steam treated for 2 or 4 h[a]

Treatment	'Falco'	'Favori'	'Murano'	'Sonsation'	Mean
0 h	2.9 a[b]	1.1 a	19.4 a	0.6 a	6.0 a
2 h	4.9 a	3.7 a	5.9 a	2.0 a	4.1 ab
4 h	1.5 a	0.5 a	2.3 a	0.0 a	1.1 b
P-values	0.3	0.14	0.02	0.04	0.018

[a]No treatment (0 h) or pre-treatment at 37 °C in aerated steam, 1 h in ambient air, then either 2 (2 h) or 4 (4 h) at 44°C in aerated steam.

[b]Columns with mean values followed by different letters are significantly different at $P \leq 0.05$ by Fisher's LSD test for each cultivar and by Tukey's HSD test for mean.

Table 3. Resistance profile[a] of 62 bulk conidia isolates of *Botrytis* sp. collected from cvs. Murano, Falco and Favori.

Fungicides	S[b]	LR	MR	R	R (%)[c]
Boscalid	7	29	15	11	17.7
Fenhexamid	51	0	1	10	16.1
Fludioxonil	58	0	2	2	3.2
Fluopyram	19	24	18	1	1.6
Pyraclostrobin	4	0	2	56	90.3
Pyrimethanil	16	4	20	22	35.5
Thiophanate-methyl	34	1	1	26	41.9

[a]Based on a mycelial growth assay by Schnabel et al. (2015).

[b]S = sensitive, LR = low resistant, MR = moderately resistant, R = resistant.

[c]Percentage of isolates exhibiting complete resistance (R) for a particular fungicide out of 62 isolates in total.

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